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XIII International Workshop on Chronic Lymphocytic Leukemia

16 - 18 October 2009, Barcelona, Spain

Guest Editors
Emili Montserrat, Michael J. Keating

ABSTRACT BOOK

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XIII International Workshop on Chronic Lymphocytic Leukemia

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Epidemiology (including familial CLL)

1.1

UNDERSTANDING INHERITED SUSCEPTIBILITY TO CHRONIC LYMPHOCYTIC LEUKAEMIA THROUGH GENOME-WIDE ASSOCIATION

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While chronic lymphocytic leukaemia (CLL) shows an 8-fold increased risk in relatives of cases the basis of this inherited predisposition is only recently becoming understood. We have conducted a genome-wide association study (GWAS) of 299,983 tagging SNPs based on 517 cases and 1,438 controls. Through fast-tracking validation in two additional case-control series totalling 1,024 cases and 1,677 controls we have identified six SNPs mapping to 2q13, 2q37.1, 6p25.3, 11q24.1, 15q23 and 19q13.32 as recently reported in Nature Genetics. The most significant association was with rs872071, which maps to the 3' untranslated region of interferon regulatory factor 4 (IRF4; $p = 1.91 \times 10^{-20}$). Resequencing and finemapping of the region revealed five candidate causal variants that we are pursuing further. Collectively the six loci account for ~3% of the excess familial risk of CLL. Clearly there are more loci yet to be identified and we are carrying out systematic replication of the remaining prospective SNPs from the initial phase. Three additional risk loci have been identified and are being investigated further. These results provide robust evidence for low-risk variants predisposing to a haematological malignancy and offer new insights in the etiological basis of CLL.

1.2

LITERATURE ON CLL HAS SIMILAR GROWTH CHARACTERISTICS AS THE WHITE CELL COUNT IN A PATIENT WITH PROGRESSIVE CHRONIC LYMPHOCYTIC LEUKAEMIA

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Over the last thirty years there has been increasing interest in Chronic Lymphocytic Leukaemia (CLL). The commonly used databases (PubMed, Highwire, Web of Knowledge and Google) were searched using terms: Chronic lymphocytic leukaemia OR Chronic lymphocytic leukaemia. There are now over 14,000 articles listed on PubMed. The numbers of publications is shown in the figure below and has grown to over 750 annually. However the number of total publications including books and abstracts is listed as 20,596 using Web of Knowledge.

Figure. Annual number of CLL publications in PubMed.

For the 14,722 results on ISI Web of Knowledge using the search term CLL, there were 22 books, 119 editorials, 598 reviews, 7,728 articles, 111 case reports and 30 clinical trials. Highwire and Google Scholar list 24,000 and 159,000 hits respectively. This poster / presentation will outline the most cited papers, the most prolific authors and the journals in which CLL articles are published. The interest in CLL continues to grow and is also reflected in increase in numbers attending the IWCLL. The sheer numbers of these electronic hits and publications make it very hard to identify the papers which provide novel or relevant information. How new researchers, clinicians and patients can absorb a deep understanding of the CLL literature remains a problem. Perhaps the answer is some targeted therapy.

1.3

CHRONIC LYMPHOCYTIC LEUKEMIA INCIDENCE IN SPAIN: RESULTS FROM A NATIONWIDE REGISTRY DATA

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It is a common belief that CLL is the most frequent leukemia in the U.S. and Western Europe. Patients and methods: Spanish Registry of Leukemia (SRL) is a thoroughful and systematic registry of all leukemia cases diagnosed in the participant centers, among residents in the geographic areas of study during 16 months: September 1st, 2001 to December 31st, 2002. Reference population of the registry was Spanish population at the beginning of study, aged over 14 years. Target population was those residents officially censored in the reference areas of the participating centers. Study population was all new cases of leukemia of any kind. 124 hospitals participated in the SRL with 28 millions of Spanish people. *Results.* 3595 cases of leukemia were recorded. Of note, only 4.8% (n=172) of the cases were unclassified leukemias. CLL and other related lymphoprolypherative disorders accounted for 34.2% (n=1229), being the most frequent type of leukemia. All cases were B-CLL, except 3.5% of hairy cell leukemia, 1.3% of T-cell CLL/T prolymphocytic leukemia and 0.7% B prolymphocytic leukemia. By gender, there was a slight predominance in men: 53.5%, while 46.5% were women. 80.6% of patients had more than 60 years of age, with a median age at diagnosis of 70 years (69.3-70.7 95% CI). 30.1% of CLL patients had first degree relatives with oncohematologic antecedents. Regarding with clinical staging, 60% of cases were Rai 0 stage and 19.4% were in stage I, so 79.4% were diagnosed at early or low-risk stage. 11.2% were in stage II or intermediate. 3.9 and 4.9% were in stages III and IV, respectively, accounting for 8.8% of cases diagnosed at advanced stage. At diagnosis, 60% of patients had an Eastern Cooperative Oncology Group performance status (PS) of 0, while 16.1% had a PS of more than 2. Of note for the whole group of leukemias, only 42.7% had a PS of 0 at presentation. One moth after diagnosis, 98.5% of CLL patients were alive, in contrast with 89.2% for the total group of leukemias. Estimated mean age-and population-adjusted global incidence rates of leukemia were 11.6/100.000 (10.1-13 95% CI) for men and 8.6/100.000 (7.4-9.9 95% CI) for women. The rates of CLL were 4.2/100.000 (3.3-5.1 95% CI) for men and 3.1/100.000 (2.3-3.8 95% CI) for women. *Conclusions.* CLL is the most frequent type of leukemia in Spain. This finding is consistent with the estimates of CLL incidence in the U.S.

1.4

BIRTH ORDER EFFECT IN THE INHERITANCE OF CHRONIC LYMPHOCYTIC LEUKEMIA

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A birth order effect is encountered when estimating a non-random occurrence of affected sibs in a sibship. Such a birth order effect with pleiotropy, viz. polymorphism between CLL and other lymphoproliferative disorders (LPD) in affected families, and a male predominance in CLL are seen in familial CLL, although the mode of segregation of the CLL susceptibility genes remains unknown so far. The birth order effect has also been discussed in the light of possible environmental factors, e.g. antigenic drive from mutual infections among the sibs. The purpose of the present work is to present data on birth order effect in various parent-offspring combinations in our Scandinavian families with CLL. 74 pairs of affected parent-offspring were ascertained from our database on familial LDP, identified in 59 consecutively families with two or more cases of CLL or CLL in combination with nonCLL (viz. other lymphocytic leukaemias than CLL, NHL, multiple myeloma, BL, MGUS etc.). All affected and healthy family members in at least three generations were recorded and crosschecked with the National Cancer Registries and the Civil Person Registries in Norway and Denmark. 45 (61%) CLL - CLL pairs, 24 matrilineal and 21 patrilineal pairs with a nearly equal number of affected sons and daughters in the matrilineal line (11 sons vs. 13 daughters) while sons were predominant in the patrilineal line (13 sons vs. 8 daughters) 11 (15%) CLL – nonCLL pairs, 5 matrilineal and 6 patrilineal pairs with a nearly equal number of affected sons and daughters (total 6 sons and 5 daughters). 18 (24%) nonCLL – LPD pairs, 8 matrilineal and 10 patrilineal pairs with a nearly equal distribution of affected sons and daughters in the matrilineal line (5 sons vs. 3 daughters), in contrast to the patrilineal line where all affected offspring were sons (10 sons) of whom 7 had CLL.

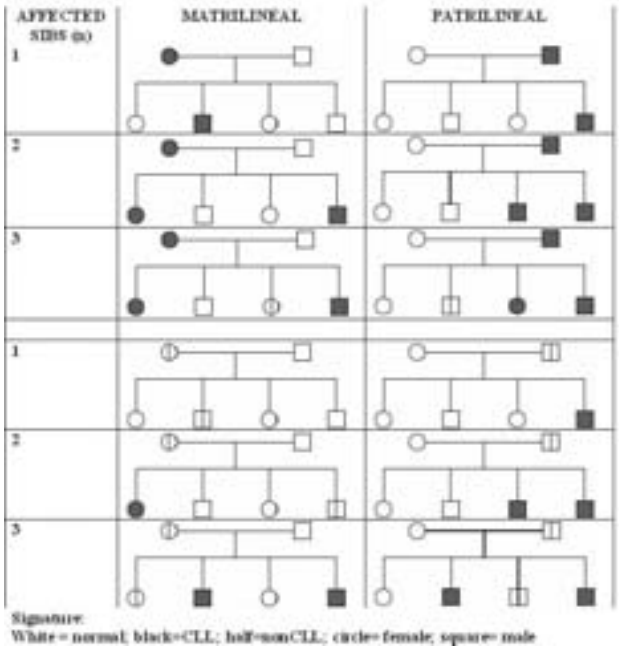

Figure. Diagram showing data conjugated to four persons per sibship as a realistic mean for the period of observation.

Vector determinant (tendency) and metric space estimation (phenetic resemblance) was based on Matrix Conjugation with Bayesian Approach calculated by means of common matrix processing. Bayesian Approach has been preferred because in contrast to traditional segregation analysis related to observed vs. expected numbers, the Bayesian Approach provides posterior probabilities no matter the mode of segregation, which is unknown in CLL, and no matter the frequencies of the susceptibility genes. Ascertainment was restricted to parent-offspring pairs to achieve ultimate commutative data, so that pairs of uncle, aunt-nephew, niece combinations as well as ascertained single affected family members were disregarded, as were the sib-sib combinations and the very few cases of combined lympho- and myeloproliferative disease. For control, all Bayesian calculations were compared with findings from Cox regression analysis. Conclusion A birth order effect is seen: -affected offspring in patrilineal lines are found late in the sibship with healthy older siblings, -while in matrilineal lines, the affected offspring are seen randomly throughout the sibship. A birth order effect seems related to father-son combinations: - affected sons compared with affected daughters were predominant in patrilineal CLL and especially in patrilineal nonCLL combinations. The low-grade B-cell follicular lymphoma was the most frequent nonCLL diagnosis. The findings are discussed in the light of non-Mendelian segregation of CLL susceptibility genes.

1.5

PATTERNS AND OUTCOMES OF INITIAL SYSTEMIC THERAPY IN ELDERLY PATIENTS DIAGNOSED WITH CLL IN THE UNITED STATES: EVIDENCE FROM SEER-MEDICARE

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Introduction. Clinical practice guidelines in the United States recommend as initial treatment for CLL, observation only, or radiation therapy to lymph nodes, or treatment with a single drug, or a combination of drugs, depending on stage at diagnosis and risk category. Presently, little is known about utilization of initial systemic therapy and outcomes in elderly patients in routine clinical practice. *Objectives.* The objectives of this study were to identify the prevalence, timing, and types of initial systemic therapy, as well as patient factors associated with receiving therapy and survival in an elderly CLL population. *Methods.* The source of data for this study was the NCI Surveillance, Epidemiology, and End Results (SEER) database linked to Medicare claims for medical services. The SEER cancer registries collect data on patient demographics, primary tumor site, morphology and stage at diagnosis (not available for CLL) and first treatment. The SEER-Medicare dataset included all Medicare eligible persons in SEER through 2002 with Medicare claims for Part A (inpatient) and Part B (outpatient and physician services) through 2005. Patients were included in this study if they were diagnosed with CLL between January 1, 1999 and December 31, 2002 and enrolled in Medicare parts A and B. Medicare claims were used to identify intravenous agents used as part of systemic therapy, including chemotherapy and immunotherapy (rituximab). Cox proportional hazards models were used to identify factors associated with receiving systemic therapy, and factors associated with improved survival in patients receiving systemic therapy. The survival analysis was based on patients who survived at least 180 days after diagnosis and were untreated during that period. Patients were included in the survival model at the time they initiated systemic therapy. *Results.* There were 3,044 patients who met the study inclusion criteria. The average age at diagnosis was 77.5 years (median 77 years); 56% were male gender; 89% were white race; and 38% had an NCI Comorbidity Index ≥ 1. Systemic therapy was provided to 1,032(34%) patients during the observation period. As initial systemic therapy, 389/1,032 (38%) received rituximab with or without chemotherapy; and 643 (62%) received chemotherapy alone. The most common chemotherapy agents were fludarabine (47%) and vincristine (14%). In multivariate analysis, age ≥80 (compared to 66-69) was associated with a significantly longer time to treatment (HR:0.71; $p<0.001$), while male gender (HR:1.41; $p<0.001$) and Hispanic race/ethnicity (compared to white: HR:1.46; $p<0.03$) were associated with shorter time to treatment. Adjusting for patient demographic and clinical characteristics, in the group of patients who began initial systemic therapy after 180 days following diagnosis (n=517), therapy that included rituximab was associated with a statistically significantly lower mortality rate (HR: 0.62; $p=0.01$) compared to chemotherapy alone. In sensitivity analyses, the results were similar for patients treated in the first 180 days after diagnosis. *Summary.* In a large cohort of elderly patients diagnosed with CLL, who were followed for up to 7 years, approximately one third received systemic therapy. Use of rituximab was associated with lower mortality in this retrospective analysis of elderly patients.

1.6

THE PREVALENCE OF MONOCLONAL B-CELL LYMPHOCYTOSIS (MBL) IN OLDER FIRST-DEGREE RELATIVES OF PATIENTS WITH SPORADIC CHRONIC LYMPHOCYTIC LEUKEMIA (CLL) IS AS HIGH AS IN OLDER FIRST-DEGREE RELATIVES OF PATIENTS WITH FAMILIAL CHRONIC LYMPHOCYTIC LEUKEMIA

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Backgrounds. Although biological similarities have been described among MBL and CLL, the relationships between these two conditions are not fully understood, and new epidemiological studies in different populations and different countries continue to be reported. *Aims.* The goal of the study was to determine the prevalence, biological characteristics and evolution of MBL in first-degree relatives of families with just one case of CLL (sporadic CLL). *Methods.* We investigated 167 individuals from 42 families by 4-color flow cytometry assay as recommended by Rawstron *et al.* (Blood. 2001;98:29-35). All subjects gave their written informed consent prior to entering the study. Purified B-lymphocytes CD19^{+} of peripheral blood were selected by magnetic sorting and used for PCR (IGHV/T-cell receptor) and FISH experiments (del 17p, del 13q, del 11q and trissomy of 12) in MBL cases.

Table.

Name	Age/Sex	Ig*	MBL phenotype	κ:λ ratio	Extended Phenotyping	PCR
LPA	46/F	L,8	CD5^{+}/CD20$^{+bright}$/CD79b^{+dim}	0,08:1	CD11a$^{+bright}$/CD23^{+}/CD38^{+} CD49c^{+}/CD49d^{+}//FMC7^{+}	Monoclonal
DRK	73/M	L,7	CD5^{+}/CD20^{+dim}/CD79b^{+dim}	0,25:1	CD11a$^{+bright}$/CD23^{+}/CD38^{+} CD49c^{+}/CD49d^{+}//FMC7^{+}	Negative
AAJ	72/M	L,3	CD5^{+}/CD20^{+dim}/CD79b^{+dim}	0,28:1	CD11a^{+dim}/CD23^{+}/CD38^{+} CD49c^{+}/CD49d^{+}/FMC7^{+}	Monoclonal*
BFR	53/M	L,3	CD5^{+}/CD20^{+dim}/CD79b^{+dim}	0,32:1	CD11a^{+dim}/CD23^{+}/CD38^{+} CD49c^{+}/CD49d^{+}/FMC7^{+}	Monoclonal
ARP	62/M	L,8	CD5^{+}/CD20^{+dim}/CD79b^{+dim}	0,35:1	CD11a^{+dim}/CD23^{+}/CD38^{+} CD49c^{+}/CD49d^{+}/FMC7^{+}	Monoclonal*
JGZ	73/M	L,1	CD5^{+}/CD20^{+dim}/CD79b^{+dim}	4,35:1	NP	NP
FCZ	61/F	4,0	CD5^{+}/CD20^{+dim}/CD79b^{+dim}	4,34:1	CD11a^{+dim}/CD23^{+}/CD38^{+} CD49c^{+}/CD49d^{+}/FMC7^{+}	Negative

Results. MBL was found in seven individuals, from five families, of a total of 167 subjects (4.1%). The prevalence according to the age was 0 (0/54) in individuals with less than 40 years, 2.5% (2/81) between 40 and 60 years and 15,6% (5/32) in individuals over than 60 years. The table shows the characteristics of MBL cases. With a median follow-up of 23 months, none individual has progressed to CLL. From the seven individuals that have been detected with MBL, six were analyzed by PCR and FISH. Clonal gene rearrangements of IGHV were detected in four subjects. In two, we also found a clonal rearrangement of the gene TCRG of T-cell receptor in the MBL population. FISH experiments did not show any abnormality. *Summary/Conclusions*. This is the first study of MBL in first-degree relatives of patients with sporadic CLL. Monoclonality was detected in all cases either by an abnormal: ratio, or by PCR, or by both. Although chromosomal lesions associated with poor prognosis are rare in MBL, 13q deletion and trissomy 12 have been found in similar proportion in MBL to that seen in CLL. Our not significant FISH results are probably related to the small number of MBL individuals studied. The overall prevalence of 4,1% found in our population is similar to that of MBL in individuals of general population, which varies between 3,5% and 5,5%. However, with regard to the prevalence in age groups, we have found a value of 15.6% (5/32) in individuals over 60 years, which is higher than values found for the same age group in general population, but very similar to the prevalence of 16,7% (3/18) found by de Tute *et al.* (Leukemia 2006;20:728-729) in familial CLL subjects over 60 years. Provided that the higher risk of emergence of clinical CLL in relatives of CLL families is probably related to the high prevalence of MBL in these subjects, our data strongly suggests that in older first-degree relatives of patients with sporadic CLL, the risk of MBL detection is as high as in older first-degree relatives from CLL families, which could render these individuals belonging to "sporadic CLL families" as susceptible as individuals belonging to "familial CLL" to the development of clinical CLL.

1.7

PREVALENCE OF MELANOMA AND NON-MELANOMA SKIN CANCER IN CHRONIC LYMPHOCYTIC LEUKAEMIA IN AUSTRALIA

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Patients with chronic lymphocytic leukaemia (CLL) are suspected to have an increased risk of both melanoma (MSC) and non-melanoma skin cancer (NMSC). Australia has one of the highest rates of skin cancer in the world. NMSC is by far the most common type of cancer in Australia. Although it is not recorded by most Australian cancer registries, it is estimated that approximately two-thirds of Australians will experience at least one NMSC during their lifetime before the age of 70 years. There have been no previous publications assessing the prevalence of either MSC or NMSC in CLL patients in Australia. We performed an audit of skin cancer prevalence (both MSC and NMSC) in two separate haematology practices in Northern Sydney and Central Coast Area Health Service, NSW, Australia over a six month period. One practice was a specialist haematology referral unit at a major metropolitan teaching hospital and the other a regional community based practice. Data from 163 CLL patients (105 regional centre, 58 metropolitan) was collected to determine the prevalence of both MSC and NMSC and any correlation with disease stage or prior therapy. Of the 163 patients, there were 94 males and 69 females with median age 70.1 years (range 22 - 96 yrs). There were 16 patients with MSC, and 96 with NMSC. The 16 (9.8%) patients diagnosed with melanoma were 12 males and 4 females with median age 72.6 (range 47-86). All but 2 melanoma patients also had NMSC. The prevalence of 9.8% melanoma in CLL patients is much higher than the Australian lifetime risk of developing melanoma (1 in 25 [4%] for men and 1 in 34 [3%] for women). Most patients with MSC had Binet stage A disease (13/16) and only 2/16 had received prior therapy for their CLL. 3 patients had more than one melanoma and 1 had metastatic disease. There was no significant difference between the metropolitan and regional practices with melanoma prevalence. The prevalence of NMSC was 58.8% affecting 96 of the 163 patients. There were 54 males and 42 females with median age 74.6 years (range 47-96). An additional 7 patients had solar keratosis without NMSC. There was significantly higher prevalence from the regional centre (69.5%) compared with patients from the tertiary referral centre (36.7%). This appears to reflect both the older age and area demographics of the CLL patients managed at the regional centre. Higher sunlight exposure of patients from the regional centre is another probable factor. There was an overall trend of increased risk of NMSC with increasing age (see Table1), in keeping with other data suggesting that the association between CLL and NMSC becomes stronger as the population studied becomes older. There was no obvious association between NMSC and prior therapy for CLL with 65/96 patients with NMSC having had no prior therapy. In conclusion, skin cancer prevalence is high in Australian patients with CLL, especially melanoma which at 9.8% appears substantially higher than the risk for the general Australian population.

Table 1. Prevalence of melanoma and non-melanoma skin cancer in CLL by age decade.

Age	*Total Patients*	*Melanoma*	*NMSC*	*NMSC % by age*
<50	6	1	1	16.6
50-59	27	1	7	25.9
60-69	35	3	16	45.7
70-79	60	6	39	65.0
≥80	35	5	33	94.3
Total	163	16	96	58.8

1.8

ANTHROPOMETRICS OVER THE LIFE COURSE AND RISK OF CHRONIC LYMPHOCYTIC LEUKEMIA

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Background. Obesity is associated with chronic inflammation and impaired immune function, and has been linked to an increased risk of certain cancers. While a recent pooled analysis from the InterLymph Consortium reported no overall association of body mass index (BMI) with risk of CLL, there was a positive association among 4 of 5 North American studies (Int J Cancer 2008;122:2062). Further, the only North America study to evaluate obesity over the life course found that excess adiposity earlier in adulthood was more strongly associated with CLL (Am J Epidemiol 2002;156:527). There are no other data on the association of childhood anthropometrics or birthweight with risk of CLL. *Methods.* We evaluated anthropometrics over the life course with risk of CLL in a clinic-based study of 297 newly diagnosed CLL/SLL cases and 1146 controls enrolled at the Mayo Clinic from 2002-2008. Anthropometric characteristics were obtained from a self-administered questionnaire, and included height, weight at various ages (including birthweight), maximum weight, relative height and relative weight at ages 7 and 12 years, and age and weight when maximum height was attained. BMI was calculated as weight (kg) divided by height (m) squared. Unconditional logistic regression was used to estimate odds ratios (ORs), 95% confidence intervals (CI), and tests for trend, adjusted for age, sex, and residence. Continuous variables were divided into quartiles based on the distribution among the controls, and results are reported for the highest versus lowest quartile, unless otherwise specified. *Results.* The mean age at diagnosis was 62.7 years for cases and 64% were male; for controls, the mean age at enrollment was 61.0 years and 53% were male. Height (OR=1.43; p-trend=0.01) and recent weight (OR=1.51; p-trend=0.01) were positively associated with CLL risk, while recent BMI showed only a weak positive association (OR=1.24; p-trend=0.3). When analyzed by the WHO categories, there was no association for a BMI of 25.0-29.9 kg/m^2 (OR=1.04; 95% CI 0.76-1.42) compared to a BMI of 18.5-24.9 kg/m^2, while there was only a small and not statistically significant excess risk for a BMI of 30.0-34.9 kg/m^2 (OR=1.18; 95% CI 0.81-1.72) and 35.0+ kg/m^2 (OR=1.24; 95% CI 0.75-2.04). The association was similar for BMI at age 50 years (OR=1.35; p-trend=0.3), but there was a stronger positive association for BMI at age 35 years with CLL risk (OR=1.72; p-trend=0.006). Weight at age 18 (OR=1.49; p-trend=0.03), BMI at age 18 (OR=1.22; p-trend=0.05), and greater weight when maximum height was attained (OR=1.63; p-trend=0.01) were all positively associated with risk, while there was no association with age when maximum height was attained. There were no associations with relative weight or height at ages 7 or 12 years or with birthweight. All associations were similar for men and women, with the exception of height, which was specific to men. *Conclusion*. These results suggest that height for men and greater BMI in late adolescence and early adulthood for both sexes may play a role in the etiology of CLL. Supported by a grant from the NIH (R01 CA92153).

1.9

HEPATITIS C VIRUS-POSITIVE CHRONIC LYMPHOCYTIC LEUKEMIA

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The association of hepatitis C virus (HCV) and B-cell chronic lymphocytic leukemia (CLL)/small lymphocytic lymphoma (SLL) is not well established. Some epidemiological studies tend to rule out such a relationship. Again, little is known about the clinic-biological features and outcome of HCV-positive patients with CLL. We retrospectively evaluated clinico-hematological characteristics of 23 HCV-positive CLL patients compared with 189 HCV-negative CLL patients seen at our Institutions aiming to evaluate clinico-biological features and outcome of HCV infected patients with CLL at diagnosis compared to HCV negative CLL patients. No differences were found with respect to sex distribution and age. The HCV genotype was known only in 4 patients: in 3 cases was found 2a/2c, in one case 1b. No difference was also found in the absolute lymphocyte count, hemoglobin level, platelet count, Rai and Binet clinical stage at diagnosis, lymphocyte doubling time. Mutational status of IgVH, CD38 expression, and ZAP-70 expression did not show differences among the two groups of patients. The major cytogenetic abnormalities, detected by means of FISH in 13 HCV-positive patients, showed 7 cases of normal karyotype,3 cases of trisomy 12, 2 cases of deletion 13q14, 1 case of deletion of 11q22.3 (ATM). Finally, overall survivals of HCV infected patients and HCV-negative patients did not show any significant difference (p 0.1). In conclusion, HCV-positive patients with B-cell CLL seem to not differ from other patients for presentation and clinical outcome as well. However, preliminary results need to be confirmed on a large cohort of patients.

1.10

SEASONAL VARIATION IN THE DIAGNOSIS OF MONOCLONAL B-LYMPHOCYTOSIS (MBL): LACK OF ASSOCIATION FOR MBL WITH CLL-PHENOTYPE BUT POSSIBLE ASSOCIATION WITH MBL WITH LYMPHOMA-PHENOTYPE

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Introduction. Some haematological malignancies, such as Acute Lymphoblastic Leukaemia (ALL) have striking seasonal variation in their presentation. There is also some data for a possible seasonal association with both Hodgkin's (HD) and non-Hodgkin's lymphoma (NHL). It is speculated that this may reflect possible infectious agent(s) implicated in the aetiology. No seasonal association has been reported for Chronic Lymphocytic Leukaemia (CLL). Monoclonal B-Lymphocytosis (MBL) can be subclassified into patients with a CLL-like phenotype accounting for 77% of MBL while 23% of MBL patients have an NHL-like phenotype. We sought to determine if there is a possible seasonal variation for the diagnosis of MBL. *Methods.* We previously identified a cohort of 414 patients diagnosed at a large non-hospital based pathology laboratory over the six year period of 2000-2005 inclusive. We analysed the month of diagnosis of MBL separating patients that had MBL with a CLL-like phenotype and those with MBL with an NHL-like phenotype.

Table 1.

Month of Diagnosis 2000-2005	*MBL[nhl] (n=92)*	*Percentage MBL[nhl] by month**	*MBL[cll]) (n=322*	*Percentage MBL[cll] by month**	*Total Phenotype Studies by month 2000-2005*	*Percentage Phenotype studies by month*
January	7	7.6	16	5.0	1255	6.9
February	5	5.4	19	5.9	1551	8.5
March	9	9.8	42	13.0	1669	9.1
April	5	5.4	29	9.0	1397	7.6
May	3	3.3	35	10.9	1544	8.5
June	6	6.5	25	7.8	1454	8.0
July	15	16.3	29	9.0	1598	8.7
August	9	9.8	29	9.0	1646	9.0
September	7	7.6	21	6.5	1607	8.8
October	5	5.4	30	9.3	1651	9.0
November	16	17.4	27	8.4	1552	8.5
December	5	5.4	20	6.2	1339	7.3

**Numbers may not add to 100 due to rounding.*

Results. Month of diagnosis for 414 patients with MBL is shown in the table. Patients are divided into those with a CLL-like phenotype (322 patients) and those wih a NHL-like phenotype (92 patients). National, state and school vacations are scheduled during the (summer) months of December, January and February and Easter. These periods typically have fewer referrals to the laboratory that other months of the year. Results by month of diagnosis for MBL show no association with season in patients with a CLL-like phenotype. MBL with a lymphoma-like phenotype shows more variation with peaks in July and November with these two months accounting for one-third of all MBL-lymphoma diagnoses. *Conclusion.* In this patient cohort, MBL with a CLL-like phenotype

has no clear seasonal variation in the timing of diagnosis that is outside of the influence of vacation related referrals and ascertainment bias. By contrast, MBL with a lymphoma-like phenotype shows more variation with peaks in July (winter) and November (late spring) that are in excess of the vacation-related referral pattern. Seasonal influences may be implicated in MBL with an NHL-like phenotype although the numbers of patients are relatively small. This might be a useful factor to consider in future studies on the epidemiology of MBL.

1.11

SMALL LYMPHOCYTIC LYMPHOMA: RETROSPECTIVE ANALYSIS FROM THE REGISTRY OF CZECH LYMPHOMA STUDY GROUP

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Background. Small lymphocytic lymphoma (SLL) accounts for 3-10% of Non-Hodgkin´s lymphomas (NHL). According to World Health Organisation classification of lymphoid neoplasms, SLL forms a common entity with chronic lymphocytic leukemia (CLL). SLL differs from CLL only by arbitrary cut-off of circulating malignant lymphocytes below 5000/mm^3. SLL is still a relatively poor-defined entity with few published data, partly because clinical studies in CLL usually exclude patients with SLL. This results in marked variability in diagnostic and especially therapeutic strategies; uniform guidelines for SLL do not exist. *Aims*. To perform a retrospective analysis of patients (pts) with SLL focused on diagnostic and therapeutic approach in real practice. *Patients and Methods.* As of April 2008, the Czech Lymphoma Study Group (CLSG) registry contained data from 161 patients (63 % males, median age 65 years [range, 29-87]) who fulfilled diagnostic criteria for SLL, i.e. coexpression of CD5/C19/23 by flow cytometry or immunohistochemistry and peripheral blood lymphocytes <5000/mm^3. Cases of SLL comprised 3.4% of all NHL in CLSG registry. *Results.* Ann Arbor stage I/II/III/IV was present in 6/2/8/84%. Splenomegaly was detected in 18%, elevated lactate dehydrogenase (LDH) in 30% and bone marrow involvement in 79% of pts. Of note, 91% pts underwent computer tomography (CT) of thorax and abdomen as part of initial staging. Mediastinal and retroperitoneal lymphadenopathy was discovered in 42% and 68%. Generalized lymphadenopathy (more than 4 involved regions) was found in 49%. Bulky lymphadenopathy (>5 cm) was present in 38%. Thirty percent of pts had B-symptoms. Age-adjusted International Prognostic Index (aa-IPI) score 0/1/2/3 was present in 7/49/38/6%. Treatment was initiated in 156 pts: chemotherapy in 94 and chemoimmunotherapy in 56 pts (rituximab, 55 pts; alemtuzumab, 1 case). Four patients were treated by radiotherapy and two by surgery. The most frequent regimens used in first line were CVP (18%), CHOP (17%), R-CVP (15%), chlorambucil (15%), FCR (11%), FC (10%) and R-CHOP (10%). Median follow-up was 37.7 months (mo). Median progression-free-survival (PFS) and overall survival (OS) were 35.3 and 63.3 mo. None of the prognostic factors (Ann Arbor stage, aa-IPI, elevated LDH, bulky lymphadenopathy) had significant influence on OS. Similarly, OS was not affected by the type of treatment (fludarabine-based vs. anthracycline-based vs. other; addition of rituximab to chemotherapy). *Conclusions.* Results from CLSG registry confirm previously published demographic data on SLL. In contrast to CLL (where routine radiological assessment is not recommended by NCI-WG criteria), nearly all SLL patients underwent CT for staging. Bulky lymphadenopathy was detected in more than a third of pts. No prognostic factors influencing overall survival were identified. Therapeutic strategies varied greatly; two thirds of pts were treated by regimens commonly used in other indolent lymphomas. Our study underscores the need for standardized diagnostic and therapeutic approach to SLL. *Supported by grant NR/ 9453-3 and research project MZO 00179906 from Ministry of Health, Czech Republic.*

1.13

WHAT DO WE KNOW ABOUT THE REAL INCIDENCE OF CHRONIC LYMPHOCYTIC LEUKEMIA (CLL) AND WHERE AND HOW ARE THE PATIENTS WITH CLL TREATED?

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Background. Currently, pathogenesis, modern prognostic factors, or modern therapy in CLL are frequently discussed, however, up-to-date data concerning the incidence and the management of CLL in "real life" are still missing. Aims. The aim of our study was to find out the actual epidemiological situation of CLL, and the diagnostic and therapeutic preferences of hematologists in the preselect area – the South Moravian Region. *Methods.* We asked 18 specialized hematological departments to fill in our questionnaire in order to get exact information about epidemiology and a treatment of patients with CLL. All hematologists filled the questionnaires out. Acquired data were merged with our databasis. According to the Czech Bureau of Statistics,1,127,718 inhabitants live in the South Moravian Region. *Results.* The total number of 540 patients (median age at the time of diagnosis: 65 years, range: 33-92; sex: 306 males, 234 females) who were followed in the year 2008 were included in the analysis. Therefore, the incidence of CLL in the South Moravia Region was 6.2 per 100,000, the prevalence 48 per 100,000. At the time of diagnosis, 274 patients (50%) had the Rai stage 0, 173 (32%) Rai I, 36 (6.7%) Rai II, 25 (4.6%) Rai III, 25 (4.6%) Rai IV, in 7 patients (1.3%) the Rai stage wasn't determined. Flowcytometry was carried out in 525 patients (97%), the others were diagnosed by a histological examination either of bone marrow or a lymph-node. Two hundred eighty-seven patients (53%) were followed up at the local hematological ambulances, 253 (47%) were treated at one main hematological center. The median follow-up was 56 months (range: 5-1262). Two hundred ninety-seven patients (54%) were cytogenetically examined (deletion of 17p (n=5); deletion of 11q (n=18); 29 trisomy of 12; 112 deletion of 13q; 94 normal karyotype). IgVH mutational status was detected in 305 (57%) patients (155 mutated, 134 unmutated, 16 polyclonal). ZAP-70 was detected in191 (35%) patients (77 positive, 114 negative). The treatment of CLL was indicated in 194 patients (36%), 93 (17%) of them also underwent the second line treatment. As the first line treatment, 64 patients (33%) were given fludarabine-based regimen, 40 (20.6%) of them achieved complete remission (CR), 74 (38%) received chlorambucil with 12 (6%) CRs, 28 (14%) CHOP-like regimen with 8 (4%) CRs, 10 (5%) alemtuzumab with 6 CRs, 18 (9%) corticosteroids with 1 CR. Ninety-three patients (17%) underwent the second line treatment: 39 (42%) fludarabine-based regimen with19 CRs, 19 (20%) chlorambucil with 1 CR, 8 (9%) CHOP-like regimen with 1 CR, 10 (10%) alemtuzumab with 4 CRs, and 17 (18%) corticoid-based treatment with no CR. Thirty patients were treated within clinical trials. *Conclusions.* According to our analysis, fewer patients with CLL were surprisingly indicated to therapy than we expected. Although the treatment was indicated in the minority of patients, the modern prognostic factors were examined in almost all patients. It remains questionable if in "real life" the examination of the modern prognostic factors is necessary in all CLL patients. *Supported in part by the Czech Leukemia Study Group for Life, and in part by Research Grant MSM 0021622430.*

1.14

CHRONIC LYMPHOCYTIC LEUKEMIA TRANSFORMATION TO HODGKIN LYMPHOMA: A REPORT OF THREE CASES

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Richter syndrome (RS) describes the development of an aggressive lymphoma, most commonly diffuse large B-cell lymphoma (DLBCL), in patients with chronic lymphocytic leukemia (CLL), which leads to shorter survival. RS affects approximately 3.3-20% of CLL patients. Moreover, CLL may rarely evolve to Hodgkin lymphoma (HL), in less than 1% of patients and is usually associated with Epstein-Barr virus infection and prior treatment with fludarabine. We present 3 CLL cases that developed HL during the course of their disease. The first patient was a 59-year-old

male who was diagnosed with Binet stage A CLL. He was asymptomatic and was followed for three years without treatment, when he presented with prolonged fever and abrupt enlargement of a single cervical lymph node. Biopsy disclosed classical HL. He received 8 cycles of ABVD chemotherapy and entered a complete remission. CLL progressed four years later for which he was treated with chlorambucil initially, followed by fludarabine with achievement of partial response. Interestingly, one year later he developed lung cancer, which was managed with surgery and chemotherapy. He is currently alive with stable CLL, active lung cancer and no evidence of HL. The second case refers to a 56-year-old female who was diagnosed with familial Binet stage B CLL and was treated with chlorambucil 3 years after diagnosis. One year after the treatment she presented with fever, abdominal lymphadenopathy and progressive cytopenias. Lymph node biopsy revealed EBV negative classical HL. She underwent therapy with 6 cycles of ABVD and achieved a complete response. She is currently alive with stable CLL and no evidence of HL. The third patient was a 59-year-old man who presented with Binet stage A CLL. Initial treatment was chlorambucil, while he received fludarabine and CVP for subsequent relapses. He developed a bulky paravertebral mass 107 months after CLL diagnosis and 18 months after last treatment. A CT-guided biopsy showed transformation of CLL to classical HL. Bone marrow was also infiltrated by Reed-Sternberg and Hodgkin cells. He received 6 cycles of ABVD with achievement of complete response. Due to increasing lymphocytosis he continued treatment for his CLL and two years later he died due to sepsis. Three cases of CLL transformation to HL are presented. All three were relatively young and their disease had a rather indolent course. At transformation, all three patients presented with isolated bulky lymphadenopathy, while 2/3 had general symptoms. HL developed 3-9 years after CLL diagnosis. Only one patient had received prior therapy with fludarabine, while one received no prior treatment. All three were treated with ABVD and remained disease free from HL. Prognosis of HL seems to be favorable compared to DLBCL transformation of CLL.

Diagnosis

2.1

PITFALL IN MULTICOLOUR FLOW CYTOMETRY EXPERIMENTS: APC-TANDEM DYES ARE DEGRADED THROUGH A CELL-DEPENDENT MECHANISM

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Flow cytometry methodology allows simultaneous measurement of distinct fluorescent and morphological parameters. Development of multicolour systems provides a large amount of information at the single cell level. Application of multicolour panels is particularly rewarding in clinical settings for routine diagnosis, prognosis evaluation and minimal residual disease monitoring in numerous diseases such as lymphoproliferative disorders and mainly Chronic Lymphocytic Leukemia. Therefore, there is an increased request for new dyes that may be used simultaneously. Performance of multiparametric analysis has been significantly enhanced with the introduction of the APC-based tandem dyes such as APC-Cy7 (allophycocyanin-cyanine7) and its analog APC-H7 (allophycocyanin-Hilite®7-BD) that exhibit similar spectral properties. However, these APC-tandem dyes allow the detection of a specific signal in the APC-Cy7/H7 channel along with an unexpected non-specific signal in the APC channel. Depending on the magnitude of the latter, it may be a drawback for interpreting the data of multicolour labelling experiments. In this study, presence of such APC signal is referred to as "decoupling phenomenon". We investigated the alteration of the APC-tandem dyes by labelling peripheral blood cells with antibodies directed toward surface proteins and by analysing APC signal on cells by flow cytometry. First, a comparison between APC-Cy7 and APC-H7 conjugated anti-CD45 antibodies showed that basal percentage of decoupling was higher for the APC-Cy7 dye than for APC-H7 fluorochrome. However, ratios of decoupling of both APC-tandem dyes had a similar variation over time. As targeting cell subsets on CD45 intensity allows discrimination between lymphocytes, monocytes and polymorphonuclear cells, we observed that APC-tandem fluorochrome alteration occurred when APC tandem dye conjugated antibodies were bound to cells but not when they were captured on passive compounds such as beads (BDcompbeads®). Moreover, APC-Cy7/H7 tandem dye alteration appeared cell type-dependent, being more striking on monocytes than on polymorphonuclear or lymphocytes. Furthermore, it also occurred on lymphocytes, regardless of targeting B or T lymphocyte surface markers. Then, we were able to enhance APC tandem dye degradation on leukocytes or trigger it on passive compounds in presence of hydrogen peroxide. In order to antagonize APC tandem degradation, we decreased the metabolic activity of labelled cells. We observed a strong inhibition of APC-Cy7/H7 tandem dye degradation in presence of a fixative buffer, when cells were incubated at low temperature or in presence of sodium azide. Finally, as our data suggested a role for the presence of free radicals in the APC-tandem degradation, we tested vitamin C for its free-radical scavenger properties. Our results showed protective effect of 1 mM vitamin C preventing apparition of the abnormal APC signal in mono- and multi-parametric flow cytometry. This study demonstrates that APC-Cy7/H7 tandem dyes are the target of cell-dependent degradation; the decoupling phenomenon is enhanced by addition of hydrogen peroxide and may be antagonized by using different reagents and procedures. Based on our data, we provide flow cytometry users with guidelines for design and optimisation of their multicolour panels containing APC-tandem dyes.

2.2

EYELID SWELLING AND CHRONIC LYMPHOCYTIC LEUKEMIA

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CLL can infiltrate any organ. Nevertheless, involvement of ocular adnexa seems rare. Case report 1 A 54 year old man consulted because of a progressive swelling of both eyelids. The blood results revealed the

diagnosis of CLL (CD38 pos, unmutated Ig VH genes, t(12), 17p13 and 13q14 deletion). Ophthalmologic examination confirmed the eyelid edema without masses in eyelids or lacrimal glands. CT imaging showed nodular infiltrative masses around the eye muscles with palpebral and periorbital soft tissue swelling, Subsequent treatment with fludarabine-cyclophosphamide, local irradiation (2x2Gy) and alemtuzumab did not ameliorate the palpebral oedema significantly. A temporary resolution of the eyelid swelling was seen after dexamethasone. Afterwards the patient entered a trial with alvocidib. The eyelid swelling disappeared during the first two treatment cycles without recurrence during the following 4 cycles. Case report 2 A 69 year old man complained of palpebral swelling 15y after the diagnosis of CLL was made. Prognostic markers: CD38 pos, mutated Ig VH genes VH3-21, 11q22 and 13q14 deletion. Ophthalmologic examination confirmed the eyelid swelling caused by expansion of the lacrimal glands. Infiltration of the pars orbitalis and palpebralis was confirmed by CT imaging. Because the eyelid swelling was socially unacceptable involved field irradiation (2x2Gy to each eye) was given with a complete resolution of the swelling. Case report 3 A 52 year old woman consulted the ophthalmologist because of epiphora and proptosis. After excluding hyperthyroidy a biopsy was taken from the soft tissue mass lying against the lateral orbita seen on CT imaging. Histology and immunohistochemistry revealed the diagnosis of a small lymphocytic lymphoma. Staging for CLL showed a monoclonal B-cell lymphocytosis in the blood of 1000/μL, CD38 neg, mutated Ig VH genes, t(12) and 13q14 deletion. Because of progressive proptosis treatment was started with chlorambucil and ofatumumab. A rapid and complete resolution of the soft tissue mass was seen. Discussion Ocular adnexal lymphoma is not frequently seen. In a large pathology study (353 cases) of ocular adnexal lymphoma (conjunctiva, eyelids, lacrimal gland and orbital soft tissue), extranodal marginal zone lymphoma constituted 52%, follicular lymphoma 23%, mantle cell lymphoma 5% and small cell lymphocytic/chronic lymphocytic leukemia only 4%. SLL/CLL in ocular adnexa can be diagnosed as part of a disseminated disease at diagnosis, at relapse but also as an isolated manifestation of the disease. The last two years three patients were seen at our institution with CLL infiltration of ocular adnexa. In 2 of the 3 patients the orbital swelling led to the diagnosis of CLL.

2.3

AN UNUSUAL WAY TO DIAGNOSE A SMALL LYMPHOCYTIC LYMPHOMA

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Introduction. A small lymphocytic lymphoma (SLL) was unexpectedly diagnosed following acute urinary retention (AUR). *Case report*. A previously healthy 64-year-old man presented to emergency room because of AUR. A rectal examination revealed a hypertrophy of the prostate and a pelvic ultrasound examination found a significant residual urine amount in the urinary bladder. A fully transurethral prostate resection (TURP) was done. *Results.* The histpathological examination of the prostate demonstrates a hyperplasia but also a focal infiltration of small and mature lymphocytes, $CD20^+/CD79a^+/CD5^+/CD23^-$. An immunophenotyping of peripheral blood (the complete blood count was normal, and the absolute lymphocyte number was normal) demonstrates the presence of a monoclonal population compatible with a variant of CLL/SLL (39% of lymphocytes): $CD19^+/CD5^+$/kappa$^+$/ $CD23^-/CD20^+/CD43^-/CD38^+/FMC7^-$. *Conclusion*. Acute urinary retention was the presenting symptom of an unexpected SLL. When lymphocytic infiltration is found concomitant with hyperplasia in the histopathological examination of the prostate, the presence of leukemia/lymphoma is known; not in this case, where the presence of SLL followed the AUR and TURP. It is not clear if the trigger of AUR was benign hyperplasia of the prostate, lymphocytic infiltration or both of them. Once considered a rare event, now the infiltration of prostate by leukemic lymphocytes is a well known fact in patients with CLL/SLL. The diagnosis of CLL/SLL following a prostatectomy, is a very rare event.

2.4

RECEPTOR-DERIVED CHRONIC LYMPHOCYTIC LEUKEMIA DIAGNOSED SHORTLY AFTER KIDNEY TRANSPLANTATION ON PROTOCOL BIOPSY

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Three months after renal transplantation, a 68-year-old man underwent a routine protocol kidney biopsy. Surprisingly, histological examination revealed infiltration with a small $CD5+CD10-CD20^+CD23^+$ $FMC7^-CD79b^-$ lymphoid population, which was also found in the bone marrow. Further prognostic stratification revealed isolated del13q14 by FISH, weak CD38 expression and a mutated IGVH status. Diagnosis of early stage CLL was made. As the patient had a normal peripheral blood count, no bone marrow examination was performed in the pre- or peritransplant period. Analysis of STR and the amelogenin locus in the bone marrow aspirate revealed only one profile of recipient origin. In order to definitely prove recipient derived CLL, we explored the status of IGH and IGK rearrangements in a sample posttransplant and in one taken before transplant. Identical rearrangements were found in both samples (Figure 1). This unequivocally establishes that the CLL was already existing before transplantation. Except for reduction of immunosuppression, a watch and wait approach was initiated. Almost three years after the diagnosis of CLL the patient remains in a good condition, without evidence of progressive disease and without signs of rejection of the transplant kidney. In case of no response after reduction of immunosuppression (RIS) or of very aggressive presentation, transplantation related lymphomas (PTLD) are usually treated with the monoclonal anti-CD20 antibody rituximab either in monotherapy or in combination with chemotherapy. In case of localized disease radiotherapy and surgery can be very effective treatment options. In our patient however, some of these therapeutic modalities would have had devastating consequences. Surgery especially seems to be very effective in case of isolated allograft localization. Since CLL is per definition a disseminated disease, removal of the transplant kidney was contra-indicated and would have returned the patient unnecessarily to a state of dialysis-dependency. Moreover, re-transplantation would have probably led to recurrence in the re-transplanted kidney. The combination of chemotherapy and immunotherapy is the standard of care in patients with CLL necessitating treatment. However, based on clinical staging and taking into account new prognostic markers, chemotherapy would have subjected our patient to unnecessary complications without proven impact on survival and to worsening of the graft function. Monotherapy with rituximab at conventional dose has proven minimal efficacy in CLL, due to the low density of CD20 antigen on CLL cells. Although treatment with rituximab has a low toxicity profile, this treatment would have not been justified in this case because of the expected low response rates. *Conclusion.* We describe the case of a renal transplant patient without apparent hematological history, showing diffuse allograft infiltration by a monoclonal small B-cel lymphoid population, leading to a diagnosis of receptor-derived CLL 3 months after transplantation. Despite the clinical context with profound immunosuppression, differential diagnosis with PTLD is extremely important in order to avoid unnecessary devastating treatment. This case underscores that it is extremely important to distinguish between a pre-existing lymphoma diagnosis after transplantation and a true PTLD as the treatment options of both can be very divergent.

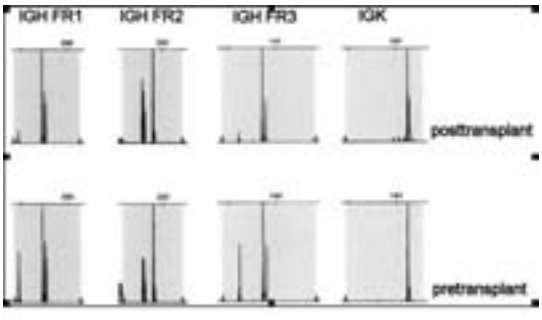

Figure 1. The status of IGH and IGK rearrangements were determined on a diagnostic blood sample obtained after transplant, and a stored pretransplant blood sample, as described in Leukemia 2003;17:2257–17. doi: 10.1038/sj.leu.2403202.

2.5

LEPTOMENINGEAL INVOLVEMENT IN CHRONIC LYMPHOCYTIC LEUKEMIA TREATED WITH DEPOCYTE

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Leptomeningeal involvement in patients with CLL is relatively rare and the prognosis is usually considered to be poor. We report a case of 73 years old male with 10 years history of untreated stage 0 CLL who presented with sudden onset of diplopia , left facial paralysis and numbness. On the basis of CSF cytology and flow cytometry (Blymphocytic population expressing CD19, DR,CD5, CD23, CD20, CD22 with k light chain restriction) the diagnosis of CLL leptomeningeal involvement was made. The tests performed on the peripheral blood (WBC count 60x10^9)\L) were remarkable for. - ZAP 70 protein detected on 55% of cells - Del (13q) detected by FISH - Unmutated IgV H MRI showed enhancement inside the left internal acoustic canal and the horizontal and descending facial nerve. Post-contrast T1 weighted images also demonstrated enhancement surrounding the anterior border of the midbrain. The patient was treated with intratecal Depocyte (50 mg x 3 times, every 21 days) together with systemic chemioimmunotherapy (Rituximab, Fludarabine, Cyclophosphamide). A complete clearance of CSF lymphoid cells was observed after the 1 st Depocyte administration; MRI performed after the 3 rd intratecal therapy showed diffuse enhancement of meninges and tentorium cerebelli. There was no enhancement of acoustic canal, the facial nerve and the midbrain. Despite the good response to systemic and intratecal therapy the patient, in poor general conditions, died of respiratory failure due to pneumonia.

2.6

CD19 COUNT IN A PRIMARY CARE SETTING IS USEFUL TO DISCRIMINATE B-CELL LYMPHOPROLIFERATIVE DISORDERS (B-CLPD) FROM BENIGN CAUSES OF LYMPHOCYTOSIS

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Background. The follow-up of an increased absolute lymphocyte count (ALC) of unknown aetiology consists of complete immunophenotyping, which leads to unnecessary referrals for complete immunophenotypings, high costs and patients discomfort, since most patients do not have a B-cell chronic lymphoproliferative disorder (B-CLPD), but a benign cause. Therefore, a simple, first line laboratory test, which can distinguish malignant from benign causes of lymphocytosis and thereby reduce the need for further complete immunophenotyping, would be of great value. Furthermore, in patients with a malignant cause, this test may provide early haematological consultation to enable risk-stratification early in the course. *Aims.* Whether CD19 count, a simple first line test, can distinguish malignant from benign causes of lymphocytosis in order to improve the diagnostic follow-up of patients with an abnormal white blood cell count (WBC) and reduce unnecessary referrals for further complete immunphenoptying. *Methods.* In the period from March 2001 until October 2006, a CD19 count was performed in all patients, living in a previously defined geographic area, aged ≥40 years with an abnormal White Blood Cell count (WBC), defined as ALC ≥6.0x10^9 cells/L, relative lymphocyte count (RLC) ≥60% or atypical lymphocytes (AL) ≥2+. Patients with positive EBV or CMV serology were excluded. CD19 counts were classified as normal (<1.0x10^9/L) or increased (≥1.0x10^9/L). In case of an increased test, referral to a haematologist for further complete immunophenotyping was advised. A geographic area in which the contingency areas of the laboratory and the referral hospitals overlapped was based on postal codes in order to link patients in whom a CD19 count was performed to newly diagnosed patients with B-CLPD in the hospitals. In October 2007, all cases of B-CLPD in this area were identified by a stepwise search-strategy with a survey among general practitioners, including two postal questionnaires and a telephone reminder and by consultation of three different hospital-based databases. All data were collected anonymously and CD19 count cases and B-CLPD cases were cross-referenced by postal code and date of birth. *Results.* A CD19 count was performed in 543 patients of 199,108 (0.3%) patients in whom a WBC was performed; 229(42%) were increased and 314 (58%) were normal. In total, 142 patients with a B-CLPD were identified; 139 in the increased group en 3 in the normal group. This resulted in a sensitivity and Positive Predictive Value (PPV) of 98%(95%CI 0.94-1.00) and 61%(95%CI 0.54-0.67) respectively and specificity and Negative Predictive Value (NPV) of 78%(95%CI 0.73-0.82) and 99%(95%CI 0.97-1.00) respectively. CD19 count improved the diagnostic follow-up of patients with lymphocytosis with a significantly better PPV and AUC of 61% and 0.92(95% CI 0.90-0.94) respectively compared to the PPV and AUC for ALC of 40% and 0.85(95% CI 0.81-0.88) respectively. CD19 count reduced unnecessary referrals for complete immunophenotyping with 37%. *Conclusion.* CD19 count improves the diagnostic follow-up of lymphocytosis and saves unnecessary referrals for further complete immnophenotyping.

2.7

WATCH AND WAIT APPROACH STILL EFFECTIVE IN THE INITIAL MANAGEMENT OF CLL

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Despite advances in the understanding of its biology Chronic Lymphocytic Leukemia remains an incurable disease. Despite a panoply of diagnostic and prognostic criteria such as analysis of molecular cytogenetics, immunoglobulin heavy-chain variable region mutations and ZAP-70 it is not evident that early treatment improves prognosis. As such extensive and expensive correlates of prognosis should be reserved for the setting of clinical trials. Most patients with progressive CLL declare themselves with a rapid rise in white blood cell count or palpable adenopathy or the development of symptoms. An analysis of data from a large hospital experience in Barcelona failed to demonstrate a survival improvement in older subjects and in those patients with lower risk disease.[1] In a multivariate analysis the only clinically significant parameter of survival were lymphocyte doubling time and other signs of active disease.[2] A conservative approach is still warranted in the great majority of patients diagnosed with CLL

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2.8

PILOT STUDY VALIDATING A NEW SCORING SYSTEM IN B-CELL CHRONIC LYMPHOCYTIC LEUKAEMIA UTILISING THE NK CELL ANTIGEN CD160

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The diagnosis of many chronic B-cell lymphoproliferative disorders (B-LPD) is based on the integration of characteristic morphology, immunophenotype and cytogenetic analysis. However, there is a degree of diagnostic uncertainty if the morphology and/or immunophenotype are atypical. Furthermore, cases of B-cell lymphocytic leukaemia (CLL), mantle cell lymphoma (MCL) and monoclonal B cell lymphocytosis (MBL) share many features in common. Throughout the past decade, considerable progress has been achieved in defining new prognostic markers and treatment options in B-cell malignancies. However, over the same period, there has been only limited work performed on the diagnostic aspects of distinguishing CLL from other B-cell malignancies. This pilot study included 163 consecutive cases of B-cell malignancies (CLL n=113; CD5- B-LPD n=13; CD5+ B-LPD n=10; MCL n=8; hairy cell leukaemia [HCL] variant n=5; HCL n=3; diffuse large B-cell lymphoma [DLBCL] n=3; splenic marginal zone lymphoma [SMZL] n=3; and lymphoplasmacytic lymphoma [LPC/WM] n=3; follicular lymphoma [FL]

n=2). Samples were analysed using multicolour flow cytometry and sequential gating strategies. Same day analysis for all antigens was performed. 95% (107/113) of CLL cases were positive for the CD160 antigen. 7% of the confirmed CLL cases had an atypical phenotype and Marsden CLL score of <3, suggestive of an alternative diagnosis to CLL. However, 75% (6/8) of these cases were CD160 positive. Using the three most robust diagnostic markers of CLL - CD5, CD23 and CD160, with each scoring one point - a 'mini CLL score' was validated against the 5 point scoring system developed by the Royal Marsden in 1997. A mini CLL score of 3 was diagnostic of CLL and excluded MCL in this cohort of 163 cases. However, 7 CLL cases with a mini CLL score of 2 could not be distinguished from other B-cell malignancies, especially $CD23^+$ MCL. By combining the mini CLL score with the ratio of CD23 to CD5 expression on malignant B-cells complete differentiation between CLL and MCL was achieved. The near universal expression of CD160 in CLL allows a simplified and accurate immunophenotypic diagnosis of B-LPD. CD160 expression in CLL had a sensitivity and specificity of 95% and 84%, respectively. Combining the CLL score with the CD23:CD5 ratio provides a complete discrimination between CLL and MCL, including cases of $CD23^+$ MCL. This study validates the previously reported expression of CD160 antigen in CLL and further justifies its use in the diagnosis of B-cell malignancies.

Table 1. Mini CLL score results by final diagnosis.

	mini CLL score'				
	0	*1*	*2*	*3*	*TOTAL*
CLL	0	0	7	106	113
MCL	0	6	2	0	8
HCL	0	1	2	0	3
SMZL/LPC/WM	5	5	1	0	11
Other BLPD	7	15	6	0	28
Total					163

Table 2. Global performance of the 5 point CLL Royal Marsden Scoring system against the mini CLL score.

	CLL Score	*Mini CLL Score*
Sensitivity	0.93	0.92
	(0.87 - 0.97)	(0.85 - 0.96)
False positive rate	0	0
	(0 - 0.07)	(0 - 0.07)
Diagnostic odds ratio	∞	∞
	(129 - ∞)	(116 - ∞)

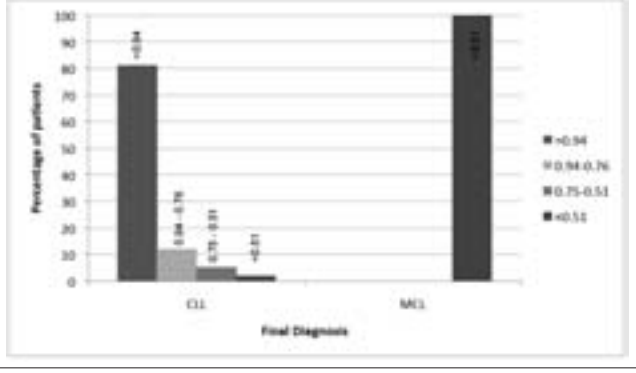

Figure 1. CD23 ratio analysis for differentiating between CLL patients and MCL patients. The $CD5^+23^+/CD5^+19^+$ ratio for further differentiating atypical cases of B-cell Chronic Lymphocytic Leukaemia and Mantle Cell Lymphoma with aberrant CD23 expression.

2.9

CHRONIC LYMPHOCITIC LEUKEMIA WITH TRISOMY 12 ASSOCIATED WITH T(14;18) OR TRISOMY 18

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Introduction. Chronic lymphocitic leukemia is a lymphoproliferative disorder. This is the most common leukemia of the adult population in Western countries. The mean age at diagnosis is 65 years and its incidence is about 2-6 cases per 100.000 person per year. Chromosomal abnormalities commonly found using conventional and molecular cytogenetic are trisomy 12, del(13)(q14), del(11)(q22-23), del(17)(p13) and del(6)(q21). Trisomy 12 is present in 20% of the cases. This abnormality is found meanly in atypical CLL. Some studies have reported the presence of trisomy 12 associated with other chromosomal abnormalities as t(14;18)(q32;q21) and trisomy 18. The t(14;18)(q32;q21) has been identified in 90% of follicular lymphomas and 20-30% of diffuse large B-cell lymphoma, but is rare in CLL. When this translocation is present, BCL2 gene (18q21.33) has a constitutive expression. This gene encodes an integral outer mitochondrial membrane protein that blocks the apoptotic death of some cells, such as lymphocytes. On the other hand, in trisomy 18, the BCL2 gene has three copies, being this oncogene overespressed. The immunophenotype in B-CLL shows the markers: CD20, CD22, CD5, CD19, CD79a, CD23, and CD43. The CD10 marker is expressed in of follicular lymphoma and is not expressed in CLL. The expression of ZAP70 and CD38 markers are both associated with a poor prognosis in CLL. *Objective.* The aim of this study was to describe the clinical and pathologic features of CLL with trisomy 12 associated with t(14;18)(q32;q21) or trisomy 18. *Materials snd Methods.* Five peripheral blood samples patients with CLL (three with atypical CLL and two with typical CLL) were studied using conventional and molecular cytogenetic. The karyotype and/or FISH studies revealed trisomy 12 + t(14;18)(q32;q21) in two cases and trisomy 12 + trisomy 18 in three cases. The ratio male: female is 1.5:1, three males with trisomy 12 + t(14;18)(q32;q21) and two females with trisomy 12 + trisomy 18. Four samples were analyzed at diagnosis and one at follow-up. The immunophenotype (CD20, CD22, CD5, CD19, CD79a, CD23, CD43, CD10, ZAP70 and CD38) was studied by flow cytometry in all the cases, except of the CD10, that was studied in three cases. *Results.* Flow cytometry analysis showed: in cases with trisomy 12 + t(14;18)(q32;q21),the ZAP70 marker was negative and CD38 was positive. CD10 was negative in 2 cases. The cases with trisomy 12 + trisomy 18 were expression CD38 and were not expression ZAP70. CD10 was negative expression in 1 case. Two patients were followed-up, one with trisomy 12 + t(14;18)(q32;q21) and one with trisomy 12 + trisomy 18, and none of them progressed. *Remarks.* CD38 was found to be positive and ZAP70 negative in all the 5 CLL patients included in the study.

2.10

IMMUNOPHENOTYPE MARKERS IN CHRONIC LYMPHOCITIC LEUKEMIA WITH TRISOMY 12 AND DELETION 13Q14.3

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Introduction. B-cell chronic lymphocytic leukemia is characterized by slow accumulation of neoplasic lymphocytes in the peripheral blood and highly variable clinical course. Conventional cytogenetic shows chromosomal abnormalities in 40-50% of the cases and fluorescence in situ hybridization (FISH) detected alterations in 80%. The most common chromosome abnormalities in CLL are 13q14.3 deletions, followed by trisomy 12 and 11q22-q23, 17p13 and 6q21 deletions. Using conventional cytogenetic the most common abnormality is trisomy 12 and by FISH analysis is 13q14.3 deletion. These aberrations can be found with as single chromosomal abnormalities or associated with further aberrations. Trisomy 12 is considered as a late event, mainly a secondary event, in the pathogenesis of B-CLL; however some studies reported that trisomy 12 can also be found in early stage. This aneuploidy correlates with atypical morphology and/or atypical immunophenotype (CD5- or $CD23^+$, $FMC7^+$ or $CD11c^+$, strong sIg or $Cd79b^+$). Deletion of 13q14.3 is associated with good prognosis and long time to progression. *Objective.* The aim of this study was to explore the differently immunophenotype expression in CLL with trisomy 12 and deletion 13q14.3. *Material and Methods.* One hundred sixty five patients of B-CLL were included in the study. The ratio male: female was 1.9:1 and the mean age was 63. Peripheral bloods were analyzed by FISH, using the following probes: CEP12, LSI D13S319 (13q14.3)/LAMP1 (13q34), LSI ATM and LSI TP53. The immunophenotype (CD20, CD22, CD5, CD19, CD79a, CD23, CD43, CD10, ZAP70 and CD38) was studied by flow cytometry in most of the cases. *Results.* Patients were distributed in three groups: A) trisomy 12 (20%), B) deletion 13q14.3 (55%), C) trisomy 12 + deletion 13q14.3 (6%). Patients with trisomy 12 found as a single abnormality (n=18), associated with deletion 13q14.3 (n=10) and with other abnormality (n=6). Patients with deletion 13q14.3 found as a single abnormality (n=55) and associated with other abnormality different of trisomy 12 (n=26). In the group of patients with trisomy 12 as a single aberration the immunophenotype showed expression of ZAP70, CD38, CD79b, FMC7 and Lambda in 92%, 66%, 94%, 61%, 55,5% of the cases, respectively. In the group of patients with deletion 13q14.3 as a single abnormality the immunophenotype showed expression of ZAP70, CD38, CD79b, FMC7 and Kappa in 33%, 14%, 55%, 55%, 62% of the cases, respectively. In the group of patients with trisomy 12 + deletion 13q14.3 the immunophenotype showed expression of ZAP70, CD38, CD79b, FMC7 and Kappa in 60%, 70%, 100%, 100%, 70% of the cases, respectively. *Conclusion.* We were found that in 6% of patients with deletion 13q14.3 was associated with trisomy 12. The most frequently abnormalities were deletion 13q14.3 (55%) and trisomy 12 (20%) either as a single abnormality or associated with other. ZAP70 was more expressed in the cases with trisomy 12. CD38 was more expressed in the cases with trisomy 12 + deletion 13q14.3. CD79b and FMC7 were expressed in all the cases with trisomy 12 + deletion 13q14.3.

2.11

APPLICATION OF THE 2008 I WCLL CRITERIA FOR THE DIAGNOSIS OF CHRONIC LYMPHOCYTIC LEUKEMIA: CORRELATION WITH CLINICAL OUTCOME

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Aims. 1.-use of the new diagnostic criteria of the IWCLL group to a series of patients diagnosed with CLL , Binet stage A at our institution in the last ten years, 2.- to analyze the prognostic value of the absolute B-lymphocyte count (BLC) in the CLL in Binet stage A, compared to other well-know prognostic parameters for progression. *Patients and methods.* We included 308 patients previously diagnosed of CLL according to the 1996 NCIWG criteria. The BLC was calculated from flow cytometric data. Median age of the patients was 71 years (range, from 34 to 90), with a 54% of males (165 out of 308 patients). *Results.* median follow-up of the series was 53.1 months (from 0.7 to 161.67= .The correlation between the absolute lymphocyte count and the B-cell count was r=0.884 (p=0.0001). 90 patients out of the 308 (29%) were classified as monoclonal B cell lymphocytosis (MBL). The main clinical characteristics of patients are detailed in Table 1. The best threshold of BLC for time to treatment was $10.8x10^9$/L, which correlated with a total lymphocyte count of $16x10^9$/L. Among the patients included in the MBL with the new criteria, we did not identify an optimal threshold clearly associated with a treatment requirement. In the multivariate analysis for the time to treatment, in the 90 patients reclassified as BLM, only the lymphocyte doubling time (LDT) retained an independent prognostic value with a hazard ratio of 18.9 (4.9 to 72.3, p<0.0001). In the CLL group, the LDT (HR 20.23) as well as the cytogenetics (p53 del, HR 3.84), 11qdel (HR 1.28) and trisomy 12 (HR 1.12) retained an independent prognostic significance. *Conclusions.* our experience support the threshold of $5x10^9$/L BLC as a better predictor of progression although a higher B-cell count ($11.6x10^9$/L) best predicts time to first treatment. In our experience, the lymphocyte doubling time is still a useful prognostic parameter for disease progression, even in the group of BCM.

Table 1.

	B-cell >=5x10⁹/L LLC-B A	*B-cell x10⁹L LBM*	*p*
Median age n=308 *	68 (34-90)	70 (42-88)	NS
Lymphocyte count x10⁹/L*	24.2 (7.8-149)	12 (5-16.9)	0.0001
BLC x10⁹/L*	9.38 (5.04-69)	3.64 (1.53-4.98)	0.0001
β2 microglobulin*	2.2 (0.1-8.9)	1.9 (0.1-8.6)	0.004
CD 38 positive (>20%)*	57/218 (26%)	25/45 (55%)	NS
ZAP 70 positive (>20%)	29/74 (39%)	31/90 (34%)	NS
LDT <1 year n =251 %**	24/157 (13.3%)	3/70 (4.35%)	0.04
Patients treated %*	65/218 (29.8%)	11/62 (12.2%)	0.001

2.13

IMMUNOPHENOTYPIC AND MOLECULAR STUDY OF A PARTICULAR EXPRESSION PATTERN OF CD13 EPITOPE 7H5 IN CHRONIC LYMPHOCYTIC LEUKEMIA

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CD13/aminopeptidase N (APN) is a membrane-bound, zinc-dependent metalloproteinase that plays a key role the control of cell growth and differentiation, as well as tumor invasion and angiogenesis, that might be strongly modified by APN inhibitors, such as the phenolic natural product curcumin. CD13 has been widely applied in leukaemia phenotyping. However, it is rarely reported in mature lymphoid malignancies and is generally correlated to poor prognosis. We developed a monoclonal antibody (Mab) 7H5 recognising a new CD13 epitope. To assess its diagnostic value, the binding pattern of 7H5 was compared to the reference CD13 antibody LeuM7 by direct immunofluorescent staining and flow cytometry and to CD13 mRNA expression detected by multiplex RT-PCR on samples from a total of 50 patients with chronic lymphocytic leukemia (CLL), as well as in 85 pts with other precursor and mature lymphoid neoplasms. Interestingly, we detected 7H5 in a significant proportion of B-CLL – 42/50 pts (84%) while LeuM7 was detected in only 10/50 pts (20%). Similar ratio was observed in other mature B-cell lymphomas – 7H5 was positive in 21/31 pts (74%) vs LeuM7 in 4 pts (13%). LeuM7 and 7H5 Mabs stained B-ALL discordantly: 7H5 in 7/37 pts (19%) and LeuM7 in 10/37 pts (27%) resulting in a total incidence of 43%. All 17 T-cell lymphoid neoplasms were negative for both Mab, except one. Molecular analysis for CD13 transcripts confirmed these data, resulting in a specific RT-PCR product in all CD13(+cases, stained either with 7H5, LeuM7 or both. Besides, *in vitro* studies of the concentration-dependent cytotoxic activity and drug interactions of curcumin with fludarabine and bendamustine in cells isolated from the blood of patients with CLL were performed. In conclusion, CLL cells are characterized by a high incidence of CD13 (APN) membrane expression. CD13 epitopes may be variably expressed by lymphoid malignancies probably reflecting different maturation/functional characteristics of malignant cells. The extensive characterization of CLL cells offers the

possibility for novel therapeutic approaches targeting tumor-specific pathways. Acknowledgement: Studies were funded by the National Research Fund, Bulgarian Ministry of Education and Science.

2.14

CLL-TYPE MONOCLONAL B-CELL LYMPHOCYTOSIS: DIFFERENCES IN MOLECULAR BIOLOGY, IMMUNE DISFUNCTION AND RISK OF DISEASE PROGRESSION ACCORDING TO PRESENTATION CLL CELL COUNT

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CLL-type Monoclonal B-cell lymphocytosis (MBL) is defined as the presence of circulating B-cells with restricted immunoglobulin expression, a CLL-like immunophenotype, an absolute B-cell count below 5,000 cells/μL and no other features of CLL. CLL-type MBL is detected in more than 10% of individuals with a lymphocytosis and also in more than 5% of adults from the general population although with a much lower absolute CLL cell count. We have reviewed data from 2196 individuals from the general population, of which 225 had CLL-type MBL, and 580 CLL-type MBL from a hematology clinic setting to examine the relationship between biology, clinical outcome and absolute CLL cell levels. Although it is difficult to identify discrete groups according to presentation CLL cell count, there are apparent biological and clinical differences between cases with less than 100 CLL cells/μL and those with more than 2000 CLL cells/μL. Over 95% of CLL-type MBL cases in the general population have an absolute CLL count below 100 cells/μL. Using 6/8-colour flow cytometry, the prevalence in the general population is 5-20% depending on assay sensitivity, race/ethnicity, and study age group. Cases with such a low count are rarely detected during routine hematology investigations (incidence <0.02/100,000 person-years). Deletions of 13q14 were frequent, even in cases with <1 CLL cell/μL. The IGHV repertoire is different to that seen in clinical CLL, and cases are often oligoclonal rather than monoclonal. The normal B cell compartment is otherwise preserved. The annual risk of developing progressive CLL is likely negligible. MBL with a CLL count between 100 and 2000 cells/μL is detected in approximately 1-2% of the general Caucasian population aged over 40 years. The incidence of new cases detected during routine hematology investigations is 0.8/100,000 person-years. Chromosomal abnormalities and IGHV repertoire are similar to IGHV-mutated CLL with a predominance of IGHV3-07, IGHV3-23 and IGHV4-34. Oligoclonal cases are infrequent. More than 25% of cases have reduced numbers of residual normal B-cells. The CLL count is typically stable but a small proportion (<5%) develop progressive disease, often with a rapid doubling time. Repeat flow cytometry analysis in 6-12 months is more informative than the total lymphocyte count in such cases. CLL-type MBL with a CLL count above 2000 cells/μL is not reported in the general population, although this may reflect screening bias. The majority (>70%) of MBL cases detected during investigations for an absolute peripheral lymphocytosis have a CLL count above 2000 cells/μL (incidence 1.9/100,000 person-years). The molecular features are similar to IGHV-mutated CLL. Most cases have depletion of residual normal B-cells and display a rising CLL cell count with time. It has been estimated that about 1% of MBL patients progress annually to CLL that requires treatment. The most significant healthcare issues are anemia, which by definition is unrelated to MBL, and immune disfunction, of which the normal B-cell or T-cell dysregulation may be related to the MBL. The absolute CLL count identifies groups of CLL-type MBL cases with different biology and risk of developing progressive disease.

Molecular biology

3.1

SNP-BASED MAPPING OF B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA REVEALS THE ROLE OF 2P GAIN IN PREDICTING CLINICAL OUTCOME

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B-cell chronic lymphocytic leukemia (B-CLL) is a genetically heterogeneous disease with a variable clinical course. Chromosomal changes have been identified by FISH in approximately 80% of patients, and specific lesions, such as trisomy 12 and 13q14, 11q23 and 17p deletions have been proven to be prognostic markers for disease progression and survival. To identify novel molecular lesions in B-CLL and test their independent prognostic value we investigated a panel of highly purified neoplastic cells (>90%) from 100 untreated, newly diagnosed patients (Binet stage A). This series was characterized by FISH for the most recurrent genomic aberrations and for the major prognostic markers. Genome-wide profiling data were generated by means of Affymetrix GeneChip® Human Mapping 250K Nsp single nucleotide polymorphism (SNP) arrays. Copy number alterations (CNA) were calculated using the DNA copy Bioconductor package, which looks for optimal breakpoints using circular binary segmentation (CBS) (Olshen *et al.*, 2004). FISH identified the presence of trisomy 12 in 21 cases; 13q14 deletion in 44 cases (present in 34 of which as the sole abnormality); 11q23, 17p13.1 and 6q23.3 in 15, 7 and 2 patients, respectively. In addition, ZAP-70 and CD38 expression were positive in 42 and 46 patients, whereas IgVH genes were mutated in 45 patients. The genome-wide analysis allowed the identification of CNAs in all cases. We identified 782 copy number alterations (CNAs) (range 1-31 per sample; mean 7.8, median 7). DNA losses were more frequent than gains (loss 365/782=46.7%; biallelic deletion 194/782=24.8%; gain 148/782=18.9%; amplification 75/782=9.6%). Additionally 18 minimally altered regions (MARs), defined as those larger than 100 kb with a frequency of more than 5%, were found. Besides well-known alterations such as trisomy 12 and 17p, 11q23 and 13q14 deletions, the MARs included the gain of part if not the entire 2p arm (11 pts), and novel alterations such as gains of 4q35.2 (5 pts) and 11q25 (6 pts), and the loss of 8q24.23 (6 pts). A high frequency of losses/gains was also found at 14q11.2 and 15q11.2, two regions affected by copy number variations. The prognostic relevance of the CNAs and MARs was evaluated in terms of time to first treatment: 2p gain (HR 2.5, p=0.021) and 17p loss (HR 3.4 p=0.03) were found to be independent risk factors. Unlike 17p loss, 2p gain maintained its independent prognostic power when IgVH mutational status but not CD38 or ZAP-70 entered into the multivariate model. Our approach allowed us to identify novel recurrent aberrations affecting B-CLL. The assessment of their prognostic significance indicates the role of the 2p gain as a novel major negative prognostic factor in patients with early stage B-CLL.

3.2

INTEGRATIVE GENOMIC APPROACH IN B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA: DEFINITION OF MOLECULARLY DISTINCT SUBGROUPS OF PATIENTS WITH 13q14 DELETION

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B-cell chronic lymphocytic leukemia (B-CLL) is characterized by a highly variable clinical course that reflects its heterogeneous genomic pattern. Genomic abnormalities such as deletions of 13q14, 11q23, and 17p13, and trisomy 12 are all recognized to be common events in B-CLL with a specific role in the pathogenesis and progression of this malignancy. Hemizygous and/or homozygous loss at 13q14 has been identified as the most frequent genomic alteration in B-CLL and associated with a favorable prognosis, if found as sole abnormality. Two microRNAs genes, miR-15a and miR-16-1 located at 13q14 have been found to be deleted and down-regulated in the majority of patient with del(13)(q14). To better define the genomic complexity of B-CLL, we applied SNP array technologies (Affymetrix GeneChip® Human Mapping 250K Nsp) in a panel of 100 newly diagnosed, untreated early stage (Binet A) patients. We performed an integrative approach between whole-genome and gene expression profiling data for 60 patients (Affymetrix GeneChip® HG-U133A) for whom RNA material was available. The miR-15a/16-1 cluster DNA CN and transcription levels of mature miR-15a and miR-16 have been detected by quantitative Real Time RT-PCR (Q-RT-PCR) using a custom TaqMan® assay and TaqMan® microRNAs assays (Applied Biosystems), respectively. A clustering analysis of genomic profiles performed my means of the non-negative matrix factorization (NMF) algorithm identified four significant groups mainly driven by del(13)(q14) and trisomy 12. Notably, patients with del(13)(q14) were grouped in two separate clusters based on the presence of a biallelic loss and the extension of the deletion. The shorter monoallelic deleted 13q14 region was found to be 635 kb in length, not encompassing the miR-15a/16-1 locus. Interestingly, the miR-15a and miR-16 expression was found to be significantly down-regulated only in patients with biallelic loss. Furthermore, a multiclass supervised analysis associated with NMF groups identified a different transcriptional signatures in the two genomic subgroups with del(13)(q14). Finally, an integrative analysis of CN and gene expression levels identified 93 transcripts, mainly mapped to chromosome 12 and 13q12-q14.3, whose expression was significantly correlated with the DNA copy number. Overall, our integrative approach further supports the notion that transcription deregulation in B-CLL could be mostly due to a gene dosage effect and underscore the presence of two well defined types of patients with 13q14 deletion with potential clinical relevance.

3.3

INCIDENCE OF CYTOGENETIC ABNORMALITIES IN NEWLY DIAGNOSED BINET STAGE A B-CLL AND RELATIONSHIP WITH PROGNOSTIC BIOMARKERS: PRELIMINARY RESULTS ON 240 PATIENTS INCLUDED IN THE PROSPECTIVE, MULTICENTER O-CLL1 GISL STUDY

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CD38 and ZAP-70 expression, IgHV mutational status and genetic abnormalities have been integrated in clinical prognostic evaluation of B-CLL. We investigated the incidence of the known major cytogenetic alterations (+12 and 13q14, 17p13, 11q23 deletions) in a series of Binet A B-CLL patients included in the prospective multicenter O-CLL1 GISL trial. The study was performed by FISH in 240 out of 310 patients enrolled to date. At least one abnormality was found in 151/240 (62.9%) cases. The most frequent was del(13)(q14) (120/240, 50%), followed by +12 (30/240, 12.5%) (one case harboring 17p13 deletion), del(17)(p13) (5/240, 2%) and del(11)(q23) (11/240, 4%). 13q14 deletion was found as a sole abnormality in 110 patients; in the remaining cases, it was combined with +12 (2 pts) and 17p13 (2 pts) or 11q23 deletions (6 pts) deletion. Among patients with 13q14 deletions, 80 were monoallelic, 10 biallelic and 30 showed a combination of the two patterns. Biomarkers data were available in all of the patients. CD38 percentages were (mean value ± sem) 8.2±1.7, 16.8±2.2, 55.9±6.3, 37.2±10.4, 31.1±10.6 for del(13)(q14), normal karyotype, +12, del(11)(q23) and del(17)(p13) alterations, respectively ($p<0.0001$). The percentages of IgVH mutations significantly correlated with cytogenetic alterations; namely, 5.6±0.3 for cases with del(13)(q14), 4.7±0.4 for normal karyotype, 2.1±0.6 in +12, 0.2±0.1 in del(11)(q23), and 2.1±1.3 in del(17)(p13) cases ($p<0.0001$). Similarly, a significant correlation was found for ZAP-70 expression: namely 30±1.9 for cases with del(13)(q14), 38.8±2.7 for normal karyotype, 50.2±4.8 for +12, 59.4±9.1 for del(11)(q22) and 35.7±7.5 for del(17)(p13) ($p<0.0001$). Finally, cytogenetic abnormalities were clustered in 3 risk groups [i.e. low del(13)(q14) and normal; intermediate (+12); and high risk del(11)(q23) and del(17)(p13)] and correlated with a scoring system in which cases were stratified in 4 different groups according to the absence (group 0) or presence of 1 (group 1), 2 (group 2) or 3 (group 3) biomarkers (Morabito et al., BJH 2009). Interestingly, 117/121 cases scoring 0, gathered in the low FISH group, whereas 12/15 high FISH risk cases clustered in scoring 2-3. Our results indicate that cytogenetic abnormalities predicting unfavorable prognosis show a relatively low incidence in newly diagnosed Binet stage A B-CLL patients and are significantly associated with negative prognostic biomarkers predictive of disease progression.

3.4

DIFFERENTIAL GENOME-WIDE METHYLATION PROFILES IN PROGNOSTIC SUBSETS OF CHRONIC LYMPHOCYTIC LEUKEMIA

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Background. Chronic lymphocytic leukemia (CLL) is a biologically heterogeneous disease, characterized by accumulation of neoplastic B-cells

due to deregulation of both apoptosis and proliferation. CLL can be classified into clinical subgroups depending on the presence or absence of somatic hypermutations in the immunoglobulin heavy-chain variable (IGHV) genes. Previous investigations have shown that aberrant DNA methylation may play an important role in tumorogenesis, however, limited knowledge exists regarding the global methylation pattern in CLL. Aim: Here we analyzed global methylation profiles in 23 CLL samples, belonging to the IGHV mutated (favorable) and IGHV unmutated/IGHV3-21 (poor-prognostic) subsets. *Methods.* We applied a high-resolution, genome-wide methylation array from Illumina, covering 28,000 CpG sites and spanning 14,000 genes. The raw data was processed using the BeadStudio software followed by bioinformatic analysis where the arcsin transformed data was used in a moderated t-test to find differentially methylated genes. Only genes with a large absolute difference between the groups were included. Methylation-specific PCR and realtime-PCR were employed to verify the array data on selected genes. Furthermore, samples were treated with methyl-inhibitors to induce re-expression of methylated genes. *Results.* Overall our results demonstrated a large number of differently methylated genes between the IGHV mutated and IGHV unmutated/IGHV3-21 subgroups. Specifically, in IGHV unmutated CLL, we identified methylation of 7 tumor suppressor genes (e.g. VHL, ABI3 and IGSF4) as well as 8 unmethylated genes enhancing cell proliferation and tumor progression (e.g. ADORA3 and PRF1 enhancing NF-kB and MAPKinase pathways, respectively). In contrast, these latter genes were silenced by methylation in IGHV mutated patients. The methylation status was confirmed for 4 genes (BCL10, PRF1, ADORA3 and IGSF4) and the expression status of 7 genes was verified using real-time RT-PCR (BCL10, PRF1, ADORA3, IGSF4, NGFR, ABI3 and VHL). The degree of methylation for two methylated tumor suppressor genes (VHL and ABI3) was confirmed in unmutated CLL samples using bi-sulfite sequencing. We also re-expressed 4 genes (e.g. NGFR, ABI3, VHL and IGSF4) by inhibiting DNA methylation using the methyl-inhibitor 5-aza-2'-deoxycytidine combined with and without the HDAC inhibitor trichostatin A. *Conclusion.* Our data reveals, for the first time, distinct methylation profiles in prognostic subsets of CLL, implying an important role for epigenetic-based silencing mechanisms in the disease pathogenesis of each subset. Specific inhibition of expression of unmethylated genes involved in facilitating tumorogenesis and re-expression of methylated tumor suppressor genes within the poor-prognostic subgroups may represent potential new targets for drug therapy.

3.5

GENOME-WIDE SCREENING REVEALS A HIGH FREQUENCY OF PATIENTS CARRYING CHROMOSOMAL ABERRATIONS AND AN ASSOCIATION BETWEEN GENOMIC COMPLEXITY AND POOR OUTCOME IN NEWLY DIAGNOSED CHRONIC LYMPHOCYTIC LEUKEMIA

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Single nucleotide polymorphism (SNP)-arrays allow simultaneous detection of copy-number aberrations (CNAs) and copy-number neutral loss-of-heterozygosity (CNN-LOH). By applying this technique, the overall genetic complexity can be assessed in patients with chronic lymphocytic leukemia (CLL). Moreover, recurrent genomic aberrations, i.e. deletions of 11q, 13q, 17p and trisomy 12, which are of great prognostic value, can be identified. In this study, we applied a 250K SNP-array and screened samples from 370 newly diagnosed CLL patients from a population-based Scandinavian cohort. The median age at diagnosis was 63 years, the male:female ratio was 2:1, and two-thirds of patients carried mutated IGHV genes. Of the 370 CLL samples investigated, 333 samples (90%) carried CNAs whereas 37 samples (10%) presented a normal karyotype. The majority of samples (71%) carried between 1 and 3 CNAs, 16% showed 4-9 aberrations, whereas only 2% were highly complex, (≥10) CNAs. Losses were more commonly detected than gains (69% vs. 31%) and the median size of these CNAs was 1.4 Mbp and 0.98 Mbp, respectively. Accordingly, a large proportion (41%) of CNAs were smaller than 1 Mbp, whereas CNAs ranging between 1-5 Mbp and CNAs >5 Mbp constituted 29% each. The known recurrent aberrations were detected in 70% of samples. The most common aberration was deletion of 13q, carried by 203 patients (55%). Among patients with del(13q), 155 displayed heterozygous deletions, while 48 (24%) showed homozygous deletion. The homozygous deletions were centered at the 13q14 region and had an average size of 1.7 Mbp, considerably smaller than the heterozygous losses, which had an average size of 5.6 Mbp. The deletions of 13q covered the miR-15/16 loci in 94% of cases and the Rb encoding region in 38% of cases. Trisomy 12 was the second most common aberration, identified in 39 patients (10.5%), followed by del(11q), which was detected in 37 samples (10%). The majority of the losses on 11q had sizes of >20 Mbp, with an average length of 29 Mbp, and encompassed the ATM encoding region in all cases. Deletion of 17p was noted in 13 samples (4%), including 2 smaller deletions with sizes of 10 Mbp and 20 Mbp, covering the TP53 coding region. Loss of 6q, which is considered as a progression marker in CLL, was only detected in 2 samples (0.05%). In addition to the known recurrent aberrations several other overlapping regions were detected in this cohort. For instance, large gains of chromosome 2p were identified in 8 samples (2%), where 5 of these samples carried a concomitant del(11q). Moreover, 8 large gains (2%) and 8 large losses (2%) was detected on chromosome 8p and 8q, respectively. Furthermore, 6 samples (1.6%) carried large losses (>17 Mbp) of chromosome 14q. Trisomies of chromosome 18 and 19 were detected in 3 and 5 samples, respectively, all of which carried trisomy 12. Despite the fact that a large proportion of aberrations were <1 Mbp, the majority of these CNAs were non-overlapping, detected only in individual samples. Analysis of CNN-LOH (n=203), revealed that 8 samples (3.9%) with homozygous del(13q) showed CNN-LOH of 13q, suggesting that chromosomal duplication is common in patients with heterozygous del(13q). No other recurrent CNN-LOH was detected. Overall survival (n=270) was investigated which verified the prognostic impact of del(13q) and del(17p), while patients with del(11q) and trisomy 12 displayed a similar risk profile. Notably, patients with homozygous del(13q) showed a significantly better survival compared to patients with heterozygous 13q deletion. An increasing number of large aberrations (>5 Mbp) was strongly associated with poor-prognostic markers, i.e. 11q-/17p- and unmutated IGHV genes, and predicted poor survival. In contrast, non-overlapping small aberrations (<1 Mbp) were commonly detected in all samples, but did not predict outcome. In conclusion, whole-genome screening with SNP-arrays revealed a high frequency of known recurrent alterations as well as additional small and large CNAs in newly diagnosed CLL patients. The fact that the majority of alterations <1 Mbp were non-overlapping indicate that these CNAs occur as random events in early CLL cells. In the survival analysis, we identified genomic complexity as a poor prognostic marker, but noted that this feature was strongly linked to established poor-risk molecular markers.

3.6

EXTENSIVE INTRACLONAL DIVERSIFICATION IN A SUBGROUP OF CHRONIC LYMPHOCYTIC LEUKEMIA PATIENTS WITH STEREOTYPED IGHV4-34 RECEPTORS: IMPLICATIONS FOR ONGOING INTERACTIONS WITH ANTIGEN

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Several studies indicate that the development of chronic lymphocytic leukemia (CLL) may be influenced by antigen recognition through the clonotypic B-cell receptors (BCRs). However, it is still unclear whether antigen involvement is restricted to the malignant transformation phase or whether the putative antigen(s) may continuously trigger the CLL clone and affect not only the progenitor cell but also the leukemic cells themselves. Evidence from several other types of B cell lymphoproliferative disorders suggests that valuable insight into these issues may be gleaned

from the study of intraclonal diversification (ID) within IG genes through ongoing mutational activity. The available data on ID within IG genes in CLL are limited and conflicting. In order to address these discrepancies and systematically explore the issue as to whether CLL cells continue to acquire hypermutations following leukemic transformation, we conducted a large-scale subcloning study of rearranged immunoglobulin heavy variable (IGHV) genes of diverse mutational status from 71 CLL cases, belonging to both the common IgM/IgD variant and the rare IgG-positive variant. PCR amplicons obtained using the high fidelity Accuprime Pfx Taq polymerase were subcloned and subsequently sequenced; overall, 1496 subcloned sequences were evaluated (median number per case: 21; range: 14-35). The interpretation of the results was based on the following definitions: (i) unconfirmed mutation (UCM) - a mutation observed in only one subcloned sequence from the same amplicon; (ii) confirmed mutation (CM) - a mutation observed more than once among subcloned sequences from the same amplicon. In order to compare mutation counts between the different rearrangements included in the analysis, mutations were normalized to both the nucleotide length and the number of subcloned sequences for each rearrangement. At cohort level, 20/71 cases (28%) carried sets of identical subcloned sequences. An additional 23/71 cases (32%) were characterized by the presence of mutations in single subcloned sequences and could not be formally assigned to the intraclonally diversified category. Finally, the remaining 28/71 cases (40%) carried intraclonally diversified IGHV-IGHD-IGHJ genes with confirmed mutations (CM) among subclones. CMs were identified in cases assigned to all mutational categories, even in rearrangements with 100% identity to the germline, suggesting that the ID process may affect IGHV genes irrespective of their original mutational status. Among 28 cases exhibiting CMs, 13 (46.4%) belonged to subset #4 with stereotyped, IgG-switched IGHV4-34/IGKV2-30 BCRs. Furthermore, a much more pronounced impact of ID was noted among subset #4 rearrangements vs. all other rearrangements included in the analysis. The strikingly different impact of ID in subset #4 cases vs. all other cases analyzed in this study could be thought to reflect an inherent mutability of the IGHV4-34 gene, or, alternatively, their mutated status or IgG-switched phenotype. However, these possibilities were ruled out by comparison to: (i) non-subset #4 IGHV4-34 rearrangements of the common IgMD variant; (ii) stereotyped IGHV4-34 rearrangements of IgG-switched cases assigned to a different subset (#16); and, (iii) both mutated and unmutated rearrangements utilizing other IGHV genes. The molecular characteristics of mutations occurring in the context of ID are compatible with the idea that the underlying causal mechanisms may generally obey the same rules as the canonical somatic hypermutation (SHM). However, especially among subset #4 IGHV4-34 rearrangements, the observed distribution patterns of CMs/UCMs differed from those reported as typical for mutations introduced by canonical SHM, leading to R/S ratios not characteristic of selection by conventional, T-dependent antigens. In conclusion, our study convincingly demonstrates that the SHM mechanism may continuously operate in certain subsets of patients with CLL, especially patients expressing stereotyped IGHV4-34 rearrangements typical of subset #4. Although it is still difficult to reach definitive conclusions with regard to the duration of exposure to and stimulation by antigen as well as the functional impact of antigen on CLL evolution, the results reported here suggest a role for persistent antigenic stimulation rather than clonal expansion promoted by nonspecific stimuli, at least for subsets of CLL cases.

3.7

ANALYSES OF SOMATIC MUTATION PATTERNS AND THREE-DIMENSIONAL STRUCTURES OF THE IMMUNOGLOBULIN VARIABLE (IGV) REGIONS IN B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA (CLL)

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CLL is characterized by the clonal expansion of CD5+ leukemic B lymphocytes. Analyses of IGH and IGL rearrangements in thousands of leukemic clones has revealed somatically mutated (M) and unmutated (U) IGHV genes, a non-stochastic use of individuals IGV genes, and the presence of quasi-identical IGHV rearrangements among CLL cases ("stereotyped B-cell receptors"), suggesting that, at some point in the pathogenesis of this disease, antigenic selection plays an important role. Notably, the mutational status of IGHV genes is an independent prognostic factor in CLL. Igs bind antigens mainly through six regions of the IgV gene segments (CDRs), three for each IgVH and IgVL gene segments, corresponding structurally to six loops that cluster in space to form the antigen binding site. Each of the six loops, with the partial exception of HCDR3, has been shown to assume only a limited number of main-chain conformations ("canonical structures") that can be identified and predicted on the basis of loop sequences, thus allowing a complete and reliable model for a large part of the structure of a binding site. We analyzed somatic mutation patterns of the Ig variable regions of a cohort of 366 CLL patients. In general, statistically significant correlation between replacement (R) mutations in the HCDRs and LCDRs and in the HFRs and LFRs was observed, with the exception of R mutations of IGKV CDRs, which were less frequently mutated compared to the paired HCDRs. To rule out that the absence of an observed correlation between the number of IGHV and IGKV mutations was due to two different populations, the sample was arbitrarily divided into two subgroups using a cut off of 3 percent units difference in mutations between the V regions (i.e., subgroup A carrying a difference between the IGHV and IGK/LV percentage of mutation <3 and subgroup B with a difference ≥3). Analyzing kappa isotype samples, no significant correlation between the number of IGHV and IGKV R mutations appeared in the CDRs of both subgroups. Conversely, for lambda isotype samples, the correlation observed between CDRs and FRs previously, when the two subgroups were analyzed together, was lost in subgroup B. This mutation pattern suggests that kappa isotype CLL Igs may be under antigenic pressure that selects for a lower number of mutations in the CDRs of the kappa chain, at variance with the mechanism observed in normal B cells. To correlate Ig features with mutational status, we used an automatic learning procedure, a random forest algorithm, based on the length and canonical structures of all loops in our dataset. Using this approach, we could predict the mutational status of CLL Igs with an out-of-bag classification error of 26.2%, i.e., these features correctly classified 75.2% of M and 72.3% of U CLL Igs. The attributes with the highest information content are: the length of H3 and the canonical structures for the H2 and L3 loops. This finding is consistent with the hypothesis that Ig mutation status, and consequently CLL patient outcome, depend upon structural properties of the antigen binding site. To further investigate this hypothesis, we analyzed the structure of the antigen binding sites of our dataset at the atomic level. We successfully built 3D models for 268 of 366 Igs in the dataset. These were clustered according to their structural distance, and a total number of 21 clusters were defined. Analyzing the clusters, we found that M and U Ig segregated with a statistically significant over-representation of either M or U Igs in 7 out of the 21 clusters. Interestingly, visual inspection of the binding sites of antibodies belonging to different clusters showed that their antigen binding sites were markedly different. It is noteworthy that M and U CLL clones were often observed within a single cluster and that known stereotyped receptors falling within a certain cluster were often accompanied by other receptors whose similarity is not obvious based solely on amino acid sequence inspection. These analyses further support a role for antigenic selection in the development of CLL leukemic clones and may represent a new molecular prognostic indicator.

3.8

INCIDENCE OF CHROMOSOMAL ANOMALIES DETECTED BY CGH METHOD IN THE GROUP OF B-CHRONIC LYMPHOCYTIC LEUKEMIA PATIENTS WITH DIFFERENTIATE PROGNOSIS ESTABLISHED BY FISH COMPARED WITH A STATUS OF THE IGH GENE

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B-chronic lymphocytic leukemia (B-CLL) is the most common adult leukemia. The most frequent chromosomal abnormalities are deletions at 13q14, 17p13 (TP53), 11q23 (ATM) and trisomy 12. Deletion of TP53 or/and ATM gene can predict rapid disease progression and inferior survival. Additionally the presence or absence of somatic mutations in the

immunoglobulin heavy chain variable regions (IgVH) of B-CLL cells provides prognostic information. Patients with unmutated (98% of homology to the germline sequence) IGVH regions often have progressive disease whereas patients with mutated IGVH regions more often have an indolent disease. The aim of this study was to establish incidence of chromosomal changes and IGVH mutation status in peripheral blood cells of B-CLL patients to evaluate its prognostic implications. We performed routine FISH on 76 patients using commercially available probes for chromosome regions 13q14 (13S272), 17p13 (TP53), 11q22.3 (ATM), 12 centromere (D12Z3), and with break apart probe for IGH gene. Moreover, chromosome comparative chromosome hybridization (CGH) was carried out to establish undetected chromosome changes in investigated patients with poor and with good prognosis established by routine FISH. The frequencies of abnormalities detected by FISH were as follows: 13q14 deletion in 47%, 11q23 (ATM) deletion in 22%, trisomy 12 in 21%, 17p13 (TP53) deletion in 8%. A sole deletion of TP53 gene was detected in 5/6 patients, while 1 case showed simultaneously deletion of ATM gene. Isolated ATM gene deletion was detected in 15/17 cases, additional changes showed trisomy 12 (1 case) and deletion TP53 (1 case). Trisomy 12 as a sole aberration was seen in 9/16 cases, in 6 cases with coexistence del(13q14). Deletion of 13q14 as a sole change was detected in 21/36 patients and deletion 13q14 with additional genetic alternations was revealed in 15/36 cases. The mutational study revealed that IGVH region was unmutated or mutated in 51 (67 %) and 25 (33%) cases, respectively. FISH analysis of IGH gene status showed gene rearrangement in 6/76 cases, whereas loss of part of IGHV locus was identified in 27/76 of cases. We have observed that patients with IGHV deletion had mainly unmutated IGVH region (23/27; 85.2%). In contrary, in patients with cytogenetically normal IGHV status we have found similar proportion of mutated and unmutated cases (19/24 – 44.2% and 24/43 – 55.8%, respectively). The difference was statistically significant (2=6.48; p=0.011). All unfavorable cytogenetic changes (deletion ATM, TP53) coexisted with unmutated status of IGVH region. Cases with the deletion 13q14 were heterogeneous in this feature: 10 of 21 (47.6%) were mutated and 11 (52,4%) had unmutated IGVH. Moreover, four cases with 13q14 deletion with trisomy 12 have mutated IGVH region. We have detected by CGH other chromosome changes, for example: +1q,+2p,-7q,+8q,+12q,+Xp. They were present in both groups of patients: with deletion ATM or TP53 and normal gene status investigated by FISH. Genomic aberrations and IGH rearrangement status appear to give additional prognostic information for individual patients early in the course of their disease. Presence of such abnormalities will be discussed in context the FISH results and IGVH status.

3.9

R-DHAP IS EFFECTIVE IN FLUDARABINE-REFRACTORY CLL, POSSIBLY VIA UPREGULATION OF PRO-APOPTOTIC P73

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Allogeneic stem cell transplantation (alloSCT) has the potential to cure high risk CLL patients including those with fludarabine refractory disease with or without a deletion of 17p. Treatment-related mortality (TRM) of this procedure is significantly reduced by the use of reduced-intensity conditioning (RIC). Risk factors for relapse after alloSCT are refractory or bulky disease. In an ongoing prospective Dutch/Belgian HOVON trial we study the efficacy of remission-induction with the R-DHAP regimen (Rituximab, cytarabine, cisplatinum and dexamethasone) prior to alloSCT in CLL patients with fludarabine refractory disease. During the initiation phase ten patients have been treated according to this protocol. The table shows their disease characteristics and response to R-DHAP. Eight of ten patients had a response: one CR and seven PR, whereas two had SD. One of the two patients with del 17p and both patients with del 11q had a response, one even a molecular complete response. Six of the eight patients with bulky disease responded. One patient developed tumor lysis syndrome. Only one patient (no 8), who had been pretreated with alemtuzumab, developed an opportunistic infection (Aspergillus pneumonia). Most responding patients subsequently underwent alloSCT. The observed response rate in these heavily pre-treated and chemo-refractory patients is remarkable. As chemorefractory disease is highly associated with a dysfunctional p53 response, we hypothesised that effects of this regimen could be independent of p53-function. Therefore the molecular basis of apoptosis induction was studied more in depth in one patient with del 17p and proven p53 dysfunction. Within weeks after the first cycle of R-DHAP peripheral blood lymphocyte counts decreased from $78x10^9$/L to $3.3x10^9$/L. Simultaneous analysis of RNA-expression levels of 30 apoptosis regulating genes by RT-MLPA in samples taken before and 24 and 48 hours after start of R-DHAP revealed significant upregulation of the pro-apoptotic BH3-only molecule Puma. Recent data indicate that Puma is not only a p53 response gene, but can also be regulated by the p53-family member p73. Indeed, detectable p73 protein levels were found already after 24 hours of treatment *in vivo*. At the functional level, we found that the pretreatment fludarabine resistance *in vitro* was abrogated after 48 hours of treatment *in vivo*. Our data indicate that the R-DHAP regimen has activity in fludarabine-refractory CLL patients, even in those with cytogenetic changes affecting the p53-response. Furthermore, in our preliminary *in vitro* studies we could show upregulation of the pro-apoptotic protein p73 and concomitant abrogation of fludarabine-resistance. This pathway should be further explored and may provide means to overcome drug-resistance in CLL.

Table.

Patient	*Age*	*FISH*	*Bulky disease[1]*	*Preceding therapies*	*No. of R-DHAP cycles*	*Response*
1	36	del 17p	+	Chl, CVP, FCR, Alem, R-CHOP	1	SD
2	65	normal	-	Chl, CVP, F	3	PR
3	71	normal	+	Chl, F, FC, FCR	1	PR
4	51	normal	+	Chl, F, FCR	4	SD
5	47	del 11q	+	FCR, Alem	3	PR
6	53	ND	-	Chl, F, Alem	3	PR
7	54	ND	+	Chl, F, FCR, R-CHOP	4	PR
8	65	ND	+	Chl, CVP, F, CHOP, Alem	3	PR
9	57	del 17p	+	R-CVP, R-CHOP	4	PR
10	50	del 11q	+	Chl, FCR, Alem	3	CR

1 lymph nodes > 5 cm

3.10

MOLECULAR APPROACH TO CHRONIC LYMPHOCYTIC LEUKAEMIA THROUGH MLPA ANALYSIS

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Background. Chronic lymphocytic leukaemia (CLL) is the commonest form of adult leukaemia in western countries. From a prognostic point of view, it is characterized by a heterogeneous course. As a consequence the characterization of prognostic factors is very important in order to prefigure evolution of disease. Nowadays it is well known that FISH analysis provides the identification of independent prognostic factors predicting outcome in CLL. *Aim*. We applied MLPA method (multiplex ligation-dependent probe amplification) to check for copy number variation in genes not included in standard FISH panel. *Methods and Patients.* CLL patients, whose diagnosis was performed according to IWG-CLL criteria, were selected for this study. Selection criteria were the following: no treatment and a disease history lasting less than 2 years. In the mean time standard cytogenetic and FISH probe set for chromosomal regions13q14, 11q22, 17p13, 6q23, 14q32 and chromosomal 12 were evaluated. Peripheral blood samples from 10 patients were collected and a genomic profiling using MLPA was performed. This method relies on the comparative quantitation of specially bound probes that are amplified by PCR with universal primers to identify intragenic, small alteration and large genomic rearrangements. Thereby this method is useful to detect copy number variation in genomic sequences. We used the SALSA P037/P038 kits. Samples analysis has been performed with sequence analyzer ABI PRISM 310 and Genescan analysis software. Cut-off levels for loss or gain of relative copy numbers were set at 0.7 and 1.3, respectively. Values included between 0.4 and 0.7 indicate a deletion, while values between 1.3 and 1.6 indicate a duplication. Each result was validated in two experiments independently. *Results and Conclusions.* Data

obtained by MLPA were compared with those from cytogenetic and standard FISH as reported in Table1. MLPA results supported the outputs obtained by FISH, and detected more information about specific chromosomal regions not included in standard FISH panel. Indeed, 13q14 deletion was detected both by MLPA and FISH in 5 cases, whereas the same deletion in Patients 5 and 9 was not detected by FISH method. Similarly, a 13q14 insertion was detected by MLPA in Patient 3 and 10 and not by FISH. In particular, Patient 3 had a complex cytogenetic that has been revealed clearly through MLPA analysis: two deletions (11q23 and 10q23) and two insertions (12p12 and 13q14). Moreover MLPA recognized variations in chromosome 12 (Patient 9) identified by cytogenetic in a low percentages of cells. In summary, our preliminary data show a good correlation between the MLPA and FISH results, however we strongly recommend integration between cytogenetic as well as FISH analysis and MLPA method for a better characterization of prognostic factors in CLL. The following step, thereby, will be to go on with this approach also to check for possible rearrangements of the patients' chromosomes during the time course of the disease.

Table 1.

Patient (n)	*Cytogenetic*	*FISH*	*MLPA*
1	47 XY+12[4] 46 XY [16]	13q14 (30%) 11q22 (2%) 17p13 (2%)	del 13q14
2	46 XX	13q14 (85%)	del 13q14 ins 2p24
3	Complex rearrangements	11q22 (27%) 3IgH- (23%)	del 11q23 del 10q23 ins 12p12 ins 13q14
4	47 XY,+12	5IgH- (78%)	ins 12p12 ins 9p21
5	46 XY	no abnormalities	del 13q14
6	46,XY,t(13;15)(q14;q26) [25]/46,XY[5]	13q14 (53,3%)5Igh- (76,6%)	del 13q14 del 2p24
7	46 XX	13q14 (85%)	del 13q14 del 2p24
8	46XX del(13)(q14q22) [2] 46XX[18]	13q14 (22%)	del 13q14 ins 2p24
9	47XY+12[8] 46XY[12]	no abnormalities	del 13q14 ins 12q23 ins 12q22 ins 12p12 ins 12p13
10	46XY	no abnormalities	ins 13q14

3.11

EXPRESSION OF THE CHEMOKINE RECEPTOR CXCR4, CXCL12G801A GENE POLYMORPHISM AND SDF1 PLASMA LEVELS IN B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA: CORRELATION WITH PBSC MOBILISATION, DISEASE CHARACTERISTICS AND OUTCOME

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The principal aim of our study was to determine the level of CXCR4 expression in lymphocyte cells and CD34+ mobilized cells, SDF1 polymorphism and SDF-1 plasma levels in CLL. The secondary objective was to check if there is a correlation of these variables with PBSC mobilisation, disease characteristics and outcome. We studied 73 CLL patients enrolled in SFGM-TC & FCLLG phase III randomized trial. Patients received 3 mini-CHOP followed by PBSC mobilisation and then 3 courses of Fludarabine. Patients were randomised between autologous HSCT versus "watch and wait" for responders and between autologous HSCT versus Fluda+cyclophosphamide in non-responders after 2 DHAP. After induction, there were 20 CR, 28 PR, 10 SD and 5 PD. Thirty-two patients were mobilized, the median number of collected CD34+ cells was $3.5x10^{xp6}$/kg [0-53.6] with a median of 15% of lymphocyte infiltration in the final product. At last follow up 63 patients are alive and 10 died. The CXCR4 expression in CLL cells (n=73) and in CD34+ mobilized cells (n=32) was evaluated by multicolour flowcytometry analysis, CXCL12 G801A gene polymorphism by PCR-RFLP and SDF-1 plasma levels by ELISA Quantikine SDF-1α kit. There was an heterogeneous expression of CXCR4 in CLL cells at diagnosis and patients were clustered into 3 groups depending of ratio MFICXCR4 vs IgG1 isotype control: 37 (51%) patients with low CXCR4 rMFI (median=39 [range 8-86]), 23 (31%) patients with intermediate CXCR4 rMFI (median 148 [range 94-216]) and 13 (18%) patients with high CXCR4rMFI (mediane 286 [range 220-534]). Concerning CXCL12-3'A polymorphism study, 5 (7%) patients showed A/A, 16 (22%) G/A and 52(71%) G/G genotype. The median of SDF1 plasma level was 1520 pg/ml (1150.4-4907.2). We did not find any correlation neither between CXCR4 rMFI in CLL cells and CXCL12-3'A polymorphism (p=0.99) nor between CXCR4 rMFI and SDF1 plasma levels (p=0.73) but CXCR4 rMFI in CD34+ mobilized cells was correlated with CXCL12-3'A polymorphism (p=0.02). In addition, we didn't find any significant correlation between Binet stage, disease status after induction and CXCR4 rMFI in CLL cells. Among the 32 mobilized patients, the median of CXCR4 rMFI in CLL cells was 124 (11-534) and 19.5 (3-38) in CD34+ mobilized cells; the CXCL12-3'A polymorphism showed 2 A/A, 7 G/A and 23 G/G. We didn't find any correlation between CXCR4 rMFI in CLL cells and CXCR4 rMFI in CD34+ mobilized cells. Among the potential impacting factors on CD34+ cell mobilization, we didn't find any correlation with age (p=0.99), lymphocyte infiltration (p=0.36), CXCL12-3'A polymorphism (p=0.8) and plasma levels SDF1 (p=0.12). There was a significant impact of disease status (p=0.006) and a negative trend of CXCR4 rMFI (p=0.07). In conclusion, the CXCR4 rMFI was very heterogeneous among our population. There was no correlation between this variable and CXCL12-3'A polymorphism. We showed a significant correlation between CD34+ mobilization and disease status, also a trend of CXCR4 rMFI. The CXCL12-3'AA genotype was associated with lower expression of CXCR4 in CD34+ mobilised cells and probably a good mobilisation; higher expression of CXCR4 in CLL cells at diagnosis was significantly correlated with poor CD34+mobilisation.

3.12

IDENTIFICATION OF NOVEL RECURRENT COPY NUMBER VARIATIONS AND REGIONS OF COPY-NEUTRAL LOSS OF HETEROZYGOSITY BY HIGH RESOLUTION GENOMIC ARRAY IN PRE-TREATMENT AND RELAPSED B-CLL

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Background. B-cell chronic lymphocytic leukaemia (B-CLL) is the most common form of adult leukaemia in the Western World. It is a heterogeneous disease and important biological and clinical differences have been identified. However, the molecular mechanisms underlying emergence and maintenance of B-CLL after treatment remain elusive. Array based comparative genomic hybridization (aCGH) has revolutionized our ability to perform genome wide analyses of copy number variation (CNV) within cancer genomes. Single Nucleotide Polymorphism arrays (aSNP) provide genotyping and copy number variation data and detect regions of copy neutral Loss of Heterozygosity (cnLOH) with the potential to indicate genes involved in leukaemia pathogenesis. Both technologies are evolving rapidly and emerging platforms are thought to allow high resolution (HR) of abnormalities down to single gene level. *Aim*. The aim of the current study was therefore to test a HR-aCGH and

a HR-aSNP platform for their ability to detect large and small CNVs and regions of cnLOH in B-CLL. More specifically, we wanted to: i) describe recurrent CNVs in B-CLL; ii) correlate them with results known from fluorescent in situ hybridization (FISH); iii) compare CNVs in pre-treatment samples and at relapse using paired samples from the same patient; iv) identify regions of cnLOH *Method.* We used a high resolution 244K aCGH platform and a 1Mio SNP array in parallel to test and characterize enriched B-CLL peripheral blood samples (>80% $CD19^+$;$CD5^+$) from 44 clinically annotated patients collected at our institution. To distinguish CNVs seen commonly in the general population the results were compared with 'in house' control data sets and the Database of Genomic Variants (http://projects.tcag.ca/variation/). *Results.* Our results show that large abnormalities, already noted by FISH, were reliably identified and the boundaries of abnormalities at 11q22.3, 13q14.2 and 17p could be defined more precisely. In addition, novel and recurrent CNVs within the sample set were identified (1p33; 3p24.3; 3p14.2; 4q12; 4q13.3; 6q21; 6q27; 8p22; 10q24; 11p15.4; 11q12; 11q13.4; 11q14.1; 11q22.1; 11q23.3; 13q14.11; 14q21.1; 15q15.1; 15q25.3; 17p13.3; 17q22; 18p11.32; 18p23; 19p13.13; 19p13.12; 19p13.32; 22q11.21; 22q11.22). Interestingly, some of these abnormalities contain single gene alterations involving oncogenes, chemokine receptors, kinases and transcription factors important in B cell development and differentiation. Assessment of smaller CNVs (less then 10 consecutive oligonucleotides) also revealed recurrent CNVs involving single genes that were clustered according to function and pathways. Comparison of paired pre-treatment and relapse samples showed differences in large CNVs in 6 out of 14 pairs with the majority being losses within the relapse sample. In particular, relapse samples contained new losses within 2q33.1-2q37.1; 4q13.2-4q13.3; 5q31.3-5q34; 7q36.3; 10q23.1-10q25.1 11q12.3 and multiple losses within 13q14.1-13q14.3. Taken together, these data indicate that genomic instability plays a role in clonal evolution and selection after treatment in at least some patients. Analysis of a bigger cohort of matched pre-treatment and relapse samples is on-going. The importance of copy neutral LOH in B-CLL has been a subject of debate. Using the 1Mio HR-aSNP, we were able to detect multiple regions of cnLOH throughout the genome. Examination of the four regions that are known to have prognostic significance when deleted identified cnLOH involving 13q11-13q34(ter) and cnLOH of 13q21.1-q34(ter) outside the FISH region. Deletions of the 17p13.1 locus including the p53 gene confer poor prognosis in B-CLL and direct treatment decisions. Interestingly, we were able to identify cnLOH involving this region in 5% of samples. In addition, we also noticed cnLOH in 17p13.2 containing genes previously implicated in cancer. The exact pathogenetic and prognostic implications of these findings remain to be established. *Conclusion.* Using HR-aCGH and HR-aSNP we have identified novel recurrent CNVs and regions of cnLOH in patients with B-CLL. Sequential analysis of the same patients over time suggests that at least in some patients, clonal complexity and dynamics are driven by genomic instability. "This work was supported by the Oxford Partnership Comprehensive Biomedical Research Centre with funding from the Department of Health's NIHR Biomedical Research Centres funding scheme. The views expressed in this publication are those of the authors and not necessarily those of the Department of Health."

3.13

A COMMON VARIANT IN THE 3' UTR OF IRF4/MUM1 ASSOCIATES WITH CD38 STATUS AND TREATMENT-FREE SURVIVAL IN B-CELL CHRONIC LYMPHOCYTIC LEUKAEMIA

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A common single nucleotide polymorphism (rs872071) in the 3' UTR of the gene encoding the interferon regulatory factor-4 (IRF4) transcription factor has been associated with risk of B-cell chronic lymphocytic leukaemia (B-CLL). We hypothesised that the disease-associated allele at the IRF4 locus would also associate with an aggressive disease course and a shorter time from diagnosis to first treatment, and that this common genetic variant contributes to the heterogeneous clinical course of B-CLL. IRF4 genotype (GG, GA or AA) was determined in a cohort of 840 B-CLL patients and genotype was correlated with established prognostic markers and clinical outcome. Carriers of the disease-associated allele at the IRF4 locus (G/G and G/A genotypes) had a significantly shorter treatment-free survival (TFS) compared to non-carriers (A/A genotype) (Hazard ratio [HR] 1.6, 95% confidence interval [CI] 1.2-2.3, p=0.006), which retained prognostic significance in multivariate Cox proportional hazards regression analysis that included age, gender, IgVH mutational status, CD38 expression and zeta-chain-associated kinase 70 (ZAP-70) expression in the model (HR 1.6, p=0.047). The IRF4 polymorphism was not associated with IgVH status (p=0.36), but the disease risk/poor prognosis allele was significantly associated with CD38 expression (p=0.004). The 3' UTR polymorphism defined by rs872071 in IRF4 is a prognostic marker in B-CLL and associates with CD38 status, identifying a high frequency genetic variant associated with both development of B-CLL and an aggressive disease course.

3.14

GENETIC MODIFICATION OF CLL CELLS *IN VITRO* USING AN OPTIMISED LENTIVIRUS-BASED DELIVERY SYSTEM

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The reproducible genetic modification of primary cells from patients with chronic lymphocytic leukaemia (CLL) represents a valuable technology that will facilitate research into this incurable disease. While the use of gene expression vectors or RNAi to modulate protein expression is well established, the non-proliferatory nature of CLL cells grown *in vitro* under standard culture conditions precludes the use of more common transfection methods such as lipid reagents or calcium phosphate. As such, our objective is to manipulate protein expression through the use of lentiviruses, which we have previously identified as being able to genetically modify CLL cells with high efficiency. Here, we investigated the factors that should be considered when generating lentivirus for transduction of CLL cells, and describe a standardised protocol used in our laboratory to genetically modify CLL cells *in vitro*. To generate lentivirus, HEK293T cells are transfected with a plasmid containing selected viral genes from the HIV-1 genome, a plasmid for the VSV-G coat protein and a transfer vector to manipulate CLL gene expression. This transfection is performed using the calcium phosphate method. The efficiency of this step is pivotal for maximum viral yield and so both the quality of the transfection reagents used and the experimental conditions applied are of great importance. To obtain the highest titre lentivirus, we recommend: 1) using transfection reagents manufactured in-house rather than commercially sourced to increase reproducibility; 2) using HEK293T cells which are 40-50% confluent at the time of transfection; 3) replacing culture medium 6hrs post-transfection to reduce cytotoxicity in HEK293T cells; 4) allowing 48hrs for the production of virus before harvesting by ultracentrifugation. Under these conditions we have generated lentivirus capable of transducing CLL cells *in vitro* with high efficiency. We have successfully applied this technology to the exogenous expression of CD38 and GFP in CLL cells, in addition to the delivery of shRNA plasmids for the knockdown of RelA. The scope of this technique could have a significant impact on all areas of CLL research. Thus, the ability to modulate gene expression in CLL cells *in vitro* may prove to be an invaluable tool in identifying and characterising the key molecules controlling this complex disease.

3.15

RECRUITMENT OF PKC-BETA TO LIPID RAFTS MEDIATES APOPTOSIS-RESISTANCE IN CHRONIC LYMPHOCYTIC LEUKEMIA ABERRANTLY EXPRESSING ZAP-70

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A defect in the programmed cell death, apoptosis, is implemented in the pathogenesis of CLL. About ten years ago, it became evident that patients with CLL can be divided into those with an indolent course of the leukaemia and those which suffer from a more aggressive disease, typically requiring frequent chemotherapy and ultimately develop a chemotherapy-refractory state. The latter group of patients aberrantly express the T-cell associated protein ZAP-70. The object of this study was to identify the molecular differences underlying the pathogenesis of these two CLL subgroups. To study differences in the apoptotic program we used primary CLL cells derived from untreated ZAP-70 negative and positive patients. Here we show that the aberrant expression of ZAP-70 enhances the signals associated with the B-cell receptor (BCR) and recruits protein kinase C-beta (PKC-β) into lipid raft domains only in patients with an aggressive variant of the disease. Subsequently, PKC-β is activated and shuttles from the plasma membrane into the mitochondria. By using co-immunoprecipitation experiments and PKC-β specific small molecule inhibitors we unravel that the anti-apoptotic protein Bcl-2 and its antagonistic BH3-protein Bim are putative substrates for PKC-β. PKC-β mediated phosphorylation of Bcl-2 augments its anti-apoptotic function by increasing its ability to sequester more pro-apoptotic Bim. In addition, the phosphorylation of Bim by PKC-β leads to its proteasomal degradation. Therefore, high levels of phospho-Bcl-2 and low levels of Bim are a hallmark of ZAP-70 positive, aggressive CLL. We demonstrate that these cells are strongly protected from chemotherapy-induced cytotoxic stress. Our data indicate that the constitutive activation of PKC-β is directly involved in the apoptotic defect in ZAP-70 positive CLL. We finally show that targeting PKC-β is an attractive approach to the treatment of CLL patients. Enzastaurin is a PKC-β specific inhibitor and currently tested in clinical phase I/II trials for cancer patients. Our data demonstrate that this compound is highly active in CLL cells and augments the cytotoxic effects of standard chemotherapeutic drugs. Our results provide evidence that the constitutive activation of PKC-β is directly implicated in the pathogenesis of aggressive CLL by altering the function of the apoptosis-regulating proteins Bcl-2 and Bim. These changes confer cells to a more anti-apoptotic state with aggressiveness of the disease. Targeting PKC-β with small-molecule inhibitors like Enzastaurin might offer a new therapeutic strategy to control or even cure CLL.

3.16

MOLECULAR CHARACTERISATION OF TELOMERE DYSFUNCTION AND CHROMOSOMAL FUSION EVENTS IN CHRONIC LYMPHOCYTIC LEUKAEMIA

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Telomere length is an important prognostic parameter in chronic lymphocytic leukaemia (CLL) and is associated with the mutation status of the immunoglobulin heavy chain (IGVH) and the presence of high risk genomic aberrations (11q(-)/17p(-)). Telomeres protect the chromosome ends from fusion, degradation and recombination. If telomeres become critically short, they can lose their end capping function and are prone to chromosome end-to-end fusions. Telomere fusion can result in the formation of dicentric chromosomes which can enter cycles of anaphase-bridging, breakage and fusion leading to genomic rearrangements such as non-reciprocal translocation (NRTs). In this present study, we have obtained the first direct evidence of telomere dysfunction and fusion in primary CLL cells. We used high resolution single molecule approaches, to detect both short dysfunctional telomeres and the resulting fusion events. We measured the telomere length of CLL samples (n=41) derived from patients in Binet stage A and stage B/C in the presence and absence of high risk genomic aberration (11q(-)/17p(-)). We observed that stage C patients exhibited extremely short telomere length distributions with a mean of 1.57kb compared to stage A with a mean of 4.28kb ($p<0.0001$); with some individuals displaying telomeres with the length ranges we had previously defined as being capable of fusion. We also found evidence of loss of heterozygosity (LOH) at the 17p telomere that was associated with LOH at the p53 locus. These findings raised the possibility that the critically short telomeres observed in CLL cells may be subject to end-to-end fusion. We therefore examined the presence of telomere fusion events in these CLL cells using a PCR-based assay that allows the detection of single telomere-telomere fusion molecules between specific chromosome ends. We detected fusion events in a small proportion of stage A samples (3/7) and a larger proportion of stage C CLL patients (12/12). Importantly some of these fusion events were shown to be clonal and the frequency of fusion events was similar to that observed in fibroblast cells (MRC5) undergoing crisis *in vitro*. Furthermore, we were able to sequence the fusion molecules and identify which chromosome partners were involved in each fusion event. We consistently demonstrated that CLL fusion events were characterised by telomeric and sub-telomeric deletion and microhomology at the fusion point providing a potential mechanism of the fusion event recombination. Taken together, our data suggested that CLL patients with short telomeres are more prone to have a high frequency of telomere fusion events that contribute the genomic instability and the development of clonal evolution during disease progression in CLL.

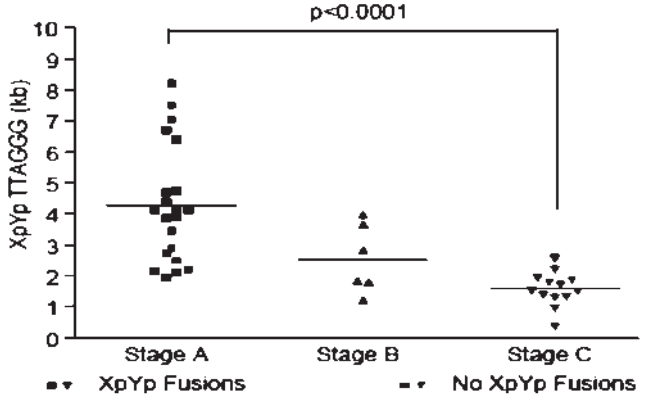

Figure 1.

3.17

GENETIC MODIFICATION OF PRIMARY CHRONIC LYMPHOCYTIC LEUKAEMIA CELLS WITH A LENTIVIRUS EXPRESSING CD38

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Chronic lymphocytic leukaemia (CLL) is a heterogeneous disease ranging from a stable condition requiring no therapy to a progressive disease that is refractory to treatment. Expression of the CD38 antigen on the surface of CLL cells is associated with poor prognosis and a reduced overall survival. Recent evidence suggests that CD38 signalling contributes to the aggressive phenotype observed in progressive disease. In order to dissect the role of CD38 in primary CLL, we have developed a technique to efficiently transduce CD38 negative CLL cells using a lentiviral vector expressing the coding sequence for CD38. A high transduction efficiency of CD38 was observed in all 17 patient samples following addition of the CD38 lentivirus (87% ±8.5) (Figure 1). In contrast, a green fluorescent protein (GFP)-expressing virus with the same backbone only caused a small increase in CD38 expression in a minority of the cells (8% ±5.8). Following transduction, cell viability was assessed using forward and side scatter and a mean survival of 74% (±17) was seen in lentivirus treated samples. No major changes in CLL cell morphology were observed following 48 hours incubation with lentivirus and there was no evidence of plasmacytoid features (CD138 negative). A key advantage is that the entire CLL population may be genetically modified with limited cell toxicity making cell sorting or selection unnec-

essary. Furthermore, over the course of this study, no patient sample has been observed to be refractory to lentiviral infection. In summary, we report the robust genetic modification of primary human CLL cells using a lentivirus a technique that should prove invaluable in the elucidation of the biological significance and role of individual genes of interest in primary CLL cells. Figure 1. Percentage of CD38 negative CLL cells expressing CD38 following the addition of lentivirus, in 17 patient samples.

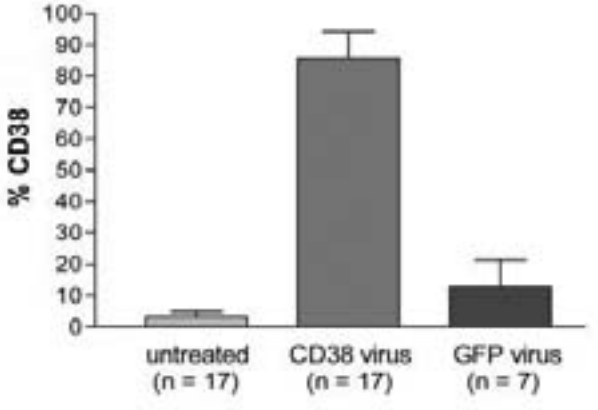

Figure 1.

3.18

THE INFLUENCES OF ZAP70 AND IGM STIMULATION ON MRNA AND MIRNA EXPRESSION PROFILES IN CLL

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Prognosis of B-cell chronic lymphocytic leukaemia (CLL) is variable, but can be predicted by a number of clinical and biological markers. The most commonly used prognostic markers include the presence or absence of certain genetic aberrations, the mutation status of the immunoglobulin variable heavy chain genes and the expression level of ZAP70. MicroRNA's (miRNA's) are small non-coding RNA molecules that regulate protein expression by targeting the mRNA of protein-coding genes resulting in repression of translation. miRNA's have been shown to be involved in cancer, apoptosis and cell metabolism. In CLL, miRNA expression profiling studies revealed different miRNA profiles associated with known prognostic factors. Since this association is not mechanistically explored and little is known about the regulation of miRNA expression, we investigated the influence of ZAP70 protein expression and/or the influence of IgM stimulation on the miRNA expression profile and correlate this with the mRNA expression profile. Recently, we demonstrated that clinically significant genes such as ZAP70 could be efficiently overexpressed in CLL cells by electroporation of mRNA (Van Bockstaele F. et al. Leukemia 2007). After electroporation we stimulated the cells with anti-IgM-polyacrylamid beads to activate the B-cell receptor (BCR) signaling pathway and to activate the introduced ZAP70 protein. As a negative transfection control we electroporated uncapped mRNA which remains untranslated in the cell, anti-IgA-polyacrylamid beads (anti-IgA) were used as negative control for stimulation. We determined the miRNA expression profile using a megaplex TaqMan miRNA assay (Mestdagh P. et al. Nucleic Acids Res 2008) together with a Human Illumina Gene Expression beadChip to investigate the differences in mRNA profile. After stimulation, expression of a lot of miRNA's is affected. The most significant differences are the upregulations in hsa-mir-132, hsa-mir-146a and hsa-miR-222. A known target of hsa-miR-146a is IRAK-1 (IL-1 receptor-associated kinase 1). In our micro-array we do see a downregulation after 24 hours of this mRNA. Hsa-miR-222 is linked to the cell cycle checkpoint p27 and stimulates cell survival (Medina et al. Cancer Res 2008). Irrespective of stimulation, the expression of hsa-miR-34a and hsa-miR-429, expressed at low level, is increased following forced ZAP70 expression. Hsa-miR-34a is a well known miRNA in CLL where it has been described as a prognostic factor. It has been shown that p53 may upregulate hsa-miR-34a which further leads the cell into apoptosis and cell cycle arrest. In analogy with the p53 data, one would expect if ZAP70 upregulates hsa-mir-34a, this would rather be associated with good prognosis. Why the opposite is true, remains to be elucidated. The role of hsa-miR-429 is not yet known. Above of this, we see a difference in miRNA expression depending on ZAP70 electroporation after stimulation for hsa-miR-98, hsa-miR-627, the hsa-let 7 family and the hsa-miR-29 family. ZAP70 induced miRNA profile could contribute to the more aggressive phenotype of ZAP70 positive CLL cells. To determine which pathways are responsible, we have to investigate in detail the targets of the affected miRNA's.

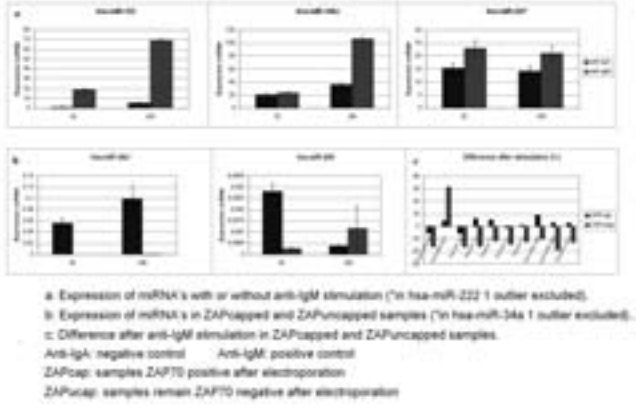

Figure 1.

3.19

C-MYC EXPRESSION IS REGULATED BY BAFF AND IS INVOLVED IN THE PROGRESSION OF CHRONIC LYMPHOCYTIC LEUKEMIA

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Chromosomal translocation involving c-Myc desregulation is a consistent feature of human Burkitt's lymphoma and multiple myeloma. The same chromosomal change rarely happens in CLL, the most prevalent leukemia in western countries. We found c-Myc differentially expressed in human CLL samples, which correlated with disease progression. In accordance with c-Myc as a factor to promote cell cycle progression as well as apoptosis, we found enhanced cell death *in vitro* in CLL cells expressing high levels of c-Myc which could be abrogated by coculture with BAFF expressing nurselike cells (NLC) or recombinant BAFF. Double transgenic iMyc(C-α)/Baff-Tg (Myc/Baff) mice developed a disease closely resembling human CLL. Myc/Baff mice had dramatically shorter mean survival than parental strains due to onset and rapid clonal expansion of a mature $CD5^+CD3$-B220low B cell population. Those cells transferred the disease into Baff-Tg (Baff) mice, with marked infiltration in lymphoid organs and bone marrow. Gene analyses revealed that among the genes altered in Myc/Baff $CD5^+CD3^-$ leukemia cells were those with known relevance to human CLL disease including elevated anti-apoptotic Bcl2 family members. Besides individual genes sub-network analysis was performed and showed apoptosis-related and stress-induced gene sets enrichment in Myc/Baff $CD5^+CD3^-$ leukemia cells. As a major target downstream of BAFF signaling, NF-kappaB gene set was also enriched in Myc/Baff $CD5^+CD3^-$ leukemia cells. Inhibition of NF-kappaB pathway significantly reduced viability of both mouse leukemia cells and human CLL cells co-cultured with NLC. Also it significantly lowered $CD5^+CD3^-$ $B220^{low}$ leukemia cell population in blood and spleen, and prevented the infiltration of leukemia cells into lymph nodes and bone marrow of transplanted mice. Beyond its anti-apoptotic role, BAFF treatment in primary human CLL cells led to dramatically enhanced expression of MYC at transcription through IKK/NF-kappaB pathway. This study clearly demonstrates the important role of c-Myc,

together with environmental factors like BAFF in the progression of CLL. Intervention of classical NF-kappaB pathways, either as single agent or in combination with others, might be helpful to treat patients with CLL.

3.20

THE ROLE OF AKT-TCL1 INTERACTIONS IN THE SURVIVAL AND CHEMORESISTANCE OF B-CLL CELLS

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Emerging evidence suggests that the survival of B-cell chronic lymphocytic leukemia (B-CLL) cells is dependent on microenvironmental influences such as antigenic stimulation and support by stromal cells. Akt, also known as protein kinase B, is a central component in pro-survival signaling downstream of these events. We investigated the role of Akt and its modulation by the proto-oncogene Tcl-1 in the survival pathways of primary B-CLL samples and B-CLL cell lines. Akt activation was increased by the protective presence of human bone marrow stromal cells and B cell receptor (BCR) mimicking signals but antagonized by direct Akt blockade with the novel specific inhibitor AiX. Determining the efficacy of Akt inhibition in different clinical risk groups, we found preferential apoptosis induction in B-CLL cells with an unmutated immunoglobulin status and high CD38 expression, both poor prognostic markers of the disease. In addition, we found a direct interaction of Akt with Tcl1 in an endogenous co-immunoprecipitating assay. Confirming the critical role of Tcl1 in modulating Akt signaling, Akt activation was enhanced by overexpressing Tcl1 in B-CLL. Vice versa, decreasing Tcl-1 levels by siRNA reduced Akt activation in the fludarabine–insensitive B-CLL cell line MEC-2 and sensitized the malignant cells to fludarabine treatment. In summary, our data reveal a significant role for the Akt-Tcl1 axis in B-CLL survival and propose a further evaluation of this interplay for targeting chemoresistance phenomenons.

3.21

A NOVEL XENOGENEIC MODEL OF CHRONIC LYMPHOCYTIC LEUKEMIA CLOSELY APPROXIMATES SEVERAL ASPECTS OF THE HUMAN DISEASE

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B-cell type chronic lymphocytic leukemia (B-CLL), an incurable disease of unknown etiology, results from the clonal expansion of a $CD5^+CD19^+$ B lymphocyte. Progress into defining the cell of origin of the disease and identifying a stem cell reservoir has been impeded because of the lack of a reproducible model for growing B-CLL cells *in vivo*. At least one possible cause for this is the murine microenvironment's inability to support B-CLL survival and proliferation. To overcome this barrier, we reconstituted the tibiae of NOD/SCID/ cnull mice by intrabone (ib) injection of $1x10^5$ $CD34^+$ cord blood cells along with ~106 bone marrow-derived human mesenchymal stem cells (hMSCs). After human cellular engraftment, a total of 108 CFSE-labeled PBMCs from individual B-CLL patients were injected into the same bones. Every two weeks thereafter, blood from the mice was examined for the presence of cells bearing CFSE, human CD45, and various human lineage markers by flow cytometry. In the presence of a human hematopoietic microenvironment, $CD5^+CD19^+$ leukemic cells underwent at least 6 cell doublings, after which CFSE fluorescence was no longer detectable. In contrast, leukemic cells injected into mice that were not reconstituted by ib injection with $hCD34^+$ cells and hMSCs or were reconstituted with only hMSCs failed to proliferate. Moreover the number of $CFSE^+CD5^+CD19^+$ cells detected in the blood of mice with a human hematopoietic microenvironment far exceeded that in mice receiving only hMSC. Robust T-cell expansion occurred in mice receiving $CD34^+$ cells and occasionally in mice (10-20%) without $hCD34^+$ cell injection. Based on genome-wide SNP analyses, the expanded T cells were of B-CLL patient origin and not from $hCD34^+$ cells. Furthermore, most of the mice with significant T-cell overexpansion died within 6 weeks of B-CLL cell injection from apparent graft vs. host disease. Therefore in subsequent experiments, we eliminated T cells by injecting an anti-CD3 antibody (OKT3); this treatment led to an inhibition of B-CLL cell proliferation. Moreover, the percentage of $CD38^+$ cells in the $CFSE^+CD5^+CD19^+$ cell fraction was similar to that in the donor patient inoculum only in the mice in which T-cell-mediated B-CLL cell proliferation occurred. The percentage and intensity of CD38-expressing B-CLL cells was higher in the spleen and bone marrow (BM), far exceeding that in the blood and peritoneum. Of note, B-CLL cells formed follicular structures in the spleen that resembled proliferation centers/pseudofollicles seen in patient lymph nodes and BM. Finally, B-CLL cells adoptively transferred into these mice exhibit kinetics similar to those observed in patients *in vivo*. In summary, these studies define conditions that permit adoptive xenogeneic transfer and clonal expansion of B-CLL cells in immune deficient mice. Factors conferring an advantage in this model include autologous T cells and an allogeneic human hematopoietic environment and. This model will be useful in discovering and understanding non-genetic factors promoting B-CLL expansion because it recapitulates several features of human B-CLL. Finally, the model may help studying the basic biology of this disease, such as if leukemic stem cells exist, and also to conduct pre-clinical tests on possible new therapeutics.

3.22

TP73 GENE METHYLATION IN CHRONIC LYMPHOCYTIC LEUKAEMIA. CORRELATION WITH CYTOGENETIC AND CLINICOPATHOLOGIC CHARACTERISTICS.

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Aberrant promoter methylation is an epigenetic DNA modification targeting CpG islands located within the regulatory regions of human genes. It results in repression of gene transcription and represents a mechanism for tumor suppressor gene inactivation. The TP73 gene, located at 1p36.3, is homologue of the TP53 tumor suppressor. TAp73 is a TP73 isoform with tumor suppressor activity that induces apoptosis and cell-cycle arrest. TP73 gene methylation has been scarcely studied in chronic lymphocytic leukaemia (CLL). In this study, we have evaluated the methylation status of TP73 gene (TAp73 isoform), in 41 patients with diagnosis of CLL (21 males; median age 67 years; Rai clinical stages: 0:41%, I:15%, II:26%, III:7% and IV:11%). Results were correlated with clinicopathologic and cytogenetic characteristics. All patients gave their informed consent and the study was approved by the local Ethics Committee. DNA samples from peripheral blood lymphocytes (PBL) of patients and controls were purified using phenol/chloroform method. Methylation status was performed using Methylation Specific PCR (MSP) technique. PBL samples from 10 normal individuals and CpGenome Universal Methylated DNA (Chemicon International) as negative and positive controls, respectively, were used. PCR products were gel purified, sequenced bi-directionally and analyzed on an automated DNA sequence analyzer (377 ABI Prism, PE Biosystem). Chromosome analysis were performed on stimulated PBL, cultured for 72-96 h. at 37°C in F-10 medium supplemented with 15% fetal calf serum. G-banding technique was used. For FISH (fluorescence in situ hybridization) analysis, slides were hybridized with the LSI p53 (17p13), LSI ATM (11q22), LSI D13S319 (13q14) and CEP12 DNA probes (Vysis-Abbott), according to manufacturer´s protocol. Four hundred interphase nuclei were evaluated for each probe. Statistical analysis was performed with the 2 test and the Student t test. Survival curves were plotted by the Kaplan-Meier method and compared by the log-rank test. Cytogenetic analysis was performed on 38 patients. Abnormal karyotypes were observed in 11 (29%) cases, three of them with complex karyotypes. By FISH analysis, 29 (71%) patients showed genomic alterations: 41% 13q14 deletion, 27% trisomy 12, 20% ATM and 15% TP53 deletions. Methylation of TP73 gene was detected in 71% of our CLL patients. MSP results were confirmed by sequencing analysis. In normal samples, no TP73 methylation was found. We investigate the possible correlation between methylation data and clinical and cytogenetic characteristics. Methylation status of TP73 was not associated with patients' age (p=0.14) or sex (p=0.72). We did not find statistical significant correlations between TP73 methylation and overall (p=0.66) or treatment free

survival (p=0.99), neither with clinicopathologic parameters: Rai stage (p=1.00), median lymphocyte count (p=0.86), β2M (p=0.96), LDH (p=0.08), haemoglobin (p=0.29), CD38 (p=0.61). When cytogenetic and FISH results were evaluated a trend to an inverse association between TP73 methylation and TP53 and ATM deletions was found (p=0.06). Our results showed a high frequency of TP73 gene methylation in CLL patients, suggesting that this abnormality represents an early event in this pathology. Simultaneously, the methylation status of TP73 had no prognostic impact in our series. As known, misbalance between cytoplasmic pro-survival and pro-death molecules appears to be one of the key factors defining B-cell longevity in CLL patients. Thus, TP73 gene inactivation by methylation may contribute to incline the balance in favor of cell survival.

3.23

MANY DIFFERENT B-CLL ANTIBODIES RECOGNIZE APOPTOTIC CELLS THAT EXPOSE NON-MUSCLE MYOSIN HEAVY CHAIN IIA

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B-cell chronic lymphocytic leukemia (B-CLL) is due to the clonal expansion of a B lymphocyte bearing a unique immunoglobulin or monoclonal antibody (mAb). Nearly 30% of B-CLL patients share mAbs with very similar sequences, suggesting common antigen reactivity. Previously, we have shown that non-muscle myosin heavy chain IIA (MYHIIA) is an antigen that binds to B-CLL mAbs characterized by a heavy (H) chain encoded by IGHV1-69, IGHD3-16, and IGHJ3. These mAbs have nearly identical, "stereotyped" sequences that form a subset (subset 6) of B-CLL patients (at least 53 worldwide). MYHIIA is a large intracellular cytoplasmic protein involved in cell shape and movement. During apoptosis, MYHIIA structurally rearranges and we have shown that it becomes exposed at the cell surface, allowing the mAb of the B-CLL cell to interact with it. Spontaneous apoptosis of a human T cell line (Jurkat) was first assessed by immunohistochemical staining with propidium iodide (PI) to detect nuclear DNA condensation. Costaining with polyclonal rabbit anti-human MYHIIA revealed that MYHIIA becomes exposed in large, punctate apoptotic structures distinct from those containing condensed nuclear DNA. Using a recombinant subset 6 B-CLL mAb and PI, we showed that this B-CLL mAb binds similar apoptotic structures as anti-MYHIIA. Finally, costaining Jurkat cells with subset 6 B-CLL mAb and anti-MYHIIA demonstrated that the B-CLL mAb colocalizes with MYHIIA containing structures. To determine at what stage MYHIIA becomes exposed during apoptosis, Jurkat cells were assayed by flow cytometry after costaining anti-MYHIIA with 7-amino-actinomycin (7AAD) and Annexin V-Phycoerytherin (AVPE). MYHIIA is exposed in a subgroup of both early (7AAD+, AVPE-) and late (7AAD+ AVPE+) apoptotic cells, but not in live (7AAD-, AVPE-) cells. Costaining subset 6 B-CLL mAb with 7AAD and AVPE demonstrated binding in a subgroup of both early and late apoptotic cells, but not live cells. Finally, costaining with anti-MYHIIA and subset 6 B-CLL mAb revealed that these antibodies bound the same subgroup of cells. Thus, subset 6 B-CLL mAbs react with a subgroup of early and late apoptotic cells exposing MYHIIA. Because we and others have observed that many CLL mAbs react with apoptotic cells, we tested a panel of 26 recombinant CLL mAbs for reactivity against these MYHIIA exposed apoptotic cells (MEACs). Interestingly, 14 of 16 CLL mAbs, which had high binding to MEACs, belonged to a characterized stereotypic CLL mAb subset; and 15/16 of these mAbs had unmutated IGHV genes. In contrast, 5 of 10 CLL mAbs, which had low binding to MEACs, belonged to a characterized stereotypic CLL mAb subset, and 6/10 had mutated (>2%) IGHV genes. Thus, unmutated CLL mAbs that belonged to a stereotypic subset tended to bind MEACs more effectively. The molecular determinants of this binding may be through MYHIIA or other uncharacterized molecules exposed during apoptosis. These results suggest that binding to a particular type of apoptotic cell may identify the source of an antigen driving the development and expansion of this leukemia.

3.24

INSIGHT INTO HCDR3 RESTRICTIONS IN CLL BY ANALYSIS OF INCOMPLETE IGHD-IGHJ REARRANGEMENTS: FURTHER EVIDENCE THAT SOMATIC SELECTION SHAPES THE EXPRESSED CLL IMMUNOGLOBULIN REPERTOIRE

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A strong sequence-based evidence supporting a role for antigen in the development of CLL is the existence of subsets of patients with highly similar, "stereotyped", heavy complementarity-determining region 3 (HCDR3) sequences, especially among cases with unmutated IGHV genes. Such stereotyped HCDR3s are often defined by the selective association of certain IGHD genes in specific reading frames with certain IGHJ genes, in particular IGHJ6. The creation of HCDR3 starts early in B cell development by the joining of IGHD and IGHJ genes, followed by attachment of an IGHV gene to the IGHD-IGHJ complex. Incomplete IGHD-IGHJ rearrangements (IDJR) are readily found in pre-B cells; they have also been reported in a proportion of precursor B-cell acute lymphoblastic leukemia (pre-B ALL) as well as in mature B cell malignancies. To gain evidence into the mechanisms used to shape the IG repertoire and also determine the developmental stage when restrictions in HCDR3 are imposed, we investigated the molecular configuration of incomplete IGHD-IGHJ rearrangements (IDJR) in a series of 830 patients with CLL. Following the BIOMED-2 protocols, IDJRs were detected in 272/830 cases (32.7%). No differences were identified regarding the frequency of IDJRs among cases with mutated vs. unmutated IGHV genes or cases utilizing certain IGHV genes in the complete IGHV-D-J rearrangements from the coding IGH allele. A trend for increased IDJR frequency was evident in specific subsets with stereotyped HCDR3s, in particular subset #1 (IGHV1-5-7/IGHD6-19/IGHJ4; 13/33 cases, 40%), #7 (IGHV1-69/IGHD3-3/IGHJ6; 10/21 cases, 48%) and #8 (IGHV4-39/IGHD6-13/IGHJ5; 5/12 cases, 41%). Sequence analysis of the IDJRs, feasible in 238/272 cases, revealed: (i) increased frequency of IGHD2 subgroup genes (115/238 cases, 48%), in particular the IGHD2-2 gene; (ii) equal distribution of the three reading frames (RF) of the IGHD genes; (iii) increased frequency of rearrangements between 5´genes of the IGHD cluster and 3´ genes of the IGHJ cluster, suggestive of secondary rearrangements on the same allele. Overall, 205/238 (86%) IDJRs were considered as potentially functional (PF), since they did not carry a stop codon at the IGHD-J junction. Interestingly, 26/28 (93%) IDJRs detected in cases from subsets #1, 7 and 8 could be assigned to the PF category. In the group of CLL cases carrying PF IDJRs, a comparative assessment of the molecular properties (repertoire, RF, hydrophobicity) of the IGHD genes in IDJRs vs. complete, expressed IGHV-D-J rearrangements (CE-VDJRs) revealed: (i) statistically significant (p<0.001) selection of the IGHD3-3 and IGHD6-19 genes in RF2 and RF3, respectively, among CE-VDJRs, especially those assigned to subsets #7 and #1, respectively; (ii) preferential usage of RFs encoding for hydrophilic peptides among CE-VDJRs, evidence of selection for functionality. At a subsequent stage, we compared the repertoire of the IDJRs from the CLL cohort to that of 174 IDJRs obtained from patients with pre-B ALL, a malignancy derived from transformation at the precursor B cell stage without involvement of any form of functional constraint. The comparative assessment of IDJRs demonstrated that except for preferential usage of the IGHD7-27 and IGHJ6 genes and the significantly increased frequency of IGHD-IGHD gene fusions in pre-B ALL, the overall configuration of IDJRs did not differ significantly between CLL and pre-B ALL. In conclusion, the results of the present study document that the early stages of IG gene rearrangements in pre-B ALL and CLL do not show intrinsic, disease-specific differences. The detailed molecular characterization and comparison of the IGHD and IGHJ gene repertoires in IDJRs vs. CE-VDJRs in CLL provides further support for the notion that CLL development is a directed rather than stochastic process.

3.25

THE ANALYSIS OF THE EXOME OF B-CLL PATIENTS WITH A HIGH NUMBER OF 13Q- CELLS REVEALS ALTERATIONS IN PI3K-MAPK PATHWAY AND CELL CYCLE CONTROL

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Patients with loss on 13q show the most favourable outcome in CLL. However, it has been reported that patients with CLL and a higher percentage of 13q- clonal lymphocytes show a more aggressive disease, and a poor prognosis than cases without cytogenetic aberrations. Aims: 1. To assess the biological differences in the group of B-CLL displaying different 13q- number of cells. 2. To analyse the alternative splicing in these groups of B-CLL. *Patients and methods*. Bone marrow from 113 patients with CLL was studied. In all cases clinical data, FISH and IGH somatic mutations were available: 75% of patients displayed citogenetic aberrations by FISH and 56% of them had IGH somatic mutation. Gene expression profile was analysed by oligonucleotide microarrays (Human Genome U133 Plus GeneChip, Affymetrix). In addition, exon array analysis (GeneChip Human Exon 1.0ST, Affymetrix) was carried out in 41 cases. Most of them had a del(13q) as a sole abnormality: 7 with a percentage of 13q- cells >80% (13qA group) and 6 with a percentage of 13q- cells<60% (13qB group). A total of 9 patients did not show any cytogenetic abnormality while the remaining 19 patients had other cytogenetic changes. To perform the analysis the following bioinformatics tools were applied: Cluster, TreeView, SAM, ASE and FIRMA (alternative splicing study). The results were confirmed by quantitative PCR. *Results.* Gene expression profile analysis by exon microarrays confirmed the results obtained with oligonucleotide microarrays. Alternative splicing analysis of both groups showed a total of 1042 genes with more than 30% of exons affected in the 13qA group. Most of these genes were involved in cell cycle regulation, which would be altered by the activation of PI3K-MAPK pathway, that releases the negative control over the cell cycle and by the alternative splicing of molecules that regulate the G1/S checkpoint: cyclins A, B, E and D; CdK1, CdK2, CdK6 and p18, as well as members of the family E2F. By contrast, no differences were observed in the expression profile or in the alternative splicing between the 13qB group and the patients without cytogenetic alterations. This could be related to a similar clinical outcome of both groups. Aditionally, in those patients with cytogenetic aberrations associated to a poor survival (del(17p) and del (11q)), alternative splicing analysis showed a deregulation in both subunits of heat shock protein 90 (HSP90), a molecular chaperone recently targeted for cancer therapy. Alterations in alternative splicing of HSP90 were also observed in 13q- group. *Conclusions.* 1. Overall the correlation between the oligonucleotide microarrays and the exon microarrays is good. 2. Patients with CLL and loss of 13q are a heterogeneous group of cases: the number of cells affected by this cytogenetic aberration could influence the clinical outcome because of the involvement of genes involved in essential cellular functions. 3. Alternative splicing analysis can contribute to a better understanding of molecular mechanisms of B-CLL in the different genetic subgroups.

3.26

IDENTIFYING POTENTIAL THERAPEUTIC TARGETS IN CLL BY EXPRESSION PROFILING OF DNA REPAIR

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The future of therapy in CLL will be an individualised choice of agents depending on the individual molecular abnormalities of each leukaemia. DNA damaging agents have activity in the treatment of CLL patients but the magnitude of clinical benefit varies markedly. The capacity to accurately identify those patients with highly sensitive disease will facilitate optimal treatment selection. We therefore set out to profile DNA repair using quantitative RT-PCR (RT-qPCR) to assay the expression of a large panel of genes involved in DNA damage recognition, DNA repair and apoptosis. This necessitated the development of protocols to efficiently isolate CLL cells to high purity (Essakali, 2008) and the development of RT-qPCR platforms to simultaneously measure the expression of multiple genes under identical thermal cycling conditions (Mikeska, 2009). Optimal RT-qPCR conditions have been determined for more than 100 (102 at abstract submission) newly designed assays that are monitored using hydrolysis probes from the Universal Probe Library (Roche). This panel currently includes 73 DNA damage recognition and repair genes, selected other genes and 10 reference (housekeeping) genes. The assays show a much higher dynamic range, and "signal-to-noise" ratio than possible with array-based methodologies. Profiling of a panel of CLL patients is currently underway and the results will be presented.

This work was supported by a grant from the CLL Global Research Foundation. *Essakali S, Carney D, Westerman D, Gambell P, Seymour JF, Dobrovic A. Negative selection of chronic lymphocytic leukaemia cells using a bifunctional rosette-based antibody cocktail. BMC Biotechnol. 2008 8:6. Mikeska T, Dobrovic A. Validation of a primer optimisation matrix to improve the performance of reverse transcription - quantitative real-time PCR assays. BMC Res. Notes 2009 2:112.*

3.27

MUTATION SCREENING OF TP53 EXONS 2-11 IN CHRONIC LYMPHOCYTIC LEUKAEMIA USING HIGH RESOLUTION MELTING ANALYSIS

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Loss of 17p13 is a prognostic marker for poor survival and chemorefractoriness to alkylating agents and purine analogues in patients with CLL. The target of the 17p13 deletion is thought to be the TP53 gene. It has been recently demonstrated that TP53 mutations are an independent predictor of poor survival and chemorefractoriness in CLL. Consequently TP53 mutation analysis is likely to become an important tool in the stratification of CLL patients for appropriate treatment. TP53 mutations are spread throughout the gene but are thought to cluster within the DNA binding region, which stretches from the middle of exon 4 to the beginning of exon 9. However, to some extent the distribution may reflect the fact that the majority of studies limit their mutation detection to exons 5-8. We developed high resolution melting (HRM) assays to allow efficient high throughput screening of mutations throughout the entire coding region of TP53 (exons 2 to 11). At the time of submission of this abstract, we have screened 25 CLL patients using these HRM assays followed by sequencing of amplicons with aberrant melting patterns.We identified TP53 mutations in 40% (10/25) of the CLL patients. Eight mutations were situated within exons 5-8 (1 nonsense, 6 missense and 1 insertion). Another nonsense mutation was detected in exon 9 outside the DNA binding domain. One patient harboured 2 alterations in exon 4, a nonsense mutation within the DNA binding domain and a synonymous change outside the DNA binding domain. In conclusion, HRM combined with sequencing of aberrantly melting amplicons is a cost-effective methodology for the rapid detection of mutations that can be used in the clinical decision making process. This work was supported by a grant from the CLL Global Research Foundation.

3.28

MONOCLONAL ANTIBODY RITUXIMAB SENSITIZES A PROPORTION OF CLL SAMPLES TO FLUDARABINE – SEARCHING FOR RESPONSIBLE GENES

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Background. Chronic lymphocytic leukemia (CLL) still constitutes an

incurable disease with a highly variable clinical course. Modern therapies, especially combined regimens of chemotherapeutics and monoclonal antibodies, may prolong progression-free survival as well as overall survival in a subset of patients. We have recently reported that monoclonal antibody rituximab sensitizes a significant proportion of CLL cultures *in vitro* to the subsequently administered fludarabine, regardless the presence of the high-risk genetic abnormalities, i.e. aberrations of the tumor-suppressors ATM and TP53 (which, however, influences the primary response to fludarabine on its own). *Aims.* The aim of the study was to define differentially expressed genes in CLL cultures sensitized by rituximab to fludarabine as opposed to cultures refractory to the sensitization. The selected genes might serve as the markers of sensitivity (or resistance) to the combination of these drugs and could further elucidate the mechanism of rituximab´s activity in CLL cells, which is poorly understood. *Methods.* Sixty primary CLL cultures were tested *in vitro* for their viability using WST-1 metabolic assay. Fludarabine was administered for 48h as a single agent (25; 6.25; 1.6 and 0.4 μg/mL) or in parallel on the cells pre-treated for 72h with rituximab in a standard *in vitro* dose (10 μg/mL). Twenty-one samples were significantly sensitized by rituximab (p<0.01). The most prominently sensitized samples were subsequently taken for the microarray analysis and compared with non-sensitized samples. In each sample the treated cells were compared with control cells on the microarray (Agilent Technologies, 4x44K microarray). *Results.* We observed up-regulation of several genes in non-sensitized samples, while the same genes were down-regulated in sensitized samples. The opposite effect has not been, surprisingly, observed. Among the identified genes were the following interesting candidates: metallothionein 2A (MT2A), which has been reported in drug efflux function; ribonucleotide reductase M2 subunit (RRM2), connected with drug resistance; topoisomerase II α (TOP2A), reported to influence a response to therapy and DNA directed polymerase theta (POLQ), implicated in cellular resistance to interstrand DNA cross-linking agents. Summary / *Conclusions.* The obtained data point to very interesting genes, which might be responsible for the observed sensitization effect. Their relevance will be further validated on the larger cohort of samples and by using quantitative real-time PCR. *Supported by grants IGA MH CR No. 9301-3/2007 and No. NR9858-4/2009.*

3.29

A BLOCK IN PRDM1 EXPRESSION PREVENTS ANTIBODY SECRETION AND PLASMA-CELL DIFFERENTIATION IN THE MAJORITY OF CLL CLONES

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CLL cells are thought to be stimulated and selected by (auto)antigen, yet their capacity to progress beyond their state of continuous activation and undergo differentiation appears to be defective. This abnormality may be central to the pathogenesis of CLL, as failure to induce antibody secretion and target and eliminate the stimulating antigen is likely to perpetuate the disease. Furthermore, inducing plasma-cell differentiation causes cell cycle arrest in normal B-cells and such induction may consequently be of interest as a therapeutic approach to the malignancy. Several older studies have shown that a number of stimuli (usually non-physiological) induce immunoglobulin (Ig) secretion and plasma-cell morphology in a proportion of CLL clones. However, these studies were performed before recent discoveries concerning the transcriptional control of Ig secretion/plasma-cell differentiation in normal B cells. Therefore, the aim of this study was to characterise the transcriptional control of CLL-cell differentiation. We initially decided to focus the study around the transcription repressor PRDM1 and the cytokine IL-21. PRDM1 has been described as the master regulator of plasma-cell differentiation and has not been examined in CLL. Furthermore, the cytokine IL-21 is the most potent physiological inducer of plasma-cell differentiation and its effects on CLL-cell differentiation have not been previously studied. When purified CLL cells are treated with IL-21 for 5 days, the cytokine was able to induce Ig secretion in some clones but not others. Directly ex vivo, CLL-cells expressed little or no PRDM1 mRNA and protein. Treatment for 48 hours with IL-21 caused detectable levels of PRDM1 protein in 14/40 purified CLL clones and only those that up-regulated PRDM1 expression increased their Ig secretion (termed 'responsive'). It is known that co-stimuli greatly enhance the ability of IL-21 to induce PRDM1 protein in normal B cells, and this was also true for responsive CLL clones. Culturing responsive CLL cells with IL-21+co-stimuli induced a pattern of transcription factor expression indicative of plasma-cell differentiation (IRF4(high), Pax5(neg), XBP-1(s)(high)) and, this was similar to that induced in normal B-cells. Conversely, the same stimuli still did not induce substantial amounts of PRDM1 in non-responsive CLL clones, and these cells failed to induce other transcription factors associated with plasma-cell differentiation. When CLL-cells were treated with a range of T-independent and non-physiological stimuli that induce PRDM1 in normal B-cells, these stimuli also induced PRDM1 only in those clones that responded to IL-21. In the limited number of cases studied, the ability to induce PRDM1 did not correlate significantly with prognostic markers for the malignancy, but may be related to the IgV(H) gene usage implying a link to their specific *in vivo* antigen (e.g. 7/7 clones with V(H)1-69 were non-responsive, while 3/3 V(H)3-21 were able to up-regulate PRDM1). To conclude, the present work has identified a block in PRDM1 expression in approximately 65% of CLL clones. This block is likely to be of importance in the pathophysiology of CLL and work aimed at identifying the cause of this block is ongoing.

3.30

GLIOTOXIN IS A POTENT NOTCH2 TRANSACTIVATION INHIBITOR AND EFFICIENTLY INDUCES APOPTOSIS IN CLL CELLS AND IN NOTCH2 ASSOCIATED MALIGNANCIES

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CLL is characterized by the clonal expansion of mature $CD5^+$ B-cells which seem to be resistant to apoptosis *in vivo*. We have shown that NOTCH2 signaling is involved in the overexpression of CD23 in CLL lymphocytes and there is emerging evidence that NOTCH2 has also an antiapoptotic role in the leukemic cells. NOTCH2 belongs to a highly conserved family of transmembrane receptors that regulate a wide variety of cellular differentiation processes including stem cell renewal, binary cell fate decisions, and adult tissue homeostasis. In murine B-cell development, Notch2 is involved in the development/homeostasis of self-reactive $Cd5^+$ (B-1a) whose human equivalents are considered as one of the possible counterparts of CLL cells. Therefore, targeting NOTCH2 might be of therapeutic relevance in CLL. The aim of this study was to search for compounds that interfere with NOTCH2 signaling at the transcription factor level. As a screening tool, we took advantage of electrophoretic mobility shift assays (EMSA). Using this strategy, we identified the fungal secondary metabolite Gliotoxin as a potent NOTCH2 transactivation inhibitor. 0.2 μM Gliotoxin completely blocked the formation of DNA-bound NOTCH2 complexes in CLL cells. The inhibition of NOTCH2 signaling by Gliotoxin was associated with downregulation of surface CD23 expression (mean±SD: 42±33 vs. 86±14; n=20) and induction of apoptosis (mean±SD: 70±29 vs. 13±14). In contrast, normal PBMCs and NOTCH negative leukemic cell lines (Jurkat, KL562, SH3, RL7) were less sensitive to Gliotoxin. The NOTCH2 inhibiting and apoptosis inducing effect of Gliotoxin was not restricted to CLL lymphocytes and was also observed in NOTCH2 positive cell lines derived from melanoma (MelJuso, 518A2) and pancreas-carcinoma (Panc1). In a melanoma xenotransplant mouse model, a single dose of Gliotoxin was able to reduce the tumour volume up to 50%. In summary, we identified Gliotoxin as natural NOTCH2 transactivation inhibitor and showed that Gliotoxin possesses a strong antitumour activity in primary CLL cells and in cell lines derived from melanoma and pancreas-carcinoma.

3.31

COMPARATIVE STUDY OF COPY NUMBER VARIATIONS IN B-CLL USING BAC ARRAYS AND OLIGONUCLEOTIDE MICROARRAYS

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The presence of genetic abnormalities is associated to the prognosis in B-CLL. Array comparative genomic hybridization (aCGH) is a genome-wide screening method providing in a single experiment, a general view of genomic gains and losses. Therefore the use of aCGH could detect new genetic lesions in B-CLL. *Aims.* 1. To compare two arrays platforms: BAC and oligonucleotide microarrays. 2. To study the copy number variations in B-CLL cytogenetic subgroups. *Patients and methods.* Seventy seven patients with B-CLL at diagnosis were selected; 75% of patients displayed cytogenetic aberrations by FISH and 56% of them had IGH somatic mutation. All samples were hybridized with a 1Mb BAC array which contains 3299 clones, kindly provided by the Sanger Institute, Cambridge (UK). The data analysis was carried out with GenePixPro 4.0. To study the gains and losses a binary segmentation method was applied. Ten additional DNA samples from pure CD19$^+$ cell populations, isolated by magnetic beads, from B-CLL patients were also hybridized. In a selected group of patients, the results were confirmed by oligonucleotide arrays (NimbleGen), MLPA, and FISH. *Results.* i) The B-CLL patients with a clonal B-cell infiltration >30% and an optimal quality sample and chip hybridization (SD<0.5) showed an excellent correlation between FISH, MLPA and both microarray platforms data. In the group of patients with del(13q), the alteration was detected only in the 50% of cases. The analysis of enriched CD19$^+$ cells improved the results in all the techniques, as well as the correlation of the results. ii) Almost all the cases analyzed showed aberrations by aCGH. Besides the alterations reported by FISH (11q-, +12, 11q-, and 17p-), other recurrent changes were detected. Thus gains in 1q31-qter, 17q21.2, 20q13 and 22 were observed in more than >35% of patients, whereas losses were observed in 2q21.2-q24, 5q14-q23, 11q14-q23 and 14q32.11-q32.33 (>30%). These aberrations were also detected in the 40% of B-CLL cases displaying normal FISH. *Conclusion.* i) Our study stresses the importance of sample quality in the use of genomic arrays techniques, demonstrating that the relevance of the use of enriched CD19$^+$ cell populations. ii) Genomic arrays detect new recurrent alterations in B-CLL, which can be also observed in patients with normal FISH. Our data confirms the heterogeneity of B-CLL.

3.32

CD38 GENE POLYMORPHISM IN CHRONIC LYMPHOCYTIC LEUKEMIA PATIENTS WITH RICHTER TRANSFORMATION

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Background. The clinical course of chronic lymphocytic leukemia (CLL) is associated in 2-8% cases with transformation to Richter syndrome (RS), represented in most cases by diffuse large B-cell lymphoma. Molecular markers of the risk as well as mechanisms involved in the transformation of CLL to RS are still poorly studied. Some recent findings suggest that CD38 genotype and usage of specific immunoglobulin variable (IgHV) genes might be risk factors of RS development. Aims: To evaluate association of CD38 single nucleotide polymorphisms (SNP) - intronic rs6449182 (184C>G) with transformation to RS in patients with CLL, and to analyze IgHV/D/HJ rearrangement patterns. *Methods.* CD38 rs6449182 (184C>G) polymorphism was genotyped using polymerase chain reaction (PCR) and restriction fragment length polymorphism analysis (RFLP). Genomic DNA was amplified in PCR using primers designed by Aydin *et al.,* 2008, followed by enzymatic digestion of PCR products. The digested products were resolved on 4% agarose gel and analyzed. *Results.* We examined the frequency of CD38 rs6449182 (184C>G) polymorphism in a total of 178 CLL patients (25 patients with RS and 153 patients without transformation as a control CLL group), and in the group of 74 health controls. We found that the frequency of variant allele CD38 rs6449182 G in control CLL group was comparable to health control (0.28 and 0.26 correspondingly), while in CLL with RS it was significantly higher (0.46). At the same time the frequency of homozygous CD38 rs6449182 GG genotype was found higher in both CLL groups in comparison with health control, though CLL with RS differed more significantly (13,1% and 36% vs. 4,1%, correspondingly, $p<0.05$). Since RS developed in previously treated patients only, we evaluated the CD38 rs6449182 (184C>G) genotype distribution in previously treated patients from CLL control group with follow up period at least 3 years (72 cases). That patients were uncommon homozygous for CD38 G variant allele in comparison with RS patients (9,7%, $p<0.01$). The IgHV/D/J rearrangement pattern data were available for 24 of 25 CLL patients with RS. Most cases (18/24) expressed unmutated IgHV genes, and 6 cases were considered mutated. Seven cases (29.2%) expressed stereotyped complementarity-determining region 3 (HCDR3) and were referred to 7 different stereotyped CLL clusters. The most frequently used IgHV gene in CLL group with RS similar to control CLL group was IgHV1-69 gene (8 cases). It was of interest that 5 of 6 mutated RS cases were homozygous for CD38 rs6449182 G allele and one case was heterozygous. *Summary*. Our results suggest about possible role of CD38 gene polymorphism in Richter syndrome development in CLL. Patients homozygous for CD38 rs6449182 G variant allele may be considered as a risk group for Richter transformation under chemotherapy.

Reference

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3.33

IGHV3-21/SUBSET-2 CLL ANTIBODIES BIND TO GASTRIC MUCOSAL ANTIGENS

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The IGHV gene mutational status is an important prognostic factor in CLL. Patients with mutated IGHV genes show significantly longer survival than unmutated cases. CLL patients with mutated IGHV3-21 genes, however, have a shorter survival compared to other mutated CLL cases. CLL cells have been shown to react with autoantigens exposed on apoptotic cells including oxidized epitopes. We recently showed that IGHV3-21M recombinant IgM belonging to homology subset 2 (rVH3-21) binds to glands in human gastric mucosa. To further characterize the antigen specificity of this CLL Ab, we collected corpus biopsies from seven *H. pylori*$^+$ patients with non-atrophic gastritis and from seven healthy controls. The binding pattern of rVH3-21 to human gastric mucosa corpus biopsies was analyzed by immunohistochemistry. Abs from another IGHV3-21M case (patient ID-23) belonging to subset 2, was also tested on two of the biopsies. rVH3-21 IgM bound to glands in three of seven corpus biopsies from *H. pylori*$^+$ patients, and showed staining of the connective tissue in all of the biopsies. In the biopsies from healthy controls, rVH3-21 bound to glands and connective tissue in six of seven biopsies. The ID-23 IGHV3-21M IgM did not bind to any of the two normal gastric mucosa biopsies tested, possibly due to a slightly different HCDR3 sequence (and thereby epitope specificity) as compared to rVH3-21 Ab. The results from this study indicate that there is an antigen present in gastric mucosal glands that is recognized by Abs from some but not all subset 2 CLL patients. This autoantigen is present in both normal and non-atrophic *H. pylori*$^+$ gastric mucosa from corpus. The exact nature of the antigen is currently under detailed structure analysis.

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THE NORMAL IGHV1-69-DERIVED B CELL REPERTOIRE CONTAINS "STEREOTYPIC" PATTERNS CHARACTERISTIC OF UNMUTATED CHRONIC LYMPHOCYTIC LEUKEMIA

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The cell of origin of Chronic lymphocytic leukemia (CLL) has long been sought and immunoglobulin gene analysis provides an important key to understanding pathogenesis and behavior of B cell tumors. The immunoglobulin heavy variable gene (IGHV) status has clinical relevance in CLL, where two subsets, delineated by the absence or presence of somatic mutation, have markedly different prognoses. The unmutated subset (U-CLL), of inferior prognosis, appears to derive from a pre-germinal center B cell. In the unmutated subset (U-CLL), there is strikingly increased usage of the 51p1-related alleles of the IGHV1-69 gene, often combined with selected IGHD genes and with IGHJ6. Shared sequence "stereotypic" characteristics of the HCDR3 result, and suggest antigen selection of the leukemic clones. We have now analyzed 147 51p1/IGHJ6 combinations in normal blood B cells from 3 healthy individuals (>51yr) for parallel sequence patterns. A high proportion (49/147, 33.3% of sequences) revealed stereotypic patterns, several (22/147, 15.0%) being similar to those described in U-CLL. Additional CLL-like stereotypes, not yet described, were detected in 7/147 sequences (4.8%). CLL-independent stereotypes were also found in 20/147 (13.6%) 51p1/IGHJ6 combinations. The HCDR2-IGHJ6 sequences were essentially unmutated. Junctional amino acid analysis revealed that patterns shared between cases of CLL were also evident in normal B cells. Normal B cells expressing 51p1-derived IgM (4.8% of all B-cells) had a phenotype of naïve B-cells, similar to 51p1-negative ($CD27^-$) B cells, i.e. IgM^+ IgD^+ $CD23^+$ $CD38^+$, and had a small percentage of $CD5^+$ B cells, not found in the memory B-cell subset. This snapshot of the naïve B-cell repertoire reveals subsets of B cells closely related to those characteristic of CLL. Conserved patterns in the 51p1-encoded IgM of normal B cells suggest a restricted sequence repertoire shaped by evolution to recognize common pathogens. Proliferative pressure on these cells is the likely route to U-CLL.

3.35

THE DLEU2/MIR-15A/MIR-16-1 LOCUS CONTROLS B-CELL COMPARTMENT EXPANSION AND ITS DELETION LEADS TO CHRONIC LYMPHOCYTIC LEUKEMIA IN MICE

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Chronic lymphocytic leukemia (CLL) is an indolent disease characterized by the malignant transformation of mature B cells. The monoallelic or biallelic deletion of the chromosomal region 13q14 is the most common genetic aberration found in CLL cells, and is also found in monoclonal B lymphocytosis (MBL), which is occasionally preceding CLL, in B-cell non-Hodgkin lymphoma (B-NHL) and in other malignancies, suggesting the presence of a tumor suppressor. The minimal deleted region (MDR) at 13q14 contains two genetic elements: DLEU2, and the microRNAs miR15a/miR16-1. The DLEU2 gene encodes for a long (1-1.8Kb) spliced cytosolic non-coding RNA whose transcription is necessary for miR-15a/miR16-1 expression. To investigate the respective roles of DLEU2 and miR-15a/miR-16-1 in tumor suppression, we studied the consequences of eliminating these genetic elements *in vivo* by establishing two transgenic mouse lines that carry deletions of either the ~120 kb large MDR, including DLEU2 and miR-15a/miR-16-1, or of the miR-15a/miR-16-1 cluster only. In addition, we tested the phenotypic effects of the re-introduction of DLEU2 or miR15a/miR-16-1 in a 13q14-/- CLL human cell line. Young MDR-/- and miR-15a/miR-16-1 -/- mice did not display phenotypic abnormalities, and showed normal lymphoid development. However, between 12 and 18 months, both MDR-/- and miR-15a/miR-16-1 -/- mice developed MBL, CLL with multiorgan involvement, or CD5- NHL subtypes. Analogous lymphoproliferations were observed, although at lower frequencies in heterozygous mice, suggesting a haploinsufficient mechanism for the 13q14 locus. While the deletion of only the microRNA cluster was sufficient to cause disease, the MDR-/- mice succumbed to their disease significantly earlier than miR-15a/miR-16-1 -/- mice, suggesting that the deletion of the entire DLEU2 locus, as usually found in human CLL, is also contributing to malignancy. In order to investigate the biological role of miR-15a/miR-16-1 deletion in B cells, we first examined the phenotype of mice miR-15a/miR-16-1 -/- B cells *in vitro* upon stimulation of the BCR with anti-IgM. A higher percentage of cells in the S phase of the cell cycle, as measured by incorporation of BrdU, as well as a faster phosphorylation of the retinoblastoma protein, along with other signs of cell cycle progression, was observed in the miR15a/miR16-1 -/- B cells when compared to wild type B cells, suggesting a direct role of miR15a/miR16-1 in impairing B cell proliferation. To further confirm this role we studied the effects of miR15a/16-1 expression in a human 13q14 -/- CLL cell line. Their inducible expression resulted in impaired proliferation and in an accumulation of cells at the G0/G1 phase of the cell cycle. In order to identify the mechanisms by which miR-15a/miR-16-1 control B cell proliferation, we identified their target genes by analyzing the protein levels of computationally predicted targets upon miR15a/16-1 expression in the 13q14 -/- CLL cell line as well as after anti-IgM stimulation of murine miR15a/16-1 -/- B cells. In conclusion, these results indicate that the 13q14 region contains a locus controlling the expansion of the B-cell compartment that includes both CD5 + and CD5 -B cells. The DLEU2/miR15a/miR16-1 locus functions as a tumor suppressor, whose deletion leads to the development of indolent lymphoproliferative disorders in mice, which recapitulate the spectrum of CLL-associated phenotypes in humans. Thus these mice may represent a useful model for identifying lesions associated with CLL progression and for the preclinical testing of novel therapeutic modalities.

3.36

TRANSCRIPTIONAL SILENCING OF FOXD3 IS AN EARLY EVENT MEDIATING EPIGENETIC SILENCING IN TCL1 POSITIVE CHRONIC LYMPHOCYTIC LEUKEMIA

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Epigenetic alterations are a hallmark of almost every malignancy. Alterations can be measured as DNA methylation and are either gains of methylation on DNA sequences normally unmethylated or losses of DNA methylation on sequences methylated in the healthy tissues. Epigenetic alterations change gene expression but they are reversible therefore now are under investigation as potential therapeutic targets. In our previous study, we utilized mouse model of chronic lymphocytic leukemia (CLL), Eu-TCL1 mice to determine the timing and patterns of aberrant DNA methylation. The results suggest that CLL cells from this mouse model recapitulate epigenetic alterations seen in the human disease. The accumulated number of methylated genes correlates with disease development. And aberrant methylation of promoter sequences is observed as early as three months of age in these animals, well prior to disease onset. In this study, we aimed to understand the mechanisms that lead to aberrant DNA methylation to seek for the potential therapeutic targets. The western analysis results on B cell samples from TCL1 mice revealed the increased level of DNA methyltransferase (DNMT) 3A and 3B comparing with that in the B cells from age-matched wild type mice. Since DNMT3A and 3B are essential de novo DNMTs in normal development, the targeted sequences of aberrant DNA methylation were further studied. Using MATCH program, we searched the differences in predicted transcription factor binding sites within promoter regions of methylated genes as compared to unmethylated genes. Our data showed that genes methylated in murine and human CLL were significantly enriched with Foxd3 promoter sites (5'-A[AT]T[AG]TTTGTTT-3') (p=0.007), these results were verified by chromatin-immunoprecipitation (ChIP) assay. Importantly, the expression of FOXD3 is negatively regulated by TCL1. We therefore hypothesized that the loss of FOXD3 might contribute to some

of the methylated targets identified in mouse and human CLL. In the support of this hypothesis, transfection of FOXD3 shRNA in Jurkat T lymphocytes resulted in the silencing and methylation of FOXD3 targets. The elevated expression of targets was only shown in FOXD3 overexpressed Raji cell line after the removal of DNA methylation by demethylation agent. In order to understand the regulation of FOXD3 by TCL1, we interrogated mouse and human Foxd3 promoters for binding site of transcription factors that could be involved in signaling pathways relevant to TCL1 and to CLL pathogenesis. We identified a highly conserved putative NF- B binding site in both mouse and human FOXD3 proximal promoters. Furthermore, we found the silenced Foxd3 in one month old TCL1 mice by a deregulated NF B p50:HDAC1 repressor complex as a consequence of the over-expressed TCL1. In contrast, normal B-cells expressed Foxd3 and had no evidence of this co-repressor binding to the Foxd3 promoter. Similar silencing of FOXD3 by this same co-repressor complex was also found in human CLL but not in normal B-cells. Reversal of this complex with inhibitors of NF- B was noted to promote significant re-expression of Foxd3 in CLL cells but not normal B-cells providing rationale for early targeting of this specific re-repressor complex in human CLL.

Cellular biology

4.1

OXIDIZED LDL AND CPG INDUCE CLONAL EXPANSION OF SUBSET-1 CLL CELLS

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Immunoglobulin VH gene usage in B-cell chronic lymphocytic leukemia (CLL) is biased and distinct from normal B cells. Subgroups of patients express B-cell receptors with remarkably similar amino acid sequences implying a role for antigens in the leukemogenesis. The nature of these antigens has however until recently been unknown. Studies from our group have shown that CLL antibodies bind to oxidized low density lipoprotein (LDL) and oxidized epitopes on apoptotic cells and bacteria. Whether these antigens can activate and drive the cells into proliferation and differentiation is, however, yet unknown. Subset-1 is the largest defined CLL subset and patients included in this group suffer from bad prognosis. IgM from subset-1 CLL cells bind specifically to malondialdehyde (MDA)-LDL. In the present study, proliferation and differentiation of five subset-1 CLL patients and two control patients were analysed after the addition of MDA-LDL and/or CpG ODN. After 72 h stimulation, the amount of BrdU incorporation was measured with flow cytometry, and IgM secretion was analyzed using ELISA. The antigen, MDA-LDL, alone gave no response at all. Noteworthy, the combined treatment with MDA-LDL and CpG gave a significant increase in proliferating $CD5^+/CD19^+$ cells and a significant increased IgM-secretion compared to CpG alone. CLL mAbs can bind to proteins complexed with DNA that translocate to the apoptotic cell membrane. Upon endocytosis of the BCR, the nucleic acid is delivered to toll like receptor-9 (TLR-9). In this way BCR and TLR9 co-signalling enhance CLL cell survival and expansion. In conclusion these preliminary data indicate an important role for the antigen, ox-LDL when combined with CpG in the clonal expansion of subset-1 CD5+B-CLL cells.

4.2

HS1 HAS A CENTRAL ROLE IN LEUKEMIC B CELLS TRAFFICKING AND HOMING

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In Chronic Lymphocytic Leukemia (CLL) the relationships between cell proliferation and accumulation within lymphoid organs are unclear, as are the rules that control CLL cell migration and re-circulation. We previous demonstrated that Hematopoietic cell specific Lyn substrate 1 (HS1) is involved in the natural history of CLL (Scielzo et al JCI 2005). Furthermore, we have shown that HS1 interacts with distinct cytoskeleton adapters and is likely involved in cytoskeleton reorganization (Muzio *et al*., Leukemia 2007). Given the role played by the cytoskeleton in controlling cellular shape, mobility and homing, we hypothesized that HS1 could be potentially relevant in the regulation of CLL cells infiltration into lymphoid tissues and re-circulation between peripheral blood and tissues. To study HS1 function *in vitro*, we silenced its expression in a CLL cell line (MEC1), and we tested its capacity to migrate in an *in vitro* and *in vivo* assay. We also analysed B cells from HS1 KO mice (Fukuda *et al.*, EMBO J. 1995) and we showed that in both cellular systems HS1-deficient B cells are severely impaired in their spontaneous migration capacity. A decrease in F-actin polymerization and an increased aggregation ability was also evident in cells lacking HS1. We then observed that HS1 is central for the initiation and maintenance of B-Cell Receptor (BCR) signalling events. Indeed in the absence of HS1,

cells failed to form actin-myosin complexes thereby leading to an instability of the cell signalling complex, in particular of the signalosome complex proximal to the BCR. Lyn, VAV, HIP-55, SHP-1, RAC1/2 and Erk were directly affected by the absence of HS1 while Syk, PLC- 2, Blnk and NFAT appear to be unaffected. To test *in vivo* HS1 function, we injected MEC1 cells silenced for HS1 subcutaneously in RAG2-/- c-/- mice and observed that cells lacking HS1 spread and localized preferentially in the BM and in the lymph nodes as compared to control cells. To further investigate its role played in the onset and progression of CLL, we crossed HS1 KO mice with Eμ-TCL1 transgenic animals (Bichi R *et al.*, PNAS 2002). The double HS1 KO/TCL-1 interestingly show a preferential accumulation of monoclonal $CD19^+CD5^+$ cells, beside all other lymphoid organs, in the bone marrow where they are usually observed at low frequencies in the Eμ-TCL1 mouse. These findings suggest that HS1 has a crucial role in controlling cell migration and tissue invasion by leukemic B cells, likely through its involvement in cytoskeleton organization. This points at HS1 as a target for development of novel cancer treatments, aiming at interfering with the lymphoid tissue infiltration and invasion which is characteristic of the disease.

4.3

A NOVEL RAG2-/-γC-/–XENOGRAFT MODEL OF HUMAN CHRONIC LYMPHOCYTIC LEUKEMIA

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Easily reproducible animal models that allow to study the biology of chronic lymphocytic leukemia (CLL) and to test new therapeutic agents have been very difficult to establish. In contrast to previous studies where MEC-2 transplanted nude mice failed to develop B-CLL (Loisel S 2005, L Res), we have developed a novel transplantable xenograft murine model of CLL by engrafting the cell line MEC1 into rag2-/-γc-/- mice. These mice lack B, T and NK cells, and, in contrast to nude mice that retain NK cells, appear to be optimal recipient for MEC1 cells, which were successfully transplanted through either subcutaneous or intravenous routes. The engraftment efficiency was 100% and tumor growth was very rapid; it resulted in a systemic involvement of the spleen, BM, liver and lungs closely resembling aggressive human CLL. In addition, administration of fludarabine alone or in association with cyclophosphamide showed a therapeutic efficacy, indicating that this CLL xenograft model could act as a reliable preclinical tool for drug investigation. This model could be very useful for evaluating the biological basis of CLL growth and dissemination as well as the efficacy of new therapeutic agents.

4.4

THE FUNCTIONAL RESPONSE TO CD40 LIGATION REFLECTS A DIFFERENT CLINICAL PROGNOSIS IN CLL PATIENTS

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Chronic lymphocytic leukemia (CLL) is characterized by the accumulation of monoclonal $CD5^+$ B cells in lymphoid organs. There is substantial evidence that CLL cells retains the capacity to respond to microenvironment-derived stimuli similar to those occurring with normal B lymphocytes, including signals through the CD40 molecule. CD40 stimulation rescues CLL cells from apoptosis and/or induces their proliferation through the up-regulation of apoptosis-regulatory proteins and modulates the expression of chemokines and surface molecules. That notwithstanding, not all CLL clones respond to CD40 stimulation suggesting the existence of functional subsets of the disease. We investigated the functional response of CLL cells exposed *in vitro* to soluble CD40 ligand (sCD40L) and its clinical significance in a cohort of 62 patients. Cells from 34/62 (55%) patients showed extended viability, upregulation of CD95 and CD80, induction of an RT-PCR band specific for CCL22/MDC and CCL17/TARC, secretion of CCL22/MDC (CD40L-responders). The remaining 28 patients showed no modifications or marginal changes in only one assay and they were defined CD40L-non-responders. Such difference was also confirmed at biochemical level, by the analysis of the signaling pathways originating from CD40 as CD40L stimulation induced upregulation of Bcl2 and increase of IKK phosphorylation in responder cases only. Interestingly, non-responders had a worse clinical outcome as compared to responder CLL, in terms of clinical progression. These evidences link leukemic cells to microenvironment and indicate that those cases maintaining responsiveness to external stimuli behave more favorably, likely because of a tighter control by physiological signals normally acting on B cells. Our results may help to distinguish patients with opposite clinical prognosis and, in particular, to predict patient response to CD40-based therapies, currently tested in clinical trials.

4.5

NURSE-LIKE CELLS PROMOTE DIFFERENTIATION OF CLL CELLS VIA GALECTIN-1-DEPENDENT MECHANISMS

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Accumulation of neoplastic cells in CLL is conditioned by a variety of signals delivered by accompanying cells in lymphoid tissues, which provide an appropriate niche for differentiation and survival. We examined the relevance of galectin-1 (Gal-1), a glycan-binding protein with immunoregulatory activity, within the CLL microenvironment. *in vitro* exposure to Gal-1 (1-10 uM) promoted the differentiation of CLL cells in a saccharide-dependent manner by up-regulating the expression of CD86 and CD25, and favoring the release of IL-10 (n= 6, $p<0.01$). Of note, Gal-1 did not alter these parameters when added to tonsillar normal B lymphocytes although it markedly bound to these cells. As Gal-1 could affect leukemic cells by acting in an autocrine fashion or could be secreted by cells present in the CLL microenvironment, we further examined these posibilities. By flow cytometry analysis, we found no considerable expression of Gal-1 in freshly isolated CLL cells. However, we found high expression of Gal-1 in nurse-like cells (NLC) differentiated from $CD14^+$ peripheral leukocytes by co-cultured with CLL cells for two weeks. Both confocal microscopy and immunoblot analysis revealed selective expression of Gal-1 in NLC but not in leukemic cells. Remarkably, silencing of Gal-1 in NLC using siRNA-mediated strategies significantly reduced the expression of co-stimulatory molecules in CLL cells and the release of IL-10 to supernatants ($p<0.05$). In addition, using quantitative real-time RT-PCR analysis we found markedly reduced levels of the chemokine CCL3 but not CCL4 in leukemic cells co-cultured for 48 h with Gal-1-interfered NLC ($p<0.05$) and considerable reduction of the cytokine BAFF but not APRIL in NLCs ($p<0.05$). To determine whether Gal-1 is produced by cells surrounding leukemic lymphocytes *in vivo*, we analyzed bone marrow samples from CLL patients by immunohistochemistry and found high expression of Gal-1 in $CD68^+$ myeloid cells. Collectively, these results indicate that accompanying myeloid cells deliver differentiation signals to CCL cells via Gal-1-dependent mechanisms, suggesting that manipulation of Gal-1 expression in NLC or signaling in CLL may influence CLL survival, a critical effect with profound implications in the design of novel anti-leukemic therapies.

4.6

CD74 REGULATES B-CLL SURVIVAL AND MIGRATION TO THE BONE MARROW

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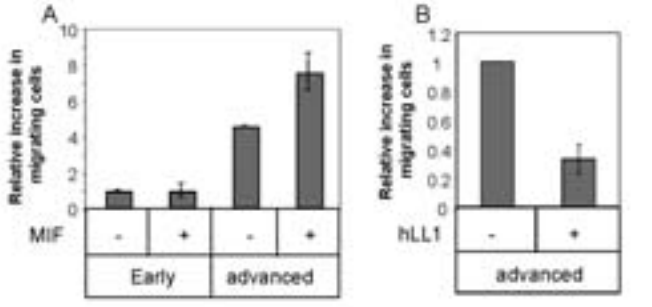
Background. CD74, an MHCII-related protein, is uniformly over-

expressed in CLL, and we have shown that it has a significant role in the increased survival of CLL cells from the very early stages of the disease (PNAS 104, 13408, 2007). In normal murine B cells, survival is induced by stimulation of cell-surface CD74 via expression of a transactivation-domain-containing isoform of p63 (TAp63). Recently, it was shown that, in addition to its known role in cell survival, this p53-family protein also modulates cellular adhesion via regulation of integrin expression. Integrins play a significant role in the survival and migration of CLL cells. Interaction of the integrin VLA 4 with its ligand fibronectin leads to decreased apoptosis and even resistance to fludarabine. A possible role for the alfa4 chain of VLA4 in disease progression has been suggested by the increased expression of this integrin in advanced-stage disease. Taken together, these facts led us to asses whether the effect of CD74 on CLL cell survival also involves increased expression of TAp63, and if this translates into increased migration and survival of the cells. Methods: B cells were purified from the peripheral blood of CLL patients in various stages of the disease. The following studies were performed, with and without activation of CD74 by its ligand MIF: the level of the alfa4 chain, transwell migration, and FACS analysis of population of cells migrating to the BM in a mouse xenograft model. *Results.* A statistically-significant difference was found in the level of alfa4 between early- and advanced-stage disease, in accordance with previous studies. Stimulation of CD74 by its ligand, MIF, led to increased expression of TAp63, resulting in elevated expression of α4. Accordingly, MIF also increased migration of CLL cells to the BM in a xenograft mouse model, an effect that was much more pronounced in advanced stage disease (figure 1A). In addition, TAp63 increased expression of bcl-2 with resultant decreased apoptosis. Interestingly, milatuzumab, a humanized monoclonal antibody directed at CD74, blocked all those effects, leading to increased apoptosis, decreased expression of CD49d and decreased migration to the BM (Figure 1B). *Discussion.* The results of our studies suggest an essential role for CD74 both in the initial pathogenesis of CLL, and in its progression. Although there is an increase in the baseline expression of the alfa4 chain of the integrin VLA4 in advanced as compared to early stage disease, this is much augmented following activation of CD74 with its ligand MIF. The result is a significantly-accelerated migration of CLL cells to the BM. We believe that this increased migration leads to the gradual occupancy of normal hematopietic niches, providing further survival signals to the malignant cells from the supportive bone marrow environment. The impressive effect of CD74 on both survival and migration of CLL cells makes milatuzuamb an attractive therapeutic option. A phase I-II clinical trial addressing its potential as a novel therapeutic agent in CLL is underway in our Institute.



Figure

4.7

CHRONIC LYMPHOCYTIC LEUKEMIC/NURSE-LIKE CELLS INTERACTIONS: RELEASE OF SOLUBLE FACTOR(S) THAT INHIBIT RITUXIMAB-DEPENDENT NORMAL B CELL DEPLETION, AND REVERSION BY LENALIDOMIDE

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Aims. Rituximab does not yield significant lymphocytic clearance of CLL cells in lymph nodes and bone marrow, due to putative Environment Mediated-Drug Resistance mechanisms (EM-DR). Among these, the impact of Nurse-Like Cells (NLC), a monocytic-derived population of CLL bystander cells, has never been adressed. METHODS 15 CLL patients (before treatment) were included in this study. According to NLC outgrowth kinetics (under culture conditions published by Burger J *et al.* : 10e.7 Peripheral Blood Mononuclear Cells/ml), conditioned medium was harvested after 15 days of CLL/NLC co-culture (CM NLC+CLL). For some experiments, CLL and NLC cells were separated and cultured for 2 additional days before conditioned medium (CM CLL or CM NLC) collection. In other experiments, Lenalidomide was added after 15d CLL+NLC co-culture (5 µM), and 48h later CM-LEN was collected. We used a classical normal B cell depletion test to assess the impact of the different CM on Rituximab (RTX)-dependent depletion. RESULTS As expected, nor adhesion onto NLC neither CM NLC+CLL provided a survival advantage to healthy donor derived B cells. Adding RTX to normal PBMCs triggered 40-70% B cell depletion after 24h, the extent of which correlated with NK and monocytes absolute numbers among PBMCs. B cell depletion was significantly reduced (by 20-25%, $p<0.05$) when normal PBMCs were cultured in CM NLC+CLL, but not in CM CLL or CM NLC alone, neither in CM PBMC (CM obtained from normal PBMCs cultured at high density (10e.7/ml) for 15d), thus indicating NLC+CLL co-culture was mandatory for this RTX resistance effect to occur. We hypothesized Antibody Dependent Cell Cytotoxicity (ADCC) and/or Phagocytosis modulation could happen in the presence of CM NLC+CLL. Phagocytosis of CFSE+ lymphoma cells by CD14+ normal monocytes was not inhibited by CM NLC+CLL in the presence of RTX 10 µg/mL (control 24.4% vs CM NLC+CLL 21.5%, p=ns). ADCC properties of normal NK cells were next explored. First, upon RTX treatment, ERK phosphorylation was decreased in NK cells treated with CM NLC+CLL as compared to control. Next, a significant decrease in both degranulation (CD107a expression after RTX 10µg/ml during 4h : control 50±7% vs CM 30±7%, $p<0.05$) and trogocytosis (PKH67 lipophilic dye transfer from lymphoma cells to CD3-/CD56+ NK cells : control 4000±1000 vs CM 2500±1000, $p<0.05$) were observed. This suggests that NK function was specifically hampered by CM NLC+CLL, accounting for the inhibition of B cell depletion. Indeed, ADCC of normal T lymphocytes was not significantly reduced under the same culture conditions. Yet, expression of activating/inhibitory receptors or adhesion molecules at the surface of NK cells were not modified by CM NLC+CLL. ELISA and cytokine arrays were performed on CM NLC+CLL to isolate specific chemokines/cytokines able to promote this NK inhibitory effect. Despite this approach, we were unable to find a specific «signature» of RTX resistance. Furthermore, trypsin digestion of CM proteins could not restore normal B cell depletion. Competitive saturation experiments indicated CD16 binding of RTX could be modified in CM NLC+CLL. We took advantage of this data to treat NLC+CLL after 15d co-cultures for 2 additional days with Lenalidomide, a well-known modulator of NK cells functions (with no direct cytotoxic effect on CLL or NLC *in vitro*. CM-LEN was completely unefficient in reducing B cell depletion. Our results show that Lenalidomide does not activate normal NK but rather perturbs the NLC/CLL dialogue (and the subsequent release of soluble factor(s)) to inhibit normal B cell depletion. *Conclusion*. We describe a new RTX resistance mechanism emerging from NLC+CLL interactions, involving specific NK cell-dependent ADCC inhibition, reversible upon Lenalidomide treatment. This is one of the first observations that EM-DR in CLL is a druggable target with Imids.

4.8

CYTOSKELETAL-ACTIVATION OF CLL CELLS IS NECESSARY FOR CHEMOKINE-MEDIATED MOTILITY AND DEPENDS ON SYK-MEDIATED SIGNALS DIRECTED THROUGH ABL

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Background. The neoplastic cells of chronic lymphocytic leukaemia (CLL) undergo directed migration into specific tissues where they interact with accessory cells, and where they receive signals that promote their proliferation and survival. Integrin-dependent adhesion and chemokine-directed migration are central to this process. The non-receptor protein tyrosine kinase ABL associates with the actin cytoskeleton, and has high expression in CLL cells. We therefore tested the role of ABL in controlling the adhesion and migration of CLL cells. *Methos and Results.* CLL cells adherent to fibronectin-coated surfaces form specific cytoskeletal-structures which can be observed in detail using fluorescent probes for polymerised actin, and visualised with confocal microscopy. These cytoskeletal structures may be evaluated statistically using measures of

cell shape (morphometrics). Within any CLL cell population we observed that a proportion of cells extended fine non-polar surface projections (filopodia) (Figure - panel A), while a variable proportion of cells underwent polarisation (with anterior lamellipodia and membrane ruffles) (Figure – panel B). Such responses are recognised to be linked to the phosphorylation and redistribution of ABL (ABL1 or ABL2) and its substrate protein CrkL. We found that during CLL cell polarisation, ABL2 and CrkL were both translocated to the anterior pole of the cell. Furthermore, using 1-D and 2-D SDS-PAGE and Western blotting we found that CLL cells have high levels of CrkL phosphorylation, and that CrkL phosphorylation is increased when compared with peripheral blood mononuclear cells. ABL phosphorylation of CrkL is inhibited using Imatinib at pharmacological doses (5 µmol/L). This inhibition of ABL signalling does not prevent adhesion, but does modify actin reorganisation: We found Imatinib-treated CLL cells did not form filopodia or lamellipodia ($p<0.0001$), instead the cells were smaller and adopted an essentially spherical form with numerous surface microvilli (akin to normal circulating lymphocytes) (Figure – panel C). Using a transwell migration assay, we found that the ABL-dependent cytoskeletal reorganisation was essential to allow the dose-dependent chemotactic response of CLL cells to appropriate chemokines (CXCL12 and CXCL13) ($p<0.05$). However CrkL phosphorylation, was not altered by chemokine exposure. These findings suggest that ABL-dependent pathways may "prime" CLL cells to allow them to respond to chemokines, although they may not directly mediate that response. In addition, we found that CrkL phosphorylation and the adhesion-induced shape-change of CLL cells are abolished by inhibition of Syk. *Conclusion*. We suggest that the ABL pathway mediates "cytoskeletal activation" of CLL cells. ABL links signals from the B-cell receptor associated kinase Syk (which is intrinsically activated in CLL cells) promoting specific adhesion-dependent cytoskeletal rearrangements. Those rearrangements are an essential prerequisite for chemokine-directed migration of CLL cells. Targeting the ABL-pathway therefore represents a possible approach to inhibit tissue migration and survival of CLL cells.

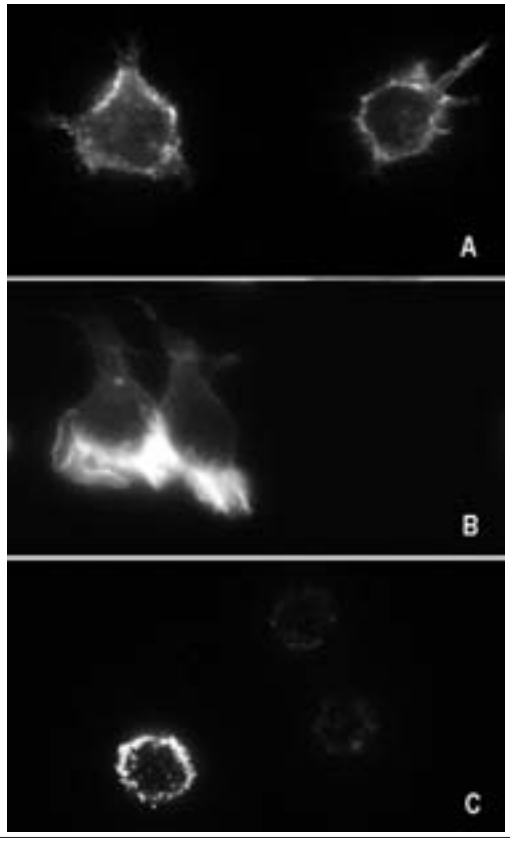

Figure 1.

4.9

INVESTIGATING THE DIRECT LINK BETWEEN NF-KAPPAB AND CELL SURVIVAL IN PRIMARY CLL CELLS

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A growing body of evidence shows that NF-kappaB, particularly the Rel A subunit, plays a role in CLL cell survival and may be useful for prognostication.[1,2] We have previously identified an inverse correlation between the levels of Rel A DNA binding and spontaneous CLL cell death.[1] By studying our patient cohort, we have now identified a strong correlation between Rel A and the cell survival protein, Mcl-1 (Figure, n=88, $r^2 = 0.41$, $p<0.0001$). In order to investigate whether there is direct link between the NF-kappaB and cell survival, we have infected primary CLL cells with lentiviruses to manipulate NF-kappaB. Using a lentivirus encoding an shRNA capable of reducing Rel A protein expression, we have investigated the role of this subunit on cell survival. Infecting primary CLL patient cells with the Rel A shRNA virus caused cell death at a higher level than that observed in cells infected with an empty virus or other lentivirus controls. This was observed for all the patient samples tested (n=8) and is the first piece of direct evidence showing the ability of Rel A to regulate primary CLL cell survival. We have observed that the Mcl-1 promoter has a very strong NF-kappaB binding site in its initiation start region and we are currently performing experiments to investigate whether there is a direct link between Rel A DNA binding and the transcription of the Mcl-1 gene.

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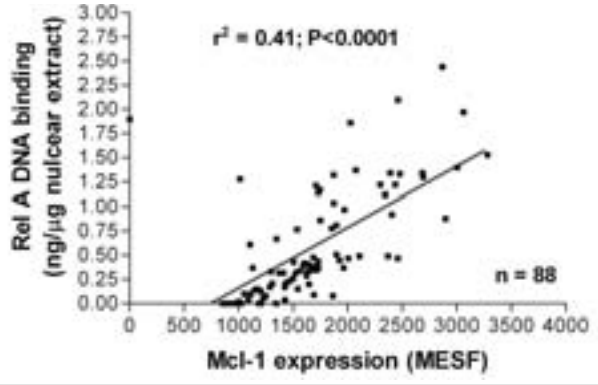

Figure 1.

4.10

RAP1 DYSFUNCTION AND THE REDUCED TISSUE HOMING OF CLL

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Lymphocyte motility and homing are necessary for an efficient immune response. It has long been known that these processes are altered in CLL and that the malignant cells usually have a reduced capacity to enter lymphoreticular tissues. The mechanisms involved in this altered homing are unknown. Since Rap is central to integrin activation by chemokines,[1] and such activation is necessary for lymphocyte motility, we have recently focussed on this GTPase. We have shown, that in the majority of CLL clones, Rap1 is constitutively GTP loaded and that the levels of Rap1-GTP are not increased in response to chemokine.[2] In addition, we found that stimuli which activate the GTPase via other GEFs (guanine nucleotide exchange factors) do not induce the GTP loading of Rap1. Culture of CLL cells for 48hrs resulted in a decrease in the levels of GTP-Rap1 and a restoration of chemokine responsiveness.

These data indicate that the Rap1-GTP loading of CLL cells is the result of stimulation *in vivo*.[2] We now show that the other molecular form of Rap, Rap2, is either absent in CLL cells, or is expressed at very low levels. Furthermore, when Rap2 is present, it is not GTP loaded either constitutively or in response to stimulation. We are therefore now concentrating our investigations on identifying the mechanisms responsible for the Rap1 dysfunction of CLL. Rap1-GTP loading is reversed by GAPs (GTPase activating proteins) of which SPA-1 is the principal form in lymphocytes. It therefore seemed possible that the altered Rap1 function of CLL cells is as a result of impaired GAP activity, more especially since a proportion of SPA-1 knockout mice develop CLL.[3] For this reason we examined levels of the GAP in CLL cells using RT-PCR and Western blotting. We found that, although the expression of SPA-1 varied between CLL clones, there was no correlation between amounts of the GAP and the ability of cells to GTP load their Rap1. We next hypothesised that chronic activation of the BCR is responsible for the inability of CLL cells to GTP load their Rap1, since signalling though the BCR is thought to be central to the pathogenesis of CLL,[4,5] and since such stimulation induces Rap1-GTP.[6] We found that prolonged BCR cross-linking of CLL clones which are able to GTP load their Rap1 resulted in these cells becoming unable to increase their Rap1-GTP and undergo transendothelial cell migration in response to chemokine. In conclusion, our studies indicate that chronic in-vivo stimulation, probably via the BCR, results in the reduced homing of CLL cells through a mechanism involving Rap1.

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4.11

HYPERFORIN INDUCES APOPTOSIS OF CHRONIC LYMPHOCYTIC LEUKEMIA CELLS THROUGH STIMULATION OF THE BH3-ONLY PROTEIN NOXA AND THE PRO-APOPTOTIC PROTEIN BAK

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We previously reported that hyperforin, a natural phloroglucinol purified from Hypericum perforatum, is able to re-establish *in vitro* the mitochondrial pathway of caspase-dependent apoptosis which is deficient in leukemic cells from the peripheral blood of CLL patients (Quiney *et al*., Leukemia 2006, 20:491). Here, we investigated the effets of hyperforin on the expression of several members of the Bcl-2 family which regulate the formation of pores at the mitochondrial membrane allowing the release of apoptogenic factors and the subsequent cascade of caspase activation (Willis & Adams, Cur Opin Cell Biol 2005, 17:617). The pro-apoptotic member Bak is known to be sequestered as an inactive complex with the anti-apoptotic protein Mcl-1, that is overexpressed by CLL cells and plays a crucial role in their apoptosis deficiency. Noxa is a pro-apoptotic BH3-only member which specifically interacts with Mcl-1. When the BH3 domain of Noxa inserts within the hydrophobic groove of Mcl-1, this displaces Mcl-1/Bak interaction, resulting in Bak release and activation. Noxa levels were reported to be inversely related to CLL cell survival capacity (Smit *et al.*, Blood 2007, 109:1660). Our results show that treatment of primary CLL cells from 12 patients with hyperforin induces the stimulation of Noxa without affecting notably Mcl-1 expression. Noxa stimulation is time and dose-dependent, and studies of mRNA expression are in progress. The stimulatory effect of hyperforin is accompanied by the formation of Mcl-1/Noxa complex and by the increased expression of the active form of Bak. In contrast, no Noxa stimulation is observed when apoptosis is triggered by flavopiridol which both inhibits Mcl-1 and increases active Bak. Our results indicate that hyperforin-promoted apoptosis of CLL cells is associated with the induction of the BH3-only Noxa, favoring the concept that hyperforin and agents capable of inducing or mimic Noxa represent a novel attractive strategy for CLL. The data also suggest different mechanisms of action for hyperforin and flavopiridol.

4.12

DICHOTOMY IN NF- B SIGNALING, BCL-XL EXPRESSION AND CHEMORESISTANCE IN IGVH MUTATED VERSUS UNMUTATED CLL CELLS UPON CD40L/CPG STIMULATION

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Chronic lymphocytic leukemia (CLL) cells circulating in peripheral blood (PB) differ in important aspects from the leukemic fraction in lymph nodes (LN). While PB CLL cells are prone to apoptosis, sensitive to various drugs and arrested in the G0/G1 phase of the cell cycle, LN cells have an anti-apoptotic profile, are drug-resistant and are dividing. CD40 stimulation of PB CLL cells *in vitro* results in chemoresistance and provides a partial model for the LN microenvironment. In cells derived from PB the TLR9 ligand CpG induces proliferation in IgVH unmutated CLL, but apoptosis in mutated CLL. In order to juxtapose proliferative with anti-apoptotic signals, we here analyzed the effect of CpG in the context of CD40 ligation in prognostic CLL subgroups. Time course analyses of CD40 ligation alone demonstrated classical followed by alternative NF- B activity in both subgroups, correlating with enhanced Bfl-1 levels and Bcl-XL levels, respectively. Blockade of classical NF- B activation by BAY-11-7082 resulted in abrogation of fludarabine resistance in mutated but not in unmutated CLL cells. A further dichotomy in NF- B signaling was observed by addition of CpG, which rendered mutated CLL sensitive to cytotoxic drugs, concomitant with declining alternative NF- B (p52) and Bcl-XL levels. The pivotal contribution of Bcl-XL to chemoresistance was demonstrated by the BH3 mimetic ABT-737 and also via specific RNAi. Finally, in ex vivo LN samples p65, p52, Bfl-1 and Bcl-XL levels were variably expressed but in general much higher than in PB samples. This corroborated the validity of the *in vitro* findings and showed that *in vivo* both classical and alternative NF- B signaling occur in LN. Thus, a novel distinction in NF- B activation and drug susceptibility in mutated versus unmutated CLL was uncovered, which was linked with the level of Bcl-XL expression.

4.13

PROTEOMIC ANALYSIS OF PROTEIN EXPRESSION FOLLOWING RITUXIMAB TREATMENT IN NORMAL AND CHRONIC LYMPHOCYTIC LEUKAEMIA (CLL) B-LYMPHOCYTES

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Introduction. As a single agent, the anti-CD20 monoclonal antibody rituximab shows dramatic clinical activity in many B-cell neoplasms, including relapsed/refractory low grade or follicular non-Hodgkin's lymphoma (NHL) and untreated follicular NHL. In contrast to the relatively high response rates to rituximab in NHL, responses to single-agent rituximab in CLL are relatively low. Reasons for this are unclear. Furthermore, the precise *in vivo* mechanism of action of rituximab is unknown. Therefore, the aim of this study was to elucidate protein pathways affected by rituximab in primary CLL cells (rituximab resistant) and normal B-cells (rituximab susceptible). Methods: PBMC from CLL patients (n=4) and normal donors (n=3) were isolated from whole blood using lymphoprep separation medium. B-lymphocytes were isolated using human CD19 MicroBeads (Miltenyi Biotech). $2x10^7$ B-lymphocytes were cultured for 6 hours ± 20 µg/mL rituximab and 50 µg/mL IgG (cross-linker). Cells were lysed, and proteins were separated into soluble and insoluble fractions by high speed centrifugation. 50 µg of treated and untreated samples were labelled with Cy3 and Cy5 dyes respectively. A pooled internal control lysate was labelled with Cy2 dye. Pools of treated, untreated and internal control lysates were mixed and separated by 2D electrophoresis. Gels were visualized using a Typhoon Variable Mode Imager, and analysed using DeCyder software (GE Healthcare). Differentially regulated proteins following treatment with rituximab were identified by mass spectrometry. *Results.* In the cytoplasmic fraction, filtering of the data identified 19 proteins as being differentially regulated following rituximab treatment in normal lymphocytes, and 18 proteins in CLL lymphocytes (≥10% change, $p<0.05$). Raf kinase inhibitor protein (RKIP) was up-regulated in CLL lymphocytes follow-

ing treatment with rituximab. Glutathione-S-transferase and superoxide dismutase (reactive oxygen species [ROS] scavengers) were up-regulated in CLL samples in response to rituximab, but were unchanged in normal lymphocytes. In contrast, peroxiredoxin 1, glutaredoxin-3, leukotriene A-4 hydrolase, isocitrate dehydrogenase, spermine synthase, glutathione-S-transferase and glutathione peroxidase were down-regulated following rituximab treatment in normal, but not CLL lymphocytes. *Conclusions.* RKIP is a negative regulator of the ERK1/2 signalling pathway. Up-regulation of RKIP following treatment with rituximab may contribute to the sensitisation of cells to further chemotherapy by decreasing expression of the anti-apoptotic protein Bcl-xL. An increase in ROS production due to the down-regulation of glutathione related enzymes may be an important determinant to the susceptibility (or resistance) to rituximab-mediated apoptosis *in vitro*. Rituximab-mediated cell death may be dependent on the generation of ROS. *Acknowledgements*. This work was supported by PhD scholarship from the Leukaemia Foundation of Australia for M. Sulda. Dr. Kuss is a member of the CLL Australian Research Council (CLLARC).

4.14

EXPRESSION AND FUNCTION OF TOLL-LIKE RECEPTORS IN CHRONIC LYMPHOCYTIC LEUKEMIA CELLS

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Mature B-lymphocytes can recognize microbial antigens via B-cell-receptor (BCR) in a specific way and via Toll-like receptors (TLR) in a costimulatory manner. TLR are best known for their role in host defence from infection; nevertheless, they have also been shown to play a role in tumor development. A wealth of information is gathering on the possible role of antigenic stimulation in the natural history of Chronic Lymphocytic Leukemia (CLL). However little is known regarding the repertoire and function of TLR in CLL cells. The TLR family includes 10 different transmembrane proteins devoted to recognize specific pathogen-associated molecular patterns and to alarm immunocompetent cells to trigger an immune response. Here, we studied fresh leukemic cells for the expression pattern of TLR1 to TLR10, NOD and SIGIRR (also known as TIR8). CLL cells were found to express several pattern recognition receptors including TLR1, TLR2 and TLR6. The specific TLR expressed by CLL cells were functional. Leukemic cells, upon stimulation with TLR1/2/6 ligands, such as bacterial lipopeptides, activated the nuclear factor-kappaB signalling pathway, expressed CD86 and CD25 activation molecules, and were protected from spontaneous apoptosis. These findings further support the hypothesis that CLL cells resemble antigen-activated B-cells and suggest a potential role of TLR in modulating CLL cell response in the context of specific antigen recognition.

4.15

CD38 AND CD49D ARE PHYSICALLY ASSOCIATED ON B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA CELL MEMBRANE AND COOPERATE IN SUPPORTING SURVIVAL OF NEOPLASTIC CELLS

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Background. Recent studies have documented that the simultaneous over-expression of CD38 and CD49d (integrin $\alpha4$) is part of the molecular signature characterizing a CLL subgroup with bad prognosis. Aim: The high correlation between CD38 and CD49d expression levels in CLL, and the known propensity of both CD38 and integrins to form supramolecular complexes, prompted us to investigate the relationship between CD49d and CD38 on the CLL membrane. The working hypothesis is that CD38 and CD49d are physically and functionally linked with the final effect of keeping CLL cells in lymphoid organs, where the microenvironment favors growth and survival. *Methods.* Confocal microscopy and biochemical approaches were used to study the membrane organization of CD49d and CD38 in primary CLL cells and other B cell lines. Detection of apoptosis was performed by flow cytometry. *Results.* Co-capping experiments in CLL cells and in the cell line models Raji and RPMI-8226 confirmed a membrane relationship between CD38 and CD49d. Anti-CD49d monoclonal antibodies (mAbs) induced capping in about 75% of CLL cells, with a 80% redistribution of CD38 in the context of the capping area. The same capping area stained for CD29 (integrin $\beta1$) indicating that CD49d is part of the $\alpha4\beta1$ (CD49d/CD29) complex. It also contained CD81, confirming the known association between this tetraspanin and $\alpha4$ integrins. Similar results were obtained with the cell lines Raji and RPMI-8226, both constitutively expressing CD38 and CD49d at high levels. The lateral association between CD38 and CD49d was next confirmed at the biochemical level by co-immunoprecipitation approaches. The results indicate that CD49d and CD38 are physically associated; furthermore, these experiments also evidenced that CD38/CD49d complexes are present both inside and outside the membrane cholesterol-rich raft domains. To further check whether the integrity of lipid rafts is necessary for the physical association between CD38 and CD49d, CLL cells were treated with methyl-β-cyclodestrin (MbCD) to deprive cell membranes of cholesterol, and cell lysates with octyl-D-glucopyranoside (ODG) to solubilize raft-associated proteins. Treatment with ODG revealed that a discrete amount of CD38/CD49d complexes could also be constitutively found outside raft domains. Consistently, co-capping experiments showed that CD49d-CD38 association was unaffected by lipid rafts disruption by MbCD. CD38-CD49d association was also maintained after polarization of CD49d to its natural ligand, as witnessed by a striking co-localization of CD49d and CD38 in cell uropods formed by CLL and Raji cells left to adhere and spread onto CS-1 fibronectin (FN) fragments, which specifically bind CD49d. Finally, we tested whether the simultaneous engagement of CD38 and CD49d could enhance the protection against spontaneous apoptosis of cultured CLL. Purified cells from 6 CLL patients were cultured in the presence of anti-CD38 mAb or CS-1 FN fragment, either alone or in combination, in order to engage CD38 and CD49d alone or simultaneously. Analysis of cell apoptosis after a 72-hour culture, showed 74%±1.7 viable cells in the absence of any stimuli, and a substantial improvement of cell viability (94%±0.5) after exposure to both anti-CD38 mAb and CS-1 fragment, compared to a slight protection from apoptosis obtained by CD38 (80±1.1) or CD49d (79±1.1) independent engagements ($p<0.01$). *Conclusions.* The physical association between CD38 and CD49d suggests a cooperative role for the two molecules which may explain at least in part the bad clinical outcome of the CLL subset coexpressing these molecules.

4.16

MONOCYTES/MACROPHAGES ARE THE MAJOR TARGETS OF THE CCL3 CHEMOKINE PRODUCED BY CD38+CD49D+ CHRONIC LYMPHOCYTIC LEUKEMIA CELLS

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Background. CD38 and CD49d are associated negative prognosticators in chronic lymphocytic leukemia (CLL). Gene expression profiling studies recently identified CCL3 as a gene upregulated by $CD38^+CD49d^+$ CLL cells when compared to CD38-CD49d- cases. We further demonstrated the release of CCL3 by cultured CLL cells upon CD38 triggering, and specifically found CCL3 protein in CLL cells from bone marrow biopsies (BMB) of CD38+ cases. *Aim*. Given the role of CCL3 as potent chemoattractant for different cell types, we aimed at identifying the major targets of CCL3, as produced by $CD38^+$ CLL cells. Methods: CLL BMB lymphoid infiltrates were characterized by immunohistochemistry (IHC). Expression of the CCL3 receptors CCR1 and CCR5 by PB CLL

subpopulations was evaluated by flow cytometry. T lymphocyte and monocyte migrations toward CCL3 were performed by in-vitro transwell chemotaxis assays. *Results.* IHC analysis of BMB from 16 CLL cases revealed a higher number of infiltrating $CD68^+$ cells in the context of CLL-involved areas of BMB from $CD38+CD49d^+CCL3^+$, compared to $CD38^-CD49d^-CCL3^-$ cases (p=0.01). CD3+ lymphocytes were interspersed in the CLL lymphoid aggregates, but with no significant difference between the two CLL subgroups. Evaluation of CCR1 and CCR5 in PB cell subpopulations from 14 $CD38^+$ and 26 $CD38^-$ CLL cases, showed the highest mean fluorescence intensity (MFI) levels for both CCR1 (624+60) and CCR5 (64+9) in the monocytic component, irrespective of CD38 expression by CLL cells. Conversely, both CLL cells and residual T lymphocytes showed low MFI levels for CCR1 (19+4 and 14+3) and CCR5 (21+2 and 20+2). High CCR1 and CCR5 expression levels were detected in in-vitro differentiated monocytes; accordingly, CCR1 expression was documented in macrophage-like cells in BMB from CD38+CD49d+ CLL. We next evaluated the capability of purified monocytes and T lymphocytes from 10 CLL cases to migrate in response to CCL3. In keeping with the strong expression of CCR1, monocytes migrated toward CCL3 at a concentration of 3 ng/mL (migration index, MI= 8.8+0.9, p=0.03), whereas T lymphocytes required a higher CCL3 concentration (100 ng/mL) to display slight migration capability (MI= 1.6+0.2, p=ns). The increased infiltration of macrophages in BMB from CCL3-producing $CD38^+CD49d^+$ CLL, prompted us to verify the capability of CCL3-stimulated macrophages to induce the expression by endothelial cells (EC) of the CD49d specific ligand VCAM-1. By using two different EC models (HUVEC and ADMEC), we documented a significant up-regulation of VCAM-1 by EC exposed to conditioned media (CM) collected from cultures of macrophage challenged *in vitro* with CCL3 (p=0.002). Notably, increased levels of the pro-inflammatory cytokine TNF-α were detected in CCL3-CM (p=0.006), and neutralization of TNF-α by specific antibodies reverted the capability of CCL3-CM to induce VCAM-1 by EC models. In agreement with these in-vitro data, we found a more prominent meshwork of $VCAM\text{-}1^+$ stromal/endothelial cells in lymphoid infiltrates from $CD38^+CD49d^+$ CLL compared to CD38-CD49d- cases (p=0.002), and engagement of CD49d by VCAM-1 was able to significantly delay the spontaneous apoptosis observed in cultured CLL cells. *Conclusions.* $CD68^+$ monocytes/ macrophages are likely to be the main targets for the CLL3 chemokine produced by $CD38^+/CD49d^+$ CLL cells, and are active in determining, through the release of TNFα and other yet unidentified cytokines, the overexpression of VCAM-1 by endothelial cells. Experiments aimed at investigating further roles of $CD68^+$ monocytes/macrophage in CLL are currently matter of study.

4.17

B-CLL SIDE POPULATION: CHARACTERIZATION AND IMPLICATION IN CHEMO-RESISTANCE

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Background. B-CLL is characterized by progressive accumulation of quiescent and clonal CD19, CD5, CD23 B lymphocytes in blood, bone marrow, lymph nodes and spleen/liver. Despite new therapeutic treatments, combining chemotherapy and monoclonal antibodies, this disease remains incurable suggesting the existence of a chemotherapeutic-resistant population maybe associated with relapse. Side Population (SP) is now well identified in different normal tissues but also in malignant diseases and linked to chemo-and radio-resistance. This population, defined by Hoechst exclusion in flow cytometry, represents a very low fraction of whole cell population, expresses high levels of various ABC transporters, and could be enriched in cancer stem cells or precursors contributing to relapse and/or disease progression even after chemotherapeutic treatment. Our aim is to analyze SP in B-CLL patients in terms of phenotypic markers, clonality, proliferative capacities and *in vitro* chemoresistance. *Methods.* 24 blood samples B-CLL from B-CLL at diagnosis (stages A-C) were analyzed by flow cytometry on Hoechst 33342 efflux criteria with or without ABC transporter inhibitors (verapamil, fumitremorgin C or reserpin), and co-staining with anti-phenotypic antibodies. To assess the clonality of SP in B-CLL, SP samples from patients with identified chromosomal aberrations were purified by flow cytometry and analyzed by FISH. Moreover, SP was analyzed after treating B-CLL samples *in vitro* by chemotherapeutic agents (Fludarabine, Bendamustine) or Rituximab. Proliferative capacities were assessed by culture of SP or NSP with DSP30 and IL-2 during 7 days and analyze for CFSE dilution and Hoechst efflux. *Results.* Our data show that SP represents less than 0.05% of whole blood cells in all B-CLL samples (range 0.01-4.85%). This population is $CD19^+$, $CD5^+$, $CD23^+$, $CD20^+$. By FISH analysis, we demonstrate that SP as well as non-SP (NSP) cells from one given patient has the same chromosomal aberrations in the same frequency, strongly demonstrating that this population belongs to the leukemic clone. Moreover, B-CLL SP cells are highly resistant *in vitro* to chemotherapeutic drugs (Fludarabine, Bendamustine) as well as Rituximab and can regenerate NSP compartment under *in vitro* DSP-30 + IL-2 proliferative stimulus. Finally, chemo-resistant purified SP cells, compared to B leukemic cells, overexpress Bmi-1, and ABCG-2 at the protein level, strongly suggesting that this population could represent B-CLL progenitors. *Conclusion.* Our data demonstrate that B-CLL SP cells are clonogenic precursors, highly resistant to conventional treatment and represent the major compartment to target for effective eradication of B-CLL disease.

4.18

DOWN-REGULATION OF CXCR4 AND CD62L IN CHRONIC LYMPHOCYTIC LEUKEMIA CELLS IS TRIGGERED BY B-CELL RECEPTOR LIGATION AND ASSOCIATED WITH PROGRESSIVE DISEASE¶

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Most progressive cases of Chronic Lymphocytic Leukaemia (CLL) are associated with lymphadenopathy, highlighting a critical role for signals originating from tumor microenvironment in malignant B-cells accumulation. CXCL12/CXCR4 axis and CD62L (L-selectin) play an important role in directing B-cell trafficking within secondary lymphoid organs and in the process of B-lymphocyte emigration from lymph nodes. Considering the emerging role of B-cell receptor (BCR) activation in CLL, we hypothesized that BCR stimulation may contribute to the accumulation of CLL cells in secondary lymphoid organs by regulating/modulating the expression of CXCR4 and CD62L at the cell membrane. Therefore, we investigated on CLL cells from 30 untreated patients, the *in vitro* impact of BCR activation on cell surface expression of CXCR4 or CD62L and its correlation with disease progression. In 24/30 cases (group 1), BCR stimulation promoted a significantly coordinated down-regulation of CXCR4 and CD62L membrane expression. The extent of BCR-dependent decrease of the proteins was variable among patients and was strikingly representative of the heterogeneous capacity of CLL cells to respond to BCR engagement in a given patient. BCR-induced decrease of CXCR4 occurred through a clathrin-mediated internalization of the protein while decrease of CD62L resulted from a shedding of the protein ectodomain. Functionally, cells down-regulating CXCR4 and CD62L in response to BCR engagement displayed a reduction in both migration toward CXCL12 and adhesion to lymphatic endothelial cells. Conversely, CLL cells from the 6/30 remaining cases (group 2) did not show any down regulation of CXCR4/CD62L upon BCR stimulation and no modification of their migration and adhesion capacities. Remarkably, the ability of CLL cells to respond to BCR ligation was correlated with unfavorable prognostic markers and short progression-free survival. Majority of cases from group 1 displayed unmutated IGVH sequence, ZAP-70 expression, BCR-dependent survival response and were at risk of disease progression. In contrast, patients from group 2 corresponded to stable stage A cases with favourable prognostic markers and no patient experienced any disease progression. In conclusion, BCR signaling promotes decrease of CXCR4 and CD62L membrane expression in progressive cases only. These results are consistent with the hypothesis that BCR signaling pathway stimulation favors accumulation of a proliferative pool within the lymph nodes in progressive CLL cases.

Reference

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4.19

C-ABL REGULATES MCL-1 EXPRESSION IN CHRONIC LYMPHOCYTIC LEUKAEMIA CELLS

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Expression of the anti-apoptotic protein Mcl-1 is an important factor for malignant cell survival in chronic lymphocytic leukaemia (CLL). Recent studies have shown that high Mcl-1 expression in CLL cells correlates with poor disease prognosis and resistance to chemotherapeutic agents such as fludarabine and chlorambucil. Therefore, drugs which reduce the expression of Mcl-1 in CLL cells may be therapeutically useful in the treatment of this disease. One protein that may regulate Mcl-1 expression in CLL cells is the non-receptor tyrosine kinase c-Abl. This is because inhibitors of the kinase activity of c-Abl, such as imatinib and dasatinib, have been shown to down-regulate the expression of Mcl-1 in CLL cells. Our published work has demonstrated that c-Abl is overexpressed in CLL cells, and that high expression of this protein strongly correlate with markers of poor disease prognosis. Since it is known in other cells that c-Abl can activate STAT3 and that active STAT3 can stimulate Mcl-1 gene transcription, we hypothesized that c-Abl may play a direct role in the regulation of Mcl-1 expression in CLL cells. In the present study we show that there is a strong correlation between c-Abl and Mcl-1 expression levels in CLL cells. Furthermore, we also show that, like Mcl-1, cellular c-Abl levels can predict patient survival in CLL, indicating the possibility of a link between Mcl-1 and c-Abl. We next investigated the mechanism of how c-Abl controls Mcl-1 expression, and show that spontaneous STAT3 phosphorylation in CLL cells is inhibited by the presence of imatinib. Moreover, we show that the presence of imatinib also inhibits Mcl-1 protein and mRNA expression in CLL cells. Taken together with the demonstrated role of STAT3 in regulating the expression of the Mcl-1 gene, these results suggest that c-Abl stimulates Mcl-1 gene transcription through a mechanism that likely involves the activation of STAT3. However, c-Abl additionally regulates Mcl-1 protein levels post-translationally, as incubation of imatinib-treated CLL cells with zVAD-FMK partially restored Mcl-1 protein levels, particularly in CLL cases bearing high expression levels of c-Abl. This result suggests that c-Abl may regulate Mcl-1 protein levels in CLL cells by controlling the activation of caspases. Taken together, our data suggest that c-Abl plays a pivotal role in CLL cell survival by regulating Mcl-1 gene transcription and by stabilising Mcl-1 protein expression. These findings therefore provide important insight into the mechanisms controlling Mcl-1 expression and apoptotic resistance in CLL, and show that inhibition of c-Abl may be a useful therapeutic approach for the treatment of this disease.

4.20

MICROVESICLES IN B CELL CHRONIC LYMPHOCYTIC LEUKEMIA MODULATE THE LEUKEMIC MICROENVIRONMENT

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B-CLL has predominantly been characterized as a clonal B-cell disorder where the defective apoptosis of the leukemic B cells is ascribed not only to intrinsic defects of the neoplastic cells but also to extrinsic factors that influence their behavior in the tissue microenvironment. A number of recent studies indicate that microvesicles (MV) released by malignant cancer cells constitute an important part of the tumor-microenvironment, able to transfer various messages to the target cells, and could be critical to initiation of disease progression. The content of microvesicles and their biological function depend on the cell of origin. Here, we report increased levels of circulating microvesicles in plasma of CLL patients. Further characterization of these MV exhibit a phenotypic shift from predominantly platelet-derived microvesicles in early stage to leukemic B-cell derived at advanced stage of the disease. Thus, we interrogated whether microvesicles can modulate the bone marrow stromal cells (BMSC), known to provide a "homing and nurturing" environment for CLL B cells. Indeed, CLL-MV activated the AKT/mTOR/p70S6K axis in CLL-BMSC with subsequent increased production of HIF-1α and vascular endothelial growth factor (VEGF), an important survival factor for CLL B cells. Importantly, we found that CLL-BMSC are capable of producing more VEGF than their normal BMSC counterparts, and MV preferentially induce production of more VEGF from CLL-BMSC. This latter finding implies that the CLL stroma is inherently different than the stroma obtained from healthy controls and the MV are able to further modulate the CLL-microenvironment in favor of CLL survival and resistance to chemotherapy. Finally, we found that MV were able to transfer a phosphorylated receptor tyrosine kinase designated as Axl directly on the BMSC in association with the activation of AKT, at least in part. This study demonstrates the existence of various *in vivo* phenotypes of microvesicles in CLL and their ability to modify the tumor stroma. These findings we believe underscore the involvement of MV in the process of disease progression, irrespective of tumor types, at least in part by modulating the tumor-microenvironment.

4.21

HIGHER EXPRESSION OF SURVIVIN, AKT-1, P53 AND BCL-2 IN LYMPH NODES COMPARED TO PERIPHERAL BLOOD AND BONE MARROW IN B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA

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Background. B-cell chronic lymphocytic leukemia (B-CLL) is characterized by clonal expansion and slow but progressive accumulation of monoclonal $CD5^+CD19^+$ B lymphocytes which accumulate in peripheral blood, bone marrow and lymphoid organs due to imbalance between birth and death rates. It was shown that circulating B-CLL cells are mostly suspended in G0/G1 phase of cell cycle which leads to the conclusion that accumulation of B-CLL cells is caused by defective cell death signaling which increases the cell survival. However, aggregation of proliferating cells was shown in pseudofollicles of lymph nodes and scattered throughout bone marrow. Because of that fact we examined expression of several key factors regulating proliferation and apoptosis in B-CLL in different microenvironments. *Aims.* In this study we aimed to analyze and compare difference in expression of proliferation and apoptosis markers on B-CLL cells taken from different microenvironments, i.e. peripheral blood, bone marrow and lymph nodes. *Methods.* Peripheral blood (PB), bone marrow (BM) and lymph nodes (LN) samples from cases of 25 B-CLL patients, representing major compartments in B-CLL, were taken by conventional techniques. On the same day we analyzed $CD5^+CD19^+$ cells for the expression of survivin, p53, bcl-2, Akt-1 and Ki-67 using flow cytometry. We expressed results as percentage of positive cells and mean fluorescence intensity (MFI). Results were statistically analyzed by pair tests. *Results.* Samples were taken from 25 typical B-CLL patients with mean age of 69 years, 14 males and 11 females. Mean β-2 microglobulin was 4.65 mg, mean TTM was 9.76, with 24% of patients with lymphoma like tumor distribution (TD<0.5). There were 10, 10 and 5 patients in Binet stages A, B and C respectively. Median expression of survivin was (MFI) 1.44, 1.19 and 2.15 for PB, BM and LN respectively ($p<0.05$), median expression of bcl-2 was (MFI) 3.13, 3.21 and 3.98 for PB, BM and LN respectively ($p<0.05$), median expression of p53 was (MFI) 0.24, 0.29 and 0.51 for PB, BM and LN respectively ($p<0.05$), median expression of Akt-1 was (MFI) 1.03, 1.27 and 1.90 for PB, BM and LN respectively ($p<0.05$) and median expression of Ki-67 (MFI) was 1.17, 1.01 and 1.79 for PB, BM and LN respectively ($p<0.05$). MFI values correlate with values expressed as percent of positive cells. Expression of all analyzed markers was highest in LN compared to PB and BM including marker of proliferation Ki-67. *Conclusions.* We found significantly highest expression of all examined markers in LN compared to PB and BM. This is in line with other results (including expression of CD38 and ZAP-70) indicating higher activity of B-CLL cells in lymph nodes.

4.22

THE OXIDATIVE STRESS IN MONOCLONAL B LYMPHOCYTOSIS AND CHRONIC LYMPHOCYTIC LEUKEMIA

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Monoclonal B-cell lymphocytosis (MBL) is a new clinical entity, recently recognized by the IWCLL (2008). The prevalence of MBL is age-related and increases to 5% or more in patients over the age of 60 years. Furthermore, individuals with MBL develop CLL at a rate of about 1% per year. This observation led to study its molecular and biologic characteristics. On the other hand, CLL also appear to have a specific susceptibility to oxidative stress, common feature of transformed tumor cells. The aim of this study was to investigate the oxidative stress status in MBL and CLL and analyze their possible etiopathogenic implications. *Patients and methods.* We analyzed peripheral blood and urine from 29 patients with MBL, 55 with CLL and 31 normal controls. The oxidative stress markers 8-oxo-2'-deoxiguanosine (8-oxo-dG), malondialdehyde (MDA), oxidized/reduced glutathione ratio (GSSG/GSH) were measured by HPLC. *Results.* MBL and CLL patients showed an oxidative damage significantly increased as compared with control subjects (p<0.001). However, there were no differences in the oxidative status between MBL and CLL patients. Thus, the obtained values (MBL vs CLL) were: mutagenic base 8-oxo-dG (lymphocytes: 43.93 vs 47.76 8-oxo-dG/10(6) dG; urine: 21.74 vs 19.86 nmol 8-oxo-dG/mmol creatinine), lipid peroxidation product MDA (1.29 vs 1.44 nmol/mg protein), and GSSG (2.7 vs 3.11 nmol/mg protein). The 8-oxo-dG levels were higher in lymphocytes than in urine of MBL and CLL patients (43.93 8-oxo-dG/10(6) dG vs 21.74 nmol 8-oxo-dG/mmol creatinine) and (47.76 8-oxo-dG/10(6) dG vs 19.86 nmol 8-oxo-dG/mmol creatinine) respectively. Finally, the GSSG/GSH ratio was increased suggesting that the thiol redox status was elevated in MBL and CLL lymphocytes. *Conclusion.* 1) Remarkable oxidative stress exists in patients with MBL and CLL, that causes damage on DNA and lipid structures. 2) There are no differences in the oxidative status between MBL and CLL patients, these results suggest that oxidative injuries appear in a pre-clinic state of the disease. 3) High levels of 8-oxo-dG in lymphocytes and in urine indicate an important oxidation damage to DNA

4.23

THE DYNAMICS OF CD38 EXPRESSION IN B-CLL CELLS OVER TIME

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B-cell chronic lymphocytic leukemia (B-CLL) is a heterogeneous disease with a highly variable clinical outcome. A number of different molecular prognostic markers have been identified that may determine the outcome of the disease including the expression of CD38. In the present study we investigated the stability of CD38 expression on B-CLL cells of 275 patients over time and correlated it to the progression of the disease. A cut-off of ≥30% $CD38^+$ B-CLL cells was determined to discriminate between patients in terms of treatment-free survival. According to this cut-off, 40% of our cohort were high risk B-CLL patients. To evaluate the stability of CD38 expression over time, we looked at patients that were sampled at least four times with a minimum of 6 months between the first and the last examination (n = 110). We found that CD38 expression was a very stable risk parameter: only 9.1% of the patients changed their risk group over time. These patients all changed from the CD38 high risk group to the low risk group (last observed time point). Further, we found a correlation between the expression of CD38 and the treatment status of patients. Under treatment the total amount of B-CLL cells in the peripheral blood of patients was greatly reduced. This reduction correlated with a higher CD38 expression in the tumor cells. To exclude all therapy induced changes in CD38 expression we removed patient samples collected during and up to 6 months after treatment as well as samples from patients in complete remission. Interestingly, there was still a great dynamic in CD38 expression over time within each one of the risk groups. In order to describe the dynamics of CD38 expression mathematically, we chose to define the dynamics of CD38 as the standard deviation of CD38 expression (as a measure of the variance) divided by the time the patient was under observation, in order to discriminate between short and long term changes. The cut-off values for definition of risk groups (treatment free survival) were determined separately for patients with high and low CD38 expression by ROC analysis and subsequent Youden index calculation. Strikingly, patients with a high dynamic in their CD38 expression had a significantly (logrank test) shorter time to treatment than those with a stable CD38 expression. In summary, we demonstrated that CD38 expression on B-CLL cells is as a very stable prognostic marker in B-CLL. However, the percentage of CD38 expressing B-CLL cells in some patients vary greatly over time within the risk groups. This dynamic of CD38 expression correlates with a significantly shorter time to treatment independent of the CD38 risk. Since CD38 has recently been associated with proliferation of B-CLL cells, this dynamic of the CD38 expression may reflect a more dynamic and thus, more aggressive tumor clone. Uncovering the biology of this dynamic might prove to be an important step in understanding the highly variable outcome of B-CLL.

4.24

CD44 SIGNALING INCREASES MCL-1 PROTEIN EXPRESSION AND PROTECTS CLL CELLS FROM SPONTANEOUS AND FLUDARABINE-INDUCED APOPTOSIS

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The survival of chronic lymphocytic leukemia (CLL) cells in-vivo is supported by the tissue microenvironment, while during in–vitro culture the same cells rapidly undergo apoptosis. An important component of the microenvironment is the extracellular matrix (ECM), which is composed of proteins and glycosaminoglycans. Hyaluronic acid is a glycosaminoglycan that not only fulfills structural functions in the ECM but that also affects cell behavior through binding to CD44. In this study, we explored the role of CD44 signaling as a survival factor in CLL. CD44 was more strongly expressed on the surface of IgVH unmutated CLL cells than on mutated CLL cells (mean fluorescence intensity ratio of 224±43 and 122±44, respectively, p<0.0001). Cross-linking of the CD44 receptor by anti-CD44 antibody protected CLL cells from spontaneous apoptosis; CD44 stimulated CLL cells had a 46% (range 7-181%) increase in viability compared with unstimulated CLL cells (n=20, p<0.0001). CD44 activation also protected CLL cells from fludarabine-induced apoptosis: viability 76±6% for control cells, 47±18% for fludarabine treated cells, and 69±16% for CD44 stimulated and fludarabine treated cells (p=0.005). In IgVH unmutated CLL cells CD44 activation had a stronger survival effect than in IgVH mutated cells: increase in viable cells of 21±9% vs. 13±6%, respectively (p=0.04). Ongoing studies using purified hyaluronic acid *in vitro* support the notion that this natural ligand of CD44 could have a protective effect on CLL cell survival *in vivo*. Next we investigated CD44 signaling in CLL cells and found activation of the PI3K/AKT and MAPK/ERK pathways, resulting in increased phospho-AKT and MCL-1 levels. The increase in AKT activation and MCL-1 expression after CD44 activation occurred preferentially in IgVH unmutated samples as compared to IgVH mutated cells: mean fold change in p-AKT 2.17 (n=9) vs 1.0 (n=5), respectively, p=0.04; mean fold change in MCL-1 1.88 (n=8) vs. 1.04 (n=8), respectively, p=0.006. PI3K and MEK inhibitors as well as obatoclax, an antagonist of MCL-1, blocked the anti-apoptotic effect of CD44 ligation. Consistent with the important anti-apoptotic effect of MCL-1, obatoclax was able to sensitize CLL cells to fludarabine-induced apoptosis (combination index <0.5, n=3). In conclusion, CD44 engagement can rescue CLL cells from spontaneous and drug-induced apoptosis. This anti-apoptotic effect is mediated through PI3K/Akt and MAPK/ERK activation, and at least in part, via increased MCL-1 levels. Targeting either pathways or MCL-1 with specific inhibitors antagonized the protective effect of CD44 activation in-vitro and could become a therapeutic strategy to overcome the anti-apoptotic effect of microenvironment interactions in CLL. The preferential survival gain with CD44 engagement in IgVH unmutated CLL cells suggests that increased CD44 activation through contact with ECM components in–vivo, especially hyaluronic acid, could contribute to the more aggressive clinical course of these patients.

4.25

ROLE OF CD38 IN CXCR4-MEDIATED HOMING OF CHRONIC LYMPHOCYTIC LEUKEMIA (CLL) CELLS

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Human CD38, a type II glycoprotein, is part of a large family of molecules involved in the recycling and scavenging of extra-cellular nucleotides (NAD and ATP). The enzymatic products (cADPR and ADPR) are involved in the regulation of cytoplasmic Ca^{2+} levels. CD38 functions are also regulated through the binding with the non-substrate ligand CD31, abundantly expressed by vascular and immune cells. CD38 engagement by means of agonistic monoclonal antibodies or the CD31 ligand leads to activation/proliferation signals in T, B, NK and dendritic cells. Its expression in the neoplastic context of chronic lymphocytic leukemia (CLL) B cells, together with the presence of the cytoplasmic kinase Zap70, above critical thresholds, represents a reliable marker for the identification of high-risk patients. We previously reported that CD38 is not a mere marker, but it delivers proliferation and survival signals by working in association with the kinase Zap70. The concomitant expression of CD38 and Zap70 defines a subset of leukemic cells characterized by a higher migration and signaling ability in response to CXCL12, the critical chemokine in the re-circulation of leukemic cells from blood to lymphoid organs. The aim of this work is to define, at the molecular level, the role of CD38 in the regulation of CLL homing from blood to lymphoid organs, an essential step in the maintenance and progression of the disease. The final goal is to evaluate the *in vivo* contribution of CD38 to the modulation of CXCR4 signaling pathway and chemokine-driven migration of neoplastic cells. Results obtained after analyzing a large cohort of clinically and molecularly characterized CLL patients indicate that i) CD38 signals stabilize early (ERK1/2 phosphorylation) and late (chemotaxis) functional responses to CXCL12. Furthermore ii) CD38 and CXCR4 are part of the same supra-molecular complex, that includes also specific cytoplasmic signaling and cytoskeletal molecules. iii) The CD38/CXCR4 membrane association is dynamic, being rapidly strengthened following CXCL12 exposure, as inferred by co-immunoprecipitation experiments and confocal microscopy analysis. Lastly, the association relies upon the presence of intracellular adaptor proteins as well on membrane integrity. Direct evidence of the role played by CD38 and the other partners of the complex in CXCR4-mediated chemotaxis is provided by genetic manipulation of CLL cells, using a lentiviral technique. The modulation of the expression of specific target molecules (e.g., CD38 and Zap70) confirms the functional role of CD38 in chemotaxis and the dynamic association with CXCR4. Taken together these results indicate that CD38 plays a crucial role in modulating the CXCR4 signaling pathway, exerted through a specific molecular organization and localization on CLL cells. The functional and physical association between CD38 and CXCR4 represents a novel mechanism in the regulation of neoplastic lymphocyte chemotaxis and homing.

4.26

LUMILIXIMAB INDUCES APOPTOSIS IN DRUG RESISTANT LYMPHOMA AND LEUKEMIA CELLS

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Chronic lymphocytic leukemia (CLL) is the most prevalent leukemia in the western hemisphere, and is characterized by accumulation of small, mature lymphocytes in blood, bone marrow, and lymphoid tissue. This accumulation is partly due to the apoptosis-resistant phenotype and enhanced survival signaling of CLL cells, due to the over-expression of anti-apoptotic proteins such as Bcl-2, Mcl-1 and XIAP, and activation of pro-survival kinases such as Akt. In addition to possessing an innate apoptosis-resistant phenotype, CLL patients can also develop a further, acquired resistance to several currently available therapies. Therefore, agents that function via novel mechanisms of action, and maintain efficacy in the presence of these defects, are of critical importance. Lumiliximab, an anti-CD23 monoclonal antibody currently being evaluated in a registrational trial in CLL, differentially induces apoptosis in CLL cells via activation of capsases -9 and -3. In addition, lumiliximab downregulates anti-apoptotic proteins such as Bcl-2, Mcl-1, and XIAP, and reduces activation of pro-survival kinases such as Akt, thus enhancing the apoptotic effects of a range of different agents such as fludarabine and rituximab on CLL cells. To investigate the ability of lumiliximab to induce apoptosis in drug resistant cells *in vitro*, a preclinical, *in vitro* chemo-resistance model was generated by incubating SKW cells which over-express CD23 on the cell surface, with increasing concentrations of the chemotherapeutic drug adriamycin. In the preclinical model, the drug-resistant cells (SKW-R) upregulated multi-drug resistance protein, MRP-2 and exhibited resistance to adriamycin and fludarabine as compared with parental cells (SKW-P). In addition, accompanying loss of CD20 expression rendered them insensitive to rituximab. Despite these changes, lumiliximab remained effective in inducing apoptosis in the drug-resistant cells. These results are consistent with findings in clinical trials of lumiliximab monotherapy and combination therapy: In-vitro results from pre- and post-treatment patient samples obtained from a Phase 1 lumiliximab monotherapy trial in relapsed CLL (Study 152-20) showed activation of caspase-3, along with downregulation of Bcl-2 and Mcl-1, and a reduction in Akt activation. Lumiliximab induced apoptosis in samples from patients who did not respond well to prior fludarabine or rituximab treatment and apoptosis induction was independent of ZAP70 status. Furthermore all of the fludarabine-refractory subjects showed a reduction in ALC following treatment with lumiliximab. In a Phase 1/2 study of lumiliximab in combination with FCR, clinical response (including complete response) appeared to be unaffected by the presence at baseline of markers of poor prognosis (2M levels greater than 2000 ng/mL; absence of IgVH mutation; CD38+ CLL cells greater than 30% or ZAP-70$^+$ CLL cells greater than 30%) or by the presence of markers of rituximab resistance (CD55 or CD59). These data indicate that lumiliximab induces apoptosis in CLL cells and may be beneficial in the treatment of CLL, especially in those patients that have developed resistance to rituximab or fludarabine.

4.27

OVEREXPRESSION OF CLLU1 GENE: A UNIQUE CHARACTERISTIC OF UNMUTATED CLL LYMPHOCYTES

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In chronic lymphocytic leukemia (CLL) no common molecular alteration leading to the development of the disease has been described and the clinical heterogeneity in CLL patients is profound. Molecular markers, such as the mutational status of the variable region of the monoclonal immunoglobulin receptor heavy chain genes (IgVH), 17p and 11q deletion, are currently used to define prognosis for patients that could benefit from a more aggressive and targeted treatment. *CLLU1* is a gene identified by differential display in CLL patients with mutated (MU) versus unmutated (UN) IgVH genes and has been found upregulated in patients without VH somatic hypermutations. In some other hematological malignancies tested, CLLU1 expression was reported to be very low. Recently it was suggested that CLLU1 expression level is an inherent and stable feature of the leukemic clone. However the current methodology for the quantification of CLLU1 expression varies between research groups, so the results are not easily comparable. Although various transcripts of CLLU1 have been identified, no Cllu1 protein has been found to date and the gene's function remains unknown. The aim of our study is to quantify CLLU1 transcripts in: a) CLL patients (n=69) and correlate expression levels with IgVH mutational status b) patients with other B-cell hematological malignancies such as mantle cell lymphoma (MCL, n=9), multiple myeloma (MM, n=5), acute lymphoblastic leukemia (ALL, n=10) c) peripheral blood from healthy volunteers (n=11) and tonsilar B-lymphocytes (n=9). Furthermore we investigated expression of CLLU1 after activation of B-CLL lymphocytes *in vitro* (72h) with i) anti-IgM+ IL-4, ii) CD40L+ IL-4 and iii) LPS+ PHA. Absolute quantification of CLLU1

transcripts was performed with "CLLU1 Profile Quant Kit" (Ipsogen) using 2-microglobulin as reference gene. Results were expressed as CLLU1/ 2m x105. In order to validate CLLU1/ 2m values, measurements were repeated using ABL as reference gene (CLLU1/ABL). IgVH sequencing was performed in CLL, ALL and MCL samples and unmutated were considered cases with more or equal to 98% homology to the germ line sequence. Median value of CLLU1/ 2m in unmutated CLL patients (n=38) was 2705, while in mutated ones (n=31) it was 71. Setting CLLU1/ 2m = 100 as a cut-off, IgVH mutational status of CLL patients could be correctly predicted in 90% of the cases. CLLU1 expression was low in all other hematological malignancies tested, irrespective of the IgVH mutational status. Briefly, median values of CLLU1/ 2m in MCL, MM, ALL, controls and tonsilar B-cells were 2.5, 3.23, 0.53, 0.57 and 4.1 respectively. *In vitro* culture and activation of B-CLL lymphocytes from 3 CLL patients with unmutated and 4 patients with mutated IgVH sequence did not result in an increase of CLLU1 expression. Our results show that CLLU1 is highly expressed only in unmutated CLL patients. In other B-cell hematological malignancies CLLU1 levels are low, irrespective of IgVH mutational status. The fact that CLLU1 expression did not increase during *in vitro* CLL cell activation indicates that CLLU1 is not another B-cell activation marker, but rather a unique and characteristic feature of unmutated CLL. Supported partly by Stayros Niarchos Foundation.

4.28

COEXISTENCE OF TRISOMIES 12 AND 19 IS ASSOCIATED WITH SIGG-SWITCHED CLL

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Trisomy 12 is a recurrent chromosomal aberration occurring in 10-20% of CLL (Stilgenbauer et al 2002). A small percentage, between 1.6% (Kuppers *et al.* 2007) and 5% (Schwaenen 2004) of patients with +12 also have trisomy 19 and may constitute a distinct subgroup. Recently (Athanasiadou *et al.* 2008), trisomy 12 has been reported to be associated with a certain subset of cases expressing stereotypic IGHV4-39/IGKV1-39 (1D-39) (subset 8) B cell receptors (BCRs) of the rare IgG-switched isotype. Another association, in this rare IgG-switched variant of CLL, concerns cases with stereotypic IGHV4-34/IGKV2-30 BCRs (subset 4), reported with the del13q cytogenetic profile. Our intention was to confirm these findings in a larger patient cohort by performing a detailed cytogenetic profiling and comparison of cases assigned to both the common IgMD and the rare IgG variant of CLL. All 334 cases included in the analysis were investigated by G-banded metaphase analysis and by FISH using probes for the centromeric region of chromosome 12 (CEP12, Vysis) and del13q using D13S319 and 13q34 probes (Vysis), unless del13q had been identified karyotypically; for IGHV-D-J sequence analysis and sIg heavy chain expression by flow cytometry. Table 1 documents the cytogenetic profiles, number of patients and incidence of sIgG+, IGHV4-34 and IGHV4-39 in each group. Considering IGHV gene usage in all cases: the expected bias of sIgG-switched expression was observed in the stereotypic IGHV4-34 cases (p=0.0001) and of these, 12/15 (80%) are assigned to subset 4 and 3/15 (20%) to subset 16 (IGHV4-34/IGKV3-20), respectively. Similarly, sIgG expression was also confirmed to be associated with IGHV4-39 gene usage (p=0.0350) of which 4/6 (67%) cases express stereotypic IGHV4-39 BCRs characteristic of subset 8. Considering cytogenetic profiles: there is no association between any cytogenetic subgroup and sIgG expression other than the +12; +19 with or without +18 group where remarkably, all 13 cases are sIgG switched (p=0.0001). IGHV usage across this group is heterogeneous, although 3/12 (25%) utilize the IGHV3-7 gene; 10/12 (83.3%) cases carry IGHV genes with <98% identity to the germline and, thus, can be considered as belonging to the mutated CLL subtype (in keeping with previous reports). No stereotypic IGHV sequences were identified. The lack of IGHV4-34 or IGHV4-39 in this group is not significant (too few cases). Whilst there was no association of IGHV4-34 usage with any cytogenetic profile, 7/13 of the del13q cases conformed to the published association of sIgG-switched CLL with usage of stereotypic subset 4 (IGHV4-34). However IGHV4-39 usage was associated with +12 (p=0.0161) but not specifically with +12; +18; +19. These data confirm the associations between sIgG-switched CLLs with stereotypic IGHV4-34 (subset 4) and IGHV4-39 (subset 8) BCRs with del13q and +12, respectively. Of particular interest is the highly significant association between isotype switched sIgG positive CLL and the distinct karyotypic profile +12; +19. The underlying biological mechanism may be still elusive, but the identified association is too striking to ignore.

Table 1. Cytogenetic profiles, number of patients and incidence of sIgG+, IGHV4-34 and IGHV4-39 in each group.

Chromosomal profile	*Number of cases*	*sIgG+*	*IGHV4-34*	*IGHV4-39*
Normal Karyotype and normal FISH	99 (29.6%)	16/99 (16.2%)	13	3
del 13q14 alone, defined either karyotypically or by FISH	76 (22.8%)	11/76 (14.4%)	13	2
Karyotype with structural abnormalities but not +12	80 (24.0%)	6/80(7.5%)	6	3
+12 alone or with other structural abnormalities	59 (17.7%)	7/59 (11.9%)	6	6
+12; +19 with or without +18	13 (3.9%)	13/13 (100%)	0	1
+12 with other trisomies but not +19	7 (2.1%)	1/7 (14.3%)	3	1
Overall		53/334 (15.9%)	41/334 (12.3%)	16/334 (4.8%)

4.29

FLUDARABINE INDUCES CHANGES IN PHOSPHORYLATION AND LEVELS OF P53, P63 AND P73 IN CHRONIC LYMPHOCYTIC LEUKAEMIA

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Background. Fludarabine is an important drug for CLL patients, and may be used with other drugs for treatment. Fludarabine induces strand breaks in DNA that activate ataxia-telangiectasia mutated (ATM) kinase, and ataxia-telangiectasia and Rad3-related (ATR) kinase, which both phosphorylate p53, causing it to accumulate resulting in cell cycle arrest or apoptosis. Henrich and Christopherson (2008) have shown that Raji cells (B-cell Burkitt's lymphoma) treated with fludarabine nucleoside (FdA, 3 μM) begin to undergo apoptosis within 24 h while MEC1 cells (B-cell chronic lymphocytic leukaemia) with mutated p53 do not respond to treatment with physiologically relevant concentrations of FdA (3 μM). They further observed that nuclear p53 accumulated in Raji cells as multiple phosphorylated isoforms and C-terminal truncated derivatives. The p53 family includes two other transcription factors, p63 and p73, with significant sequence homology to p53, overlapping functions and emerging roles in drug induced apoptosis in CLL cells (Alonso *et al.,* 2009). The present study aims to translate observations with Raji cells to clinical samples and ultimately to integrate the roles of p53, p63 and p73 as transcription factors in CLL cells. *Methods.* Clinical CLL samples were treated in culture with FdA (3 μM, 24 h). The cells were harvested, and total protein extracted and analysed by 1D and 2D polyacrylamide gel electrophoresis and Western blotting (PVDF membrane) with monoclonal antibodies against p53 (clone DO1), p63 (clone 4A4) and p73 (clone 5B1288). *Results.* Preliminary results with clinical samples of CLL show that p53 accumulates with low molecular weight derivatives at approximately 47 kDa and 30 kDa. This observation is consistent with data from Raji cells as well as previous studies by Okorokov *et al.* (1997) who showed that cleaved p53 has enhanced transcriptional activity. We also observed that p63 increases in abundance in some FdA-treated cells and decreases in others. Western blotting with anti- Np63 antibody showed that the Np63 isoforms decrease in abundance 6 h after FdA treatment, these isoforms have anti-apoptotic roles. Further investigation of these effects on p53 family members should enhance understanding of the mechanisms of action which may assist more effective use of FdA and development of new drugs. *Conclusions.* Treatment of

patient CLL cells with FdA induces changes in phosphorylation and the levels of p53 family transcription factors, leading to apoptosis. These observations with patient samples correlate with data from cell lines show a successful translation to clinically relevant data from cells grown in culture.

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4.30

CCL2, CXCL2, IL-6 AND IL-8 ARE EXPRESSED IN PRIMARY CHRONIC LYMPHOCYTIC LEUKAEMIA CELL CULTURES AND ENHANCE CLL CELL SURVIVAL IN VITRO.

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Aim. To investigate the role and clinical relevance of cytokines CCL2, CXCL2, IL-6 and IL-8 in the *in vitro* survival of CLL PBMC cultures. *Methods.* PBMCs from CLL patients were purified and grown in culture at high density (107 cell/mL) for seven days before supernatants were collected and cytokines detected using the Human Cytokine Antibody Array III (Chemicon). Cultures were undertaken in the presence of these exogenous cytokines and with blocking antibodies, and leukaemic cell survival was determined using trypan blue exclusion. The mRNA level of these cytokines was examined by real time PCR and results compared to age-matched normal PBMCs. In addition, expression of the these cytokines receptors was also undertaken by FACS. *Results.* The cytokines CCL2, CXCL2, IL-6 and IL-8 were found to be produced in CLL PBMC cultures. Addition of these cytokines improved *in vitro* survival of CLL cells. However, no further survival advantage resulted when these cytokines were used in various combinations. Blocking CCL2 and CXCL2 cytokines with specific antibodies resulted in the loss of this effect. Moreover, the expression of CCR2 and CXCR2 was found to decrease on CLL cells and increase on accessory cells over time in culture. Furthermore, the mRNA levels of these cytokines were elevated when compared to age-matched normal PBMCs. *Conclusion.* The role of CCL2 and CXCL2 and their receptors in contributing to CLL cell survival *in vitro* is a novel finding in CLL culture systems. These chemokines are likely part of the intricate network of soluble and cell-bound signals required to support the CLL microenvironment. No conflict of interest to disclose.

4.31

LIGATION OF CD180 IS A STRONG ACTIVATION, PROLIFERATION AND SURVIVAL SIGNAL FOR B CELLS FROM NORMAL SUBJECTS AND CERTAIN B-CLL PATIENTS

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Introduction. CD180/RP105 is a type 1 transmembrane molecule expressed on monocytes/macrophages, dendritic cells and B lymphocytes. It was shown previously that ligation of murine RP105 with monoclonal antibody (mAb) led to B cell activation, proliferation and survival. We have demonstrated that while the majority of normal human B cells express CD180$^+$, in B-CLL expression is mainly on leukemic cells with mutated IGHV. Half of CD180$^+$ B-CLL clones upregulated CD86 in response to anti-CD180 mAb, defined as responders (R), as opposed to non-responder CD180$^+$ cells (NR). Here we study whether or not CD180 ligation on control B cells and B-CLL cells leads to activation, proliferation and survival. Methods. PBMCs or magnetically isolated B cells from 20 healthy subjects and 38 B-CLL patients were stimulated for 72h with 10 mcg/mL of anti-CD180 or anti-CD40 mAbs or 15 ng/ml of rIL-4 (optimal concentrations and stimulation time). After incubation, cells were collected, stained with Cy5~anti-CD19 mAb, and: (a) counter-stained with PE~anti-CD86 mAb; (b) fixed, permeabilized and counter-stained with PE~Ki-67 mAb; (c) loaded with DiOC6 dye for 20 minutes. Percentages of CD86$^+$, Ki-67$^+$ and DiOC6(bright)(viable) cells in CD19$^+$ population were analysed by flow cytometry. Results. Control B cells and R B-CLL cells responded strongly to anti-CD180 mAb by increasing percentages of CD86$^+$, Ki-67$^+$ cells and DiOC6(bright)cells, whilst no response was detected for NR and, as expected, for CD180-ve B-CLL cells (Table 1). Ligation of CD180 appeared to be a stronger activation stimulus than anti-CD40 for control B cells (p=0.008), and a stronger activation and proliferation stimulus than rIL-4 for control B cells (p=0.02) and R B-CLL cells (p=0.014). Surprisingly NR and CD180-ve B-CLL cells did not respond either by appreciable activation, proliferation or survival to the addition of anti-CD40 mAb or rIL-4. Purification of B-CLL cells did not enhance NR or CD180-ve cell responses. Conclusions. CD180 represents a strong pro-activation, proliferation and survival signal for normal human B cells and CD180$^+$ B-CLL cells categorised as "Responders". Non-responder B-CLL cells and CD180-ve cells are unresponsive and may be anergic to CD180, CD40 and IL-4 mediated stimuli.

Table 1. Percentages of CD86+, Ki-67+ and DiOC6bright subsets in CD19+ population of control B cells and B-CLL cells in non-stimulated (NS) cultures or after the addition of anti-CD180 mAb

	% of CD19+CD86+		% of CD19+Ki-67+		% of CD19+DiOC$_6^{bright}$	
	NS	anti-CD180	NS	anti-CD180	NS	anti-CD180
Control B cells	23.9±7.2	68.1±17.0*	11.1±5.9	29.1±19.5#	71.3±10.9	89.5±6.1#
R CD180+B-CLL cells	17.9±11.4	57.9±18.8*	6.2±2.0	21.7±13.1#	65.3±9.3	88.9±13.8#
NR CD180+B-CLL cells	14.9±7.1	15.3±7.4	6.5±3.0	9.8±3.0	74.0±8.8	75.7±11.9
CD180-ve B-CLL cells	11.0±4.7	10.3±6.3	5.4±3.6	5.6±1.9	73.4±12.9	71.3±10.9

*P (paired test) - compared with NS cultures: *<0.000001; #<0.02.*

4.32

REGULATION OF BCL-2 BY EXPRESSION IN CLL BY THE LEUKEMIC MICROENVIRONMENT: ROLE OF VASCULAR ENDOTHELIUM AND THE PI3-KINASE PATHWAY

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Chronic lymphocytic leukemia (CLL) is a common B-cell malignancy that pursues a variable but generally indolent clinical course. When studied *in vitro*, peripheral blood CLL cells have a low proliferation rate and die by apoptosis. In-vivo measurements of tumor kinetics reveal a more complex situation, with a higher than expected proliferation rate and an imbalance between cell production and loss in patients with progressive disease. These differences in ex-vivo and *in vivo* behavior have focused attention on the role of the leukemic microenvironment in controlling proliferation and apoptosis of the tumor. It is now generally accepted that proliferation of the leukemic clone mainly occurs in pseudofollicles in secondary lymphoid tissues. In a recent study we demonstrated that, within pseudofollicles, high levels of CD38 were associated with an increased number of CD31$^+$ microvessels. Since CD31 is the only known ligand for CD38, we have now investigated the effect on the endothelium on primary CLL cells. Contact with the microvascular endothelial cell line HMEC-1 caused a small increase in CLL CD38 expression (MFI increased from 3693 to 4747 [n=10, p=0.001, paired t test]) but had a marked effect on viability, which was increased from 25.5-86.5% after 7 days co-culture (n=15, p<0.0001). The same conditions had no effect on the viability of purified normal B-cells. Analysis of purified CLL cells co-cultured with endothelial cells for 24 hours revealed a marked increase in the expression of the anti-apoptotic proteins Bcl-2 (6/6 patients) and Bcl-XL (5/6) as well as inhibition of PARP cleavage (6/6) compared with CLL cells incubated in control medium. Expression of Mcl-1 was variable with 4/6 patients demonstrating increased expression in the pres-

ence of endothelial cells. Viability assessed by dye exclusion at this time point was not reduced, suggesting that the observed changes in Bcl-2 and Bcl-XL levels were not due to degradation following loss of cell viability in control medium. Flow cytometric assessment of Bcl-2 expression by viable $CD19^+/CD5^+$ cells showed an increase in the presence of endothelial cells (p=0.0041, n=5), further confirming this point. Several signaling pathways are known to mediate cell survival through effects on apoptosis. These include the JAK/STAT, ERK/MAPK and PI3-kinase/AKT pathway. We analyzed Phospho-AKT, Phospho-ERK and Phospho-STAT-3 on purified CLL cells incubated in the absence and presence of endothelial cells. In all 4 cases studied, P-AKT was raised after 1 hour in the presence of endothelial cells and P-ERK within 24 hours. P-STAT-3 gave more variable results. As stimulation of AKT acts principally to attenuate the activation of the intrinsic apoptosis pathway, this data suggests that the anti-apoptotic effect of endothelial cells is via this mechanism. To investigate the clinical relevance of these results we analyzed primary patient material. Multiparameter confocal immunofluorescence microscopy was used to investigate the interactions between CLL cells and the surrounding vessels in leukemic lymph node sections from 5 different patients. Sections with variable levels of CD31 were also stained for CD23 and Bcl-2. Pixel counts showed a strong correlation between the number of $CD31^+$ microvascular cells and the level of Bcl-2 expression by CLL B-cells (r=0.77, p=0.0008). These results suggest that interactions with vascular endothelial cells inhibit the apoptosis of CLL cells, upregulate Bcl-2 family proteins via the PI3-K and the intrinsic apoptosis pathway, and have a small but significant effect on the level of expression of CD38. Although initial studies show no significant relationship between protection from apoptosis and the initial expression level of CD38, incubation with HMEC-1 cells did result in its upregulation and it is possible therefore that the CD31/CD38 axis is responsible for the effects observed. The observation that HMEC-1 supernatants also protect CLL cells from apoptosis, albeit to a lesser extent, suggest involvement of a soluble factor. Several candidate molecules can be excluded including VEGF, which is present in the control medium and CD49d, since blocking experiments with Natalizumab (anti-CD49d monoclonal antibody) at concentrations ranging from 10-1000 µg/mL had no effect on apoptosis inhibition by HMEC-1 cells. In summary these findings demonstrate that Bcl-2 expression, which is known to be increased in CLL, is modulated *in vivo* by the tumor microenvironment and PI3-kinase pathway and correlates with lymph node vascularity. Although the ligands and receptors mediating this effect remain to be defined, they present a promising target for the therapy of this common and generally incurable disorder.

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CD180-MEDIATED SIGNALLING IN NORMAL HUMAN B CELLS AND SUBSETS OF B-CLL CELLS

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Introduction. CD180, a member of Toll-like receptor family, is differentially expressed on B-CLL cells, and its ligation leads to activation, proliferation and survival of B cells from healthy subjects and some $CD180^+$ B-CLL clones' defined as "responders": R. Non-responder (NR) $CD180^+$ B-CLL clones did not respond to anti-CD180 as well as to CD40 ligation and rIL-4, suggesting anergy. Here we study activation of major intracellular signalling enzymes following CD180 crosslinking. *Methods.* B cells enriched from the peripheral blood of 13 B-CLL patients and 8 age-matched controls were incubated with 20 µg/mL of anti-CD180 monoclonal antibodies (mAbs) for 20 minutes at 37 C. Unstimulated cells were used as negative controls, and cells stimulated with Phorbol myristate acetate and Ionomycin (PMA/I) for 10 minutes were used as positive controls. After fixation, cells were incubated for 10 minutes at 37C, washed and permeabilized in chilled methanol. The cells were treated with Alexa Fluor~rabbit/mouse antibodies to phospho-Akt, phospho-ERK, phospho-MAPK, FITC~mouse mAb to phospho-Syk and unconjugated rabbit antibodies to phospho-ZAP70/Syk, the latter followed by FITC~goat anti-rabbit antibodies. Cells were analysed by flow cytometry as percentages and as relative mean fluorescence intensity (RMFI) vs isotype control antibodies. Cells were loaded with indole and Ca^{2+} flux measured up to 10 mins. *Results.* Control B cells responded to anti-CD180 mAb by a robust increase in the percentages of cells with phosphorylated enzymes (Table 1) at a level similar to that of PMA/I. R B-CLL cells also showed significant phosphorylation of these enzymes. Although percentages of pZAP70/Syk in NR B-CLL cells did not change upon CD180 ligation due to extremely high levels of constitutive expression, there was a significant increase in the intensity of phosphorylation measured by RMFI (p=0.047). Ligation of CD180 on NR B-CLL cells failed to lead to phosphorylation of MAPK and Akt indicating a possible signalling block. PMA/I induced phosphorylation of all enzymes except Akt in NR B-CLL cells. Ca^{2+} flux was not seen in the R BCLL following CD180 ligation. Conclusions. CD180-ligation on control B cells and R B-CLL cells is followed by activation of major intracellular signalling pathways. NR B-CLL clones did not respond to CD180 ligation by activation of downstream signalling enzymes, particularly MAPK and Akt, suggesting a block at this level.

Table 1. Percentages of control B cells and B-CLL cells with phosphorylated enzymes in non-stimulated samples (NS) and following CD180 ligation with mAb.

	Control B cells		*R CD180+ B-CLL cells*		*NR CD180+ B-CLL cells*	
	NS	*anti-CD180*	*NS*	*anti-CD180*	*NS*	*anti-CD180*
pZAP70/Syk, %	29.4±7.6	44.8±8.6*	59.9±23.6	78.2±12.1†	71.3±10.9	71.2±24.5
pERK, %	23.9±1 0.1	37.5±7.9#	10.2±6.3	29.0±11.6†	9.4±6.7	11.7±8.5
pMAPK, %	28.3±10.9	49.2±5.1*	22.9±11.4	37.4±9.0†	29.8±22.2	34.3±18.7
pAkt, %	19.2±9.3	39.4±4.9*	18.7±8.4	31.2±10.1†	14.5±8.4	13.4±10.5

*Paired t test, p compares with non-stimulated (NS) cells: *<0.001; #<0.02; †<0.05.*

4.34

CROSS-TALK BETWEEN CD180 AND SIGM ON B-CLL AND NORMAL B CELLS

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Introduction. CD180 is differentially expressed on B-CLL cells and its ligation on normal B cells and responder (R) $CD180^+$ B-CLL cells with monoclonal antibody (mAb) leads to activation, proliferation and survival. Since CD180 has a short intracellular domain, it presumably signals through pathways associated with other surface receptors, in particular sIgM. Priming with anti-CD180 leads to IgM-induced apoptosis of murine B cells, and engagement of sIgM and CD180 induces Lyn and Syk phosphorylation. Here we study the possible functional cross-talk between sIgM and CD180 in B-CLL cells. Methods. PBMCs from 12 B-CLL patients and 8 age-matched controls were stimulated with combinations of various concentrations (0.1-10 mcg/mL) of anti-CD180 mAb and F(ab')2 goat anti-human IgM pAbs for 24-72h, and stained with Cy5~anti-CD19 and PE~anti-CD86 mAbs to assess B cell activation. To study intracellular signalling, purified B cells were pre-treated with 20 µg/mL of anti-CD180 mAb for 10 minutes at 37C, followed by 10 minutes incubation with 20 mcg/mL of anti-IgM Ab, fixed, permeabilized and treated with Alexa Fluor~rabbit/mouse antibodies to phospho-Akt, phospho-ERK, phospho-MAPK, FITC~mouse mAb to phospho-Syk and unconjugated rabbit antibodies to phospho-ZAP70/Syk the latter followed by FITC~goat anti-rabbit antibodies.

Table 1. Percentages of normal B cells and B-CLL cells with phosphorylated enzymes after sIgM ligation or following sequential ligation of CD180 and sIgM with corresponding Abs (spontaneous phosphorylation subtracted, p= paired t test).

	Normal B cells		*CD180+ B-CLL cells*	
	Anti-sIgM Ab	*Anti-CD180 + anti-sIgM Abs*	*Anti-sIgM Ab*	*Anti-CD180 + anti-sIgM Abs*
pZAP70/Syk, %	39.0±10.8	41.8±9.7	10.3±8.3	3.5±7.3, p=0.08
pERK, %	31.2±8.6	34.4±8.1	23.2±13.8	24.4±14.1, p=0.235
pMAPK, %	30.9±7.1	30.5±6.6	22.9±7.5	14.2±3.5, p=0.049
pAkt, %	31.0±12.7	34.6±11.6	38.3±8.5	26.4±7.4, p=0.003

Unstimulated cells and cells stimulated with anti-sIgM pAbs alone were used as controls. All cells were analysed by flow cytometry. Results. No combination of anti-IgM and anti-CD180 Abs resulted in additive enhancement of the percentages of $CD19^+CD86^+$ control B cells or B-CLL cells, compared to the stimulation with each antibody alone, suggesting a possible convergence of signalling pathways. Pre-treatment of R B-CLL cells with anti-CD180 mAb significantly abrogated sIgM-mediated phosphorylation of Akt and MAPK, whilst no effect was seen in control B cells (Table 1) suggesting that CD180 is a negative regulator of downstream IgM signalling in B-CLL cells. *Conclusions.* Ligation of CD180 negatively regulates sIgM-induced phosphorylation of Akt and MAPK but not ERK. This finding might have implications for the hypothesized role of the BCR on B-CLL cells (with specificity for self/microbial antigens) in the development of this leukemia.

4.35

APOPTOTIC CELL DEATH INDUCED BY GLUCOCORTICOIDS (GC) IS HIGHER IN UNMUTATED IGVH CHRONIC LYMPHOCYTIC LEUKEMIA CELLS AND CAN BE PREDICTED BY FKBP5 EXPRESSION

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Background. The antileukemic effect of glucocorticoids (GC) in CLL is well known. GC, however, are rarely employed in CLL. The fact that GC, particularly dexamethasone (DEX), can overcome p53 mediated chemoresistance to therapy has renewed the interest in the use of GC as therapeutic agents in CLL. However, the exact mechanisms by which GCs induce apoptotic cell death are not completely understood. Patients and Methods Peripheral blood samples from 45 patients with CLL patients were selected for *in vitro* studies. Patients were analyzed for IGVH mutational status and ZAP-70 expression. Tumoral cells were cultured over 24 hours with DEX at 13.25 uM. Viable cells were determined by surface annexin V binding and propidium iodide (PI) staining flow cytometry analysis. Moreover, expression of BIM, a proapoptotic Bcl2 family member with a clear role in GC induced apoptosis, was quantified at baseline and after DEX treatment. Finally genome wide expression profile of CLL cells was done to discriminate genes predicting response to DEX. Gene expression of 13 CLL cases (7 UM-CLL, 5 M-CLL) was analyzed after 6 hours of DEX treatment and was compared to the baseline gene expression. Results After 24 hours of exposure to therapeutic concentrations of DEX, cell viability was higher in mutated cases (M-CLL) than in unmutated IGVH cases (UM-CLL) (85.6% vs 69.5% in mean, respectively; p=0.000). mRNA BIM expression after 24 hours of treatment with DEX correlated with induced apoptotic cell death (R=0.496; p=0.000). As a consequence, UM-CLL had higher levels of induced mRNA levels of BIM than M-CLL cases (p=0.036). Among other genes, gene expression analysis of genes present in GCs pathway revealed that FKBP5, a GC receptor (GR) complex protein, was highly induced by DEX. After DEX treatment, UM-CLL cases had 2 times more FKBP5 expression than M-CLL cases (p adjusted = 0.02). Of note, FKBP5 basal levels were higher in UM-CLL patients than in M-CLL (1.85 times, p adjusted = 0.027). To expand these results, FKPB5 expression was analyzed in 45 CLL patients by quantitative RT-PCR. At baseline, FKPB5 expression was higher in UM-CLL than M-CLL (0.46 vs. 0.21 arbitrary units, respectively; p=0.000). Finally, after 6 hours of treatment with DEX UM-CLL cells expressed more FKBP5 than M-CLL cells (3.65 vs 1.89, respectively; p=0.000). Conclusions Treatment with GC induces more apoptotic cell death in UM-CLL than in M-CLL. As a downstream effect, BIM expression after GC exposure correlates with GC-induced apoptosis. Moreover, GC apoptotic effect in CLL is the result of several cell pathways imbalance as revealed by gene expression analysis. FKBP5 expression, upstream of the GC pathway, correlates with cellular effect of GC and could be used to predict GC apoptotic activity in CLL.

4.36

SUSTAINED ACTIVATION OF MEK AND ERK AND ENHANCED IN VITRO SURVIVAL INDUCED BY SDF-1 (CXCL12) IN CLL ARE ASSOCIATED WITH FEATURES OF AGGRESSIVE DISEASE

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Marrow stromal cells and nurselike cells, which are thought to reside in the leukemia-microenvironment, are effective in supporting the survival of CLL cells *in vitro* through the elaboration of factors such as SDF-1 α (CXCL12). We found that isolated CLL cells that express high levels of the zeta-chain associated protein of 70 kD (ZAP-70) are more dependent on CXCL12 for survival *in vitro* than are ZAP-70-negative CLL cells. We examined the cell-signaling events induced by CXCL12 in CLL cells to study the basis for this difference. We found that CXCL12 induced phosphorylation of ERK1/2 in both ZAP-70-positive and ZAP-70-negative CLL cells, as assessed via immunoblot analyses. However, phosphorylation of ERK1/2 was relatively short-lived in ZAP-70-negative samples, a difference that was corroborated using a quantitative ELISA assay for activated ERK1/2 to examine lysates prepared from CLL cells at various times after stimulation with CXCL12. $ZAP\text{-}70^+$ CLL cells had significantly greater amounts of phosphorylated ERK1/2 (38±16, ELISA values indicating absorbance at 460 nm, n=10), and for significantly longer time periods, after stimulation with CXCL12 than did ZAP-70-negative CLL cell samples (21±7, ELISA values indicating absorbance at 460 nm, n=9; p<0.5). Using a quantitative ELISA for pMEK, we also noted similar changes in MEK phosphorylation in response to CXCL12 in CLL cells. Again, $ZAP\text{-}70^+$ CLL cells had significantly greater amounts of phosphorylated MEK (74±33, ELISA values indicating absorbance at 460 nm, n=9) and for significantly longer time periods after stimulation with CXCL12 than did ZAP-70-negative CLL cell samples (45±15, ELISA values indicate absorbance at 460 nm, n=10; p<0.5), suggesting that ZAP-70-positive CLL cells had enhanced Raf activity. For this reason, we examined CXCL12 -induced activation of MEK in CLL cells cultured in varying concentrations of Sorafenib, a small molecule inhibitor of both b- and c-Raf. We found that 2h pretreatment of CLL cells using Sorafenib (40 uM) significantly blocked CXCL12-induced activation of pMEK in ZAP-70-positive samples. ZAP-70-negative samples showed low CXCL12-induced activation of pMEK, which was also completely blocked by sorafenib. However, CXCL12 appeared to induce similar levels of phosphorylated c-Raf in all CLL samples, as assessed by phosphoflow cytometry and immunoblot analyses. This suggests that b-Raf might contribute to the CXCL12-induced activation of MEK in ZAP70-positive CLL samples, although the differential activity of phosphatases cannot be excluded. Collectively, our studies reveal that CXCL12 could induce pronounced and sustained activation of MEK and EKR1/2 in CLL cells that express ZAP-70, a characteristic associated with more aggressive disease. Moreover, the extent and duration of MEK and ERK1/2 activation and survival following treatment with CXC12 appeared significantly greater in ZAP-70-positive cases than in ZAP-70-negative cases. Conceivably, ZAP-70-positive leukemia cells might be more sensitive and/or dependent than ZAP-70-negative CLL on the survival factors, such as CXCL12, which are elaborated by the leukemia microenvironment. This feature could contribute to the relatively poor prognosis of patients with ZAP-70-positive CLL.

4.37

COMPARATIVE GENE EXPRESSION PROFILING OF LEUKEMIA CELLS IN PERIPHERAL BLOOD AND TISSUE COMPARTMENTS REVEALS A PROMINENT ROLE OF THE MICROENVIRONMENT FOR CLL PROLIFERATION AND SURVIVAL

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In vitro studies suggest that chronic lymphocytic leukemia cells critically depend on the interaction with host cells and many different mol-

ecules, signaling pathways and cell types have been reported to enhance CLL cell survival. The presence of CLL cells in three distinct tissue compartments: peripheral blood (PB), bone marrow (BM) and lymph node (LN), provides a unique opportunity to investigate the effects of the microenvironment on tumor cell biology *in vivo*. To this end, we used gene expression profiling to compare CLL cells sampled on the same day from PB, BM, and/or LN from 24 previously untreated patients. Tumor cells from blood, marrow aspirates, and single cell suspensions of LN biopsies were purified to >96% by $CD19^+$ selection (Miltenyi). A total of 62 samples were arrayed on Affymetrix HU133 plus arrays. Probe sets that had no signal above background in any sample were excluded. ANOVA analysis with a cutoff of >2-fold change and false discovery rate (FDR) <0.2 identified 151 genes that discriminated between circulating and LN resident CLL cells (n=17), most of which were more highly expressed in LN, and 27 genes that were differentially expressed in BM as compared to PB cells (n=19). Compared to PB cells, the activation marker CD69 was also more strongly upregulated in LN than in BM derived cells. Using gene set enrichment analysis (GSEA) we identified several gene expression signatures that were preferentially expressed in LN resident cells: a proliferation signature characterized by E2F and c-MYC regulated genes, signatures related to B-cell receptor, TNF and CD40 receptor signaling and a NF-kB activation profile (FDR for all <0.02, normalized enrichment scores 1.79-2.15). The gene expression based proliferation score was highest in LN, followed by BM and weak in PB. Upregulation of nuclear E2F and c-MYC in LN resident cells was confirmed by Western blotting. NF-kB signature genes upregulated in the LN serve important functions, including cell cycle regulation (c-MYC and cyclin D2), inhibition of apoptosis (survivin and GADD45), signal transduction (JUN-B, DUSP2), chemotaxis (CCL3, CCL4 and RGS1), and class switch DNA recombination (AICDA). This NF-kB signature was consistently much more prominent in LN than in BM or circulating CLL cells. Activation of the NF-kB pathway was confirmed by detection of phosphorylated IkB and enhanced p65/Rel-A DNA binding activity in cell extracts from LN resident cells as well as by demonstration of increased JUNB protein in LN cells. Likely pathways activating NF-kB in CLL cells include BCR and CD40 signaling. However, GSEA analysis indicated BCR signaling as the dominant pathway and using quantitative PCR of *in vitro* stimulated cells we validated select genes as being preferentially induced by BCR as opposed to CD40 engagement. In *Conclusion.* interactions between CLL cells and elements of the microenvironment induced leukemic cell proliferation, BCR and NF-kB activation preferentially in the LN. Based on a systems wide approach our results highlight the relevance of these pathways in-vivo and provide a roadmap for the development of targeted therapy in CLL.

4.38

LYMPH NODE B-CLL LYMPHOCYTES SHOW THE HIGHEST EXPRESSON OF CD52 AND THE LOWEST OF CD20 COMPARED TO THOSE FROM PERIPHERAL BLOOD AND BONE MARROW - IMPLICATIONS FOR THERAPY?

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Background. B-cell chronic lymphocytic leukemia (B-CLL) is characterized by variable clinical presentation with different involvement of various lymphoid compartments ie peripheral blood, bone marrow and lymphoid organs such as lymph nodes and spleen. Also there is a well documented intraclonal and interclonal variability of B-CLL cells in different microenvironments regarding a number of surface and intracellular molecules (for example CD38 and ZAP-70). This variable distribution of tumor mass has strong association with prognosis and a well documented influence on response to novel immunotherapeutics like rituximab and alemtuzumab. There is a well documented efficacy of rituximab in cases with with 11q deletion (associated with significant lymphadenopathy), and known resistance to alemtuzumab in pateints with bulky lymphadenopaty (>5 cm). Aim of this study was to evaluate level of expression of CD20 and CD52 on B-CLL lymphocytes and intra and interclonal differences dependent on different microenvironment, ie peripheral blood, bone marrow and lymph nodes. *Methods.* peripheral blood, (PB), bone marrow (BM) and lymph node (LN) samples were taken by conventional techniques (venepuncture and fine needle aspiration) on the same day. The expression level of CD20 and CD52 molecules on $CD19^+CD5^+$ B-CLL cells was analyzed by flow cytometry. Results were expressed as mean fluorescence intensity (MFI) and percentage of positive cells and analyzed by paired tests. *Results.* we have analyzed samples taken from 12 typical B-CLL patients with median age of 70.5 years. There were 7 males and 5 females. Mean β2M was 5.5 mg/L, mean TTM size was 9.8 and mean TD was 0.75. There were 7, 3 and 2 patients in Binet stage A, B and C, respectively. There were 4 previously treated patients (patients were not treated 3 months before sampling) of whom one patient was previously treated with both rituximab and alemtuzumab. Among included patients there were patients with 11q deletion and with 17p deletion. Median expression level (MFI) of CD52 was 115.5, 140, and 179 for PB, BM and LN respectively ($p<0.05$). Median expression level (MFI) of CD20 was 4.82, 2.89 and 1.81 for PB, BM and LN respectively ($p<0.05$). Results were very consistent in this clinically and cytogenetically heterogenous group of B-CLL patients, with lowest expression of CD20 in LN in all patients and highest in PB in 10 out of 12 patients, and with lowest expression of CD52 in PB in 10 out of 12 patients and highest in LN in 11 out of 12 patients. *Conclusions.* relatively unexpected results demonstrating the lowest level of expression of CD20 in lymph nodes compared to PB and BM and the highest expression of CD52 in LN compared to PB and BM is inversely related to known efficacy of agents (ie rituximab and alemtuzumab) targeting these molecules in these lymphoid compartments. These results indicate that other factors in selected microenvironment (beside number of molecules on cell surface) regulate sensitivity of B-CLL cells on rituximab and alemtuzumab *in vivo*. These results warrant further studies to indentify these factors which may evantually uncover novel therapeutic targets.

4.39

CD160 PROTECTS CELL DEATH VIA MITOCHONDRIAL PATHWAY

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B-cell chronic lymphocytic leukemia (CLL) is an incurable disease, which is at least partly attributable to most of the malignant cells being in the G0/G1 phase of the cell cycle and expressing high levels of anti-apoptotic Bcl-2 family proteins. These malignant cells exhibit genetic abnormalities which can modify their resistance to apoptosis and response to selected microenvironmental signals giving both a growth and survival advantage. Despite their prolonged survival *in vivo*, CLL cells rapidly undergo spontaneous apoptosis once removed from their *in vivo* microenvironment, suggesting that survival signals available *in vivo* have been lost *in vitro* culture conditions. CD160, a cell surface antigen, is expressed on CLL cells. The role of CD160 in CLL is unclear, but activation of CD160 by two different anti CD160 mAbs led to protection from spontaneous apoptosis *in vitro* and upregulation of the antiapoptotic proteins Bcl-2, Bcl-xL and Mcl-1. Activation of CD160 stabilised both the inner and outer mitochondrial membrane integrity, thereby inhibiting cytochrome C release and mitochondrial membrane potential (m) collapse. The PI3- kinase/AKT pathway is a well known survival pathway in cancer cells. Activation of CD160 in CLL was associated with the appearance of phosphorylated AKT. Inhibition of PI3K by wortmannin completely blocked AKT phosphorylation and the protection against spontaneous apoptosis mediated by CD160. In summary, the activation of CD160 protected CLL cells from spontaneous cell death *in vitro* via a PI3-kinase/AKT pathway. This improved survival was also associated with increased Bcl-2, Bcl-xL and Mcl-1 expression and inhibition of mitochondrial dysfunction.

4.40

CIRCULATING B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA CELLS DISPLAY IMPAIRED MIGRATION TO LYMPH NODES AND BONE MARROW

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Homing to secondary lymphoid organs and bone marrow (BM) is a central aspect of leukemic pathophysiology. Abnormalities in the expres-

sion and function of cell adhesion molecules such as integrins may account for the patterns of intranodal growth and hematogenous spread of the malignant cells. The roles of the two major lymphocyte integrins LFA-1 and VLA-4 in the migration of B-cell chronic lymphocytic leukemia (CLL) cells are largely unknown. We investigated expression and function of these integrins in extravasation and homing processes of CLL cells *in vitro* and *in vivo*. Integrin expression on peripheral blood and BM derived CLL and normal B lymphocytes was determined by flow cytometry and quantitative real time PCR. Rapid chemokine-induced integrin activation and transendothelial migration was studied *in vitro* under conditions that simulate the blood flow and was combined with short term *in vivo* homing experiments of CLL and normal human B lymphocytes to BM, spleen and lymph nodes (LNs) of immunodeficient mice. We found that the majority of CLL cells expressed significantly reduced LFA-1 due to low β2 integrin transcripts. VLA-4 expression was heterogenous due to differential α4 integrin transcription but underwent rapid activation by the BM chemokine CXCL12. The majority of CLL cells failed to transmigrate across VCAM-1, ICAM-1 and CXCL12 expressing endothelium whereas when LFA-1 expression was regained in subsets of CLL cells, these lymphocytes rapidly transmigrated the endothelium. Furthermore, when injected into tail veins of immunodeficient mice, normal B cells rapidly homed to LNs in an LFA-1 dependent manner whereas the major part of CLL cells did not. In addition, only residual CLL subsets could re-enter BM whereas, both, normal and CLL cells homed to the mice spleen in an LFA-1- and VLA-4-independent manner. Our results suggest that CLL cells have a reduced capacity to adhere and transmigrate through multiple vascular endothelial beds and poorly home to lymphoid organs other than spleen. Integrin blocking could thus be an efficient strategy to prevent circulating CLL cells from reaching prosurvival niches in LNs and BM but not in spleen.

4.41

THE ADVERSE OUTCOME ASSOCIATED WITH CD38, CD44 AND CD49D EXPRESSION IN CLL MAY BE EXPLAINED BY THEIR PARTICIPATION IN A MACROMOLECULAR COMPLEX THAT MEDIATES TISSUE INVASION

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The outcome for patients with chronic lymphocytic leukemia (CLL) can be predicted using clinical parameters that correlate with disease bulk and kinetics, such as stage and lymphocyte doubling time, as well as a range of phenotypic and genetic markers that reflect the biological properties of the tumor. In clinical practice one of then most frequently used biomarkers is the level of expression of the multi-functional membrane protein CD38 by peripheral blood leukemic cells. Although the precise explanation for the association between high CD38 levels and adverse outcome remains unknown, a correlation with tumor proliferation in the blood and tissues has been found by several groups, whilst others have reported that CD38 and ZAP70 expression identifies cells with a high migratory potential. Lymphocyte migration in CLL is mediated by a number of other factors including a macromolecular complex containing CD49d, an integrin whose level of expression also correlates with advanced disease and bulky lymphadenopathy. In the present study we investigated the hypothesis that the overlapping clinical and functional features of CD38 and CD49d may be due to their physical association in the CLL cell membrane. Using 4 colour flow cytometry we measured the level of expression of CD38 and CD49d by $CD19^+CD5^+$ cells in patients with CLL presenting to our routine diagnostic laboratory. Some correlation was noted between both the percentage positivity and mean fluorescent intensity of CD38 and CD49d expression (r^2=0.42, p=<0.0001, n=234; r^2=0.28, p=<0.0001, n=61 respectively). Expression levels were not always linked and a number of patients expressed high levels of either CD38 or CD49d with low levels of the other. Within individual patients however, a strong correlation between the two was apparent so that cells expressing high levels of CD38 also had high levels of CD49d. Previous studies have shown that CD49d and CD44 are associated in the CLL cell membrane and involved in tissue invasion. We investigated the possibility that CD38 might also form part of this complex by immunoprecipitating CLL cell lysates using anti-CD38 then western blotting for CD49d and CD44. In 9/9 $CD38^+$ cases of CLL, these immunoprecipitates contained both CD49d and CD44, whilst controls precipitated with an irrelevant antibody did not. These results were confirmed by immunofluorescence confocal microscopy. CLL cells from 5 different patients known to express both CD38 and CD49d were settled on poly-lysine coated slides, stained by indirect immunofluorescence and analyzed using confocal microscopy. Pixel counts showed that a mean of 64% of CD38 was co-localised to CD49d (SEM 5.1%) and a mean of 72% of CD49d to CD38 (SEM 4.8%). These findings suggest that the role of CD38 in CLL might be more complex than previously suspected. There is already strong evidence that signaling through CD38/ZAP70 promotes the survival and proliferation of CLL cells, presumably through interactions with its ligand CD31. The data presented here show that, in addition, CD38 may be involved in migration across the endothelial barrier by interacting with CD49d and CD44. This is in keeping with our previous observation that $CD38^+$ patients have higher numbers of lymph node microvessels. In addition, since progressive disease is invariably associated with tissue invasion, our findings may also explain why the expression level of all these molecules correlates with adverse outcome in CLL.

**These authors contributed equally to this study.*

4.42

SMALL MOLECULAR INHIBITORS OF THE POTASSIUM CHANNELS AS POTENTIAL THERAPEUTIC AGENTS FOR CHRONIC LYMPHOCYTIC LEUKEMIA

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Region q14 of human chromosome 13 harbors a critical tumor suppressor gene (TSG) for Chronic Lymphocytic Leukemia (CLL) and some other hematopoietic malignancies. Recently, we described CLL gene candidate KCNRG (K+ Channel Negatively Regulating Gene). KCNRG resembles the tetramerization domain of voltage-gated K^+ channels (Kv channels) and is capable of the suppression of Kv currents. Studies of the proliferation and apoptosis strongly suggest that KCNRG may act as a tumor suppressor gene for the development or progression of various types of cancers, including CLL. If KCNRG indeed plays a role in CLL tumorigenesis, it might define a novel class of human tumor suppressor genes with a mechanism of action that can be relatively easily reproduced by pharmacological means. We hypothesized that K^+ channel inhibitors could be used to make up for a loss of KCNRG activity in CLL. Therefore, we started an initial evaluation of the known non-protein K^+ channel blockers as apoptotic inducers in lymphocytes collected from 8 CLL patients and 4 healthy donors. Primary cells were cultivated in 2 ml of RPMI-1640 in the 24-well plates. Cytotoxicity of tested compounds was evaluated using both MTT and Chemiluminescent CaspaseGlo assays after 3 and 24 hours of exposure. At physiological concentrations, Kv channels inhibitors diltiazem hydrochloride, verapamil hydrochloride, and anandamide selectively stimulate apoptosis in CLL cells, but not in the normal lymophocytes (p<0.05, Mann-Whitney test). Riluzole, amiodarone hydrochloride and nifedipine demonstrated similar trends that have not reached significance (p<0.1). It might be possible to retarget these and other K^+ channel blockers as adjuvant therapeutic agents in CLL. The excellent records for nanomolar concentrations of K^+ channel blockers used in the management of epilepsy, stroke, and cardiac arrhythmias enhances the attractiveness of this approach. *Supported by NIH R1R15CA113331-01 "KCNRG gene as candidate tumor suppressor for CLL and MM" (2005-2009) and Russian Fund for Basic Research RFFI N07-04-12232 (ofi-a).*

4.44

THE SIGNIFICANCE OF BETA 2-MICROGLOBULIN IN B-CLL: EVIDENCE OF B2-M SECRETION FOLLOWING IN VITRO ACTIVATION OF B-CLL CELLS

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Beta 2-microglobulin (β2M) is an 11-kDa protein recognized as the light-chain component of the MHC-I molecule. It is produced by nucleated cells membranes and is detectable in the serum and other body fluids. The link between the β2M/ MHC-1 molecules and membrane structure has been associated with lymphocyte activation. Serum β2M is ele-

vated in a variety of disorders and has a prognostic value in lymphoproliferative diseases such as multiple myeloma and CLL. High β2M in CLL, has been found to correlate mainly with the advanced stage of the disease and high lymphocyte count and is routinely used as a bad prognostic factor (Keating, 1995, Montillo, 2005). Previous studies from our Institute have shown significantly higher level of β2M in CLL/PL and PLL and in cases associated with autoimmune disorders close to levels found in active myeloma (Shvidel, 1996, Duek, 2006). We suggested that β2M levels are increased during B-cell activation which not necessarily correlates with aggravation of CLL. In an attempt to further evaluate the correlation of β2M and B-cells activation we stimulated B-CLL cells *in vitro* with pokeweed mitogen (PWM) and compared the results with CLL controls after 3 and 7 days of culture. Level of β2M in supernatants was tested using a microparticle enzyme immunoassay. B-CLL cells from 6 patients (2 in stage Binet A, 3 B and 1 C) were studied. Except for stage A, the serum β2M was mildly increased. The results showed a significant *in vitro* secretion of β2M after three and seven days in supernatants of PWM-activated cells compared to unstimulated controls: mean values after 3 days were: 161 ng/mL (range 96-206) in activated B-cells versus 46 ng/mL (41-64) in controls; and after 7 days 517 ng/mL (401-785) versus 146 (67-186) in controls. Interestingly, $CD8^+$ T-cells from a T-large granular leukemia patient stimulated with PHA do not secrete significant amount of β2M *in vitro* (156 ng/mL versus 87 at day 7). Our results demonstrate that activation of B-CLL cells *in vitro* induced a marked secretion of β2M. Thus, the significance of β2M in CLL may correlate with degree of activation of B-CLL cells. This hypothesis is supported by the increase of CD38 (an activation marker) in CLL cases with high β2M serum level. Moreover, the results of our study suggest that progression of disease may be the results of in-vivo activation of the cells and lends support to previous studies showing that CLL cells are G_0 cells with a potential to progress in the cell cycle after stimulation.

4.45

LACK OF PHLPP1 EXPRESSION IN CLL B CELLS LEADS TO ENHANCED AKT ACTIVATION IN RESPONSE TO B-CELL RECEPTOR STIMULATION

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PHLPP (PH domain leucin-rich repeat protein phosphatase) is a novel family of Ser/Thr phosphatases that were recently identified as key negative regulators of the Akt signaling pathway. The PHLPP family comprises of two members, PHLPP1 and PHLPP2, which share 60% overall identity at the amino acid level. PHLPP1 exists as two alternatively spliced isoforms, PHLPP1 and PHLPP . Recent studies have shown that all PHLPP family members specifically dephosphorylate Ser473 in the hydrophobic motif of Akt, which is essential for the enzymatic activity of this kinase. Reduced or absent expression of PHLPP1 has been reported in colon cancer and glioblastoma, leading to elevated basal or agonist-induced AKT activity. Introduction of PHLPP1 in these cells resulted in decreased Akt activity, increased apoptosis and inhibition of cell-cycle progression, suggesting that PHLPP1 can act as a tumor suppressor. Given the essential role of Akt in transducing pro-survival signals from the B-cell receptor (BCR), we decided to investigate the expression of these phosphatases in a large series of primary CLL B-cell samples. Remarkably, PHLPP1 was either not expressed or was expressed at markedly reduced levels in all of the investigated CLL samples (n=31), with levels of expression ranging from 0 to 10% of the levels in normal B-cells (n=7) and lymphoma B-cell lines (n=13). In contrast, no difference was observed in PHLPP2 expression between the three series of samples. Real-time RT/PCR analysis revealed that in most cases the absence of PHLPP1 protein was due to reduced gene expression. However, the mRNA levels in 2 of the 16 investigated CLL cases were comparable to those in normal B-cells and lymphoma cell lines, suggesting that other mechanisms may also be involved. To determine what are the consequences of reduced PHLPP1 expression on Akt signaling in malignant B-lymphocytes, we downregulated PHLPP1 in the BJAB and DHL-4 lymphoma cell lines by RNA interference. A significant reduction in PHLPP1 expression was achieved in both cell lines, with levels ranging from 5-20% of the levels in cells transfected with control siRNAs. Immunoblotting analysis of protein extracts from unstimulated cells transfected with PHLPP1 siRNA did not show an increase in AKT phosphorylation on the activating Ser473 and Thr308 residues, indicating that reduced PHLPP1 expression does not affect basal AKT activity. However, an approximately 40% increase in BCR-induced phosphorylation of Akt, GSK3 and ERK was observed in PHLPP1 siRNA-transfected cells, suggesting that this phosphatase is involved in regulating the intensity of the BCR signal. To directly evaluate the role of PHLPP1 in CLL, we expressed the PHLPP1 isoform in primary leukemic cells by mRNA nucleofection. BCR stimulation of CLL cells transfected with PHLPP1 showed a 20-30% decrease in Akt phosphorylation, consistent with the results obtained from the RNA interference experiments. In conclusion, these data show that PHLPP1 regulates the activity of Akt and other important signaling molecules downstream of the BCR in CLL B-cells and suggest that the reduced or absent PHLPP1 expression is likely to be functionally relevant and to contribute to the pathogenesis of the disease.

4.46

BIOLOGICAL ANALYSIS AND PROGNOSTIC SIGNIFICANCE OF PROLIFERATIVE CELLULAR COMPARTMENT IN CHRONIC LYMPHOTIC LEUKEMIA

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Background. Historically CLL has been considered a non-proliferative disease characterized by accumulation of leukemic cells. However, recent clinical and biological observations are questioning this concept. From the clinical standpoint, although some patients have stable lymphocyte counts during the course of the disease, others exhibit a short lymphocyte doubling time, suggesting the existence of a significant cell proliferation. Furthermore, proliferation would be more prominent in some specific anatomic locations (bone marrow (BM) and lymph nodes), than in peripheral blood (PB). The amount of cell proliferation in PB and BM, and its prognostic significance has not been properly analyzed. *Patients and Methods*. Gene expression profiling of proliferation genes and the amount of cell proliferation in different tissue compartments (BM and PB) were examined in patients with CLL. Isolated CD19/CD5+ tumoral cells from 20 paired samples (BM and PB) were analyzed for gene expression profile by low-density arrays (TaqMan® Low Density Arrays / Applied Biosystems) including 95 genes involved in the initiation and development of the cell cycle. In addition, the amount of proliferative CLL cells was measured by Ki67 positivity by flow cytometry in 40 additional paired samples. *Results.* Expression of genes involved in the initiation and development of cell cycle was more pronounced in BM than in PB. The percentage of Ki67+ CLL cells analyzed by flow cytometry in BM and PB ranged from 0.3% to 4% and from 0.1% to 2.4%, respectively. Ki67+ CLL cells were significantly higher in BM than in PB (mean: 1.2% vs 0.9%; p= 0.005). When the subgroup of patients with a stable disease was analyzed (n=24), no significant differences were observed between BM and PB (mean: 0.8% vs 0.7%, respectively; p=0.093). In contrast, Ki67+ CLL cells were higher in BM than in PB (mean: BM 1.6% vs PB 1.1%; p=0.023) in those patients who presented a progression of their disease at any time (n=16). Thirty-two paired samples were obtained at diagnosis. Patients with $Ki67^+$ CLL cells ≥1% in BM at diagnosis had a shorter time to progression than those with Ki67 <1% (progression at 4 years: 46% vs 6%; p<0.0001) (Figure).

Conclusions. In CLL, expression of genes related to proliferation is significantly increased in BM compared to PB. Moreover, the number of proliferating CLL cells is also increased in BM, particularly in those patients with an aggressive disease. Finally, the amount of $Ki67^+$ CLL cells in BM correlates with a shorter time to progression. These results challenge the concept of CLL as disease more accumulative than proliferative. Bone marrow microenvironment, where the leukemic cells would find a "protector" ambience, would play an important role in the proliferation and survival of CLL cells. New insights on the proliferation pathways in CLL not only may provide a better understanding of the pathogenesis of this disease, but also would be of prognostic relevance and can support the use of new treatments aimed to inhibit proliferation in CLL.

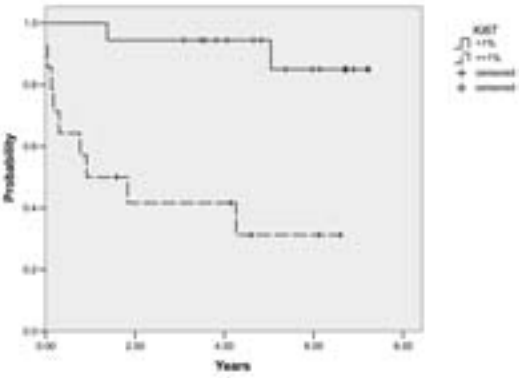

Figure 1.

4.47

ENHANCED EXPRESSION OF IL-17 IN CHRONIC LYMPHOCYTIC LEUKEMIA

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Background. The proliferation and survival of chronic lymphocytic leukemia (CLL) clones is dependent on the interplay between leukemic cells and cytokines, chemokines and the cellular microenvironment of the blood, bone marrow, lymph node and spleen. Interleukin 17 (IL-17) is a novel proinflammatory cytokine secreted by a subset of $CD4^+$ effector T cells (Th17 cells). Its role is being defined in sepsis and various autoimmune diseases but its relevance to CLL is unknown. We hypothesize that IL-17 levels are elevated in CLL patients, creating a proinflammatory microenvironment that promotes angiogenesis and augments release of pro-survival cytokines and chemokines from nurse-like and stromal cells. To decipher functional and possibly prognostic roles for Th17 cells in CLL, we have analyzed CLL cells and cells of the tissue microenvironment for expression of IL 17. *Methods.* Serum cytokines were measured using a combination of multiplex cytokine bead and protein array technologies. Constitutive and inducible intracellular IL-17A production in $CD4^+$ T cells from cryopreserved samples of peripheral blood mononuclear cells isolated from untreated CLL patients (n = 20) and age-matched healthy subjects (n = 7) was measured by flow cytometry. Flow cytometric analyses were performed at baseline (Day 0) and after 7 day culture with/without the Th17-polarizing cytokines, IL 1β and IL 23. Lastly, IL-17 expression was assessed by immunohistochemistry in spleen tissue from CLL patients and healthy age-matched subjects. *Results.* These studies revealed significantly higher levels of IL-17 in the serum of CLL patients as compared to the serum of healthy age-matched subjects. In subsequent flow cytometric studies of IL-17 expression in peripheral blood leukocytes we found that the percentage of $CD4^+$ T cells producing IL-17 (Th17 cells) was significantly higher in CLL patients as compared to healthy subjects both at baseline and after culturing cells for 7 days in the absence of Th17 polarizing cytokines. When peripheral blood leukocytes were cultured for 7 days in the presence of Th17 polarizing cytokines, the percentage of IL-17 producing $CD4^+$ T cells increased in cultures derived from both patients and healthy subjects, but again, the percentage of IL-17 expressing $CD4^+$cells was significantly higher in cultures of cells from CLL patients as compared to those from healthy subjects. Preliminary immunohistochemical analyses of spleen sections from CLL patients and healthy subjects revealed strong IL-17 production in spleen tissue from CLL patients but not in spleen tissue from healthy subjects *Conclusions.* Patients with CLL have higher levels of circulating IL-17, as well as increased numbers of Th17 cells in the peripheral blood and lymphoid tissues, as compared to healthy age-matched subjects. Enhanced production of IL-17 within the context of CLL is provocative and may contribute to disease-associated pathobiology. Further studies are in progress to demonstrate the functional role of IL-17 in the CLL microenvironment.

4.48

CD49D EXPRESSION IDENTIFIES A CLL SUBSET WITH HIGH LEVELS OF CIRCULATING CD34+ CELLS WITH ENDOTHELIAL FEATURES

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Background. In chronic lymphocytic leukemia (CLL) CD49d, often in association with CD38, has been shown to mark a disease subset with poor prognosis. Functionally, both molecules act as counter receptors for surface structures (i.e. VCAM-1/CD106 and CD31) usually expressed by the endothelial/stromal component of tumour micro-environment. We have recently identified a micro-environmental circuitry which involves CD38 triggering, and eventually determines an enrichment of the VCAM-1/CD106-expressing endothelial component detected in the context of lymphoid infiltrates of bone marrow biopsies. Data was also provided that CD49d/VCAM-1 interactions are functionally active, and can deliver pro-survival signals to CD49d-expressing CLL cells (Zucchetto *et al*., Cancer Res, 69, 4001, 2009). *Aim.* To investigate the amount of circulating progenitors with endothelial phenotype in CLL samples with different CD49d and CD38 expression levels. *Methods.* Peripheral blood (PB) samples from 91 CLL cases purposely selected with WBC >25,000/microl (B cells absolute lymphocyte count >10,000/microl), were evaluated in 4-6 colors multiparametric flow cytometry for the absolute count of the circulating $CD34^+$ cells (ISHAGE protocol in single platform). Whenever possible (i.e. if a cluster of at least 100 CD34+ cell was detectable in flow cytometric cytograms), a further characterization was performed for circulating endothelial cells (CEC), identified as a $CD34^+CD45^{low}$ cell population co-expressing one of the following endothelial markers: CD309/VEGFR-2, CD144/VE-cadherin, CD106/VCAM-1 and CD146/Muc-18. CD49d and CD38 expression by CLL cells was considered positive if exceeding the canonical cut-off value of 30% of positive cells. *Results.* PB absolute $CD34^+$ cell counts were 7.5±7.5/microl in $CD49d^+$ CLL (32 cases), vs. 3.3±2.7/microl in CD49d- CLL (59 cases; $p=2.6x10^{-4}$), or 9.4±8.7/microl in $CD49d^+$ CLL (30 cases) vs. 4.6±2.9/microl in CD49d- CLL (18 cases; $p=0.004$) when only cases phenotyped for CEC were considered. Furthermore, when samples were stratified also for CD38 expression, values of circulating $CD34^+$ cells increased to 10.6±10.1/microl in CD38+CD49d+ CLL (11 cases) vs. 3.1±2.4/microl in CD38-CD49d- (51 cases; p=1x10e-5). Regarding the absolute quantisation of CEC, a CD49d+ phenotype again marked the CLL subset with the highest CEC counts, as identified by the expression of either the CD309/VEGFR-2 (CEC counts 1.7±2.3/microl in $CD49d^+$ CLL vs. 0.5±0.5/microl $CD49d^-$ CLL; $p=0.009$) or the CD144/VE-cadherin (CEC counts 0.8±1/microl in $CD49d^+$ CLL vs. 0.3±0.5/microl in CD49d- CLL; $p=0.057$) endothelial markers on $CD34^+CD45^{low}$ cells. Notably, CEC from $CD49d^+$ CLL expressed CD106/VCAM-1 in virtually all cells (1.6±2.4/microl), while the other marker of endothelial activation CD146/Muc-18 was detected in a fraction of CEC only (0.4±0.9/microl). *Conclusions.* CD49d and CD38 expression by CLL cells identifies a disease subset with significantly higher numbers of both circulating $CD34^+$ cells and CEC. This phenomenon could be explained by considering several aspects: i) the sharing of common phenotypic markers between CLL cells and $CD34^+$ progenitors, including CD38 and CD49d, which could be responsible for a displacement of $CD34^+$ progenitors in the context of micro-environmental niches; ii) the known capacity of CLL cells, especially with a unmutated IGHV gene status and/or a $CD38^+CD49d^+$ phenotype to produce pro-angiogenic factors including Ang-2; iii) the rare PB cells expressing CD34 and CEC markers may represent CLL cell precursors with tumour-initiating cell features. Studies are currently ongoing to dissect among these intriguing hypotheses.

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THE TUMOR MICROENVIRONMENT AS A CONDITIONING FACTOR FOR THE SURVIVAL OF MALIGNANT CELLS IN IGVH UNMUTATED CHRONIC LYMPHOCYTIC LEUKEMIA

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Chronic lymphocytic leukemia (CLL) is a clinically heterogeneous disease. A very reliable prognosticator is the mutational status of the tumor immunoglobulin heavy chain variable region (IgVH): patients with unmutated (UM) IgVH have a worse prognosis than patients with mutated (M) IgVH. Soluble factors (i.e. IL-4 and CD40L) and cellular components of the local microenvironment [i.e. bone marrow stromal cells (BMSC)] are important survival factors for CLL B cells. It is currently unknown to what extent UM and M CLL cells depend on the local microenvironment for their survival. We have evaluated the spontaneous apoptotic rate of tumor cells isolated by immunomagnetic selection from the peripheral blood (PB) of M and UM CLL patients. Leukemic cells purified by negative selection from the PB of UM CLL patients showed significantly higher rates of spontaneous apoptosis after long-term *in vitro* culture as compared to CLL cells isolated from M patients. Both M and UM CLL cells showed high basal levels of Bcl-2 expression and NF-kB activity soon after purification. *In vitro* spontaneous apoptosis of purified UM CLL cells was associated with a progressive downregulation of the intracellular expression of Bcl-2 and with a complete loss of the active nuclear form of NF-kB. On the contrary, the higher long term viability of M CLL cells was paralleled by maintained Bcl-2 and NF-kB expressions. IL-4 and CD40L, used alone or in combination, as well as murine and human BMSC were capable of rescuing UM tumor cells from apoptosis. The pro-survival effect of these stimuli was exerted through the upregulation of Bcl-2 and was totally independent from the recovery of NF-kB nuclear translocation. We observed that UM CLL cells were less susceptible to spontaneous apoptosis when cultured as unfractionated peripheral blood mononuclear cells (PBMC) as compared to purified leukemic cells. This higher cell viability was associated with a retained expression of Bcl-2 and of the nuclear form of NF-kB, thus suggesting the presence of a pro-survival element in the peripheral blood of these patients. Coculture experiments showed a significant increase in the absolute number of $CD5^+/CD19^+$ viable CLL cells when purified UM cells were plated in the presence of autologous peripheral blood T cells (PBT). The prosurvival effect of circulating T cells was particularly evident at high T:B ratio, did not require a cell-cell contact and was mediated by the upregulation of Bcl-2 and the activation of NF-kB in leukemic cells. On the contrary, M cells viability as well as Bcl2 expression and NF-kB activity were totally independent from the presence of autologous PBT. These data indicate that the survival of UM tumor cells is highly dependent on the action of multiple microenviromental stimuli. Conversely, M cells are intrinsically more resistant to apoptosis and minimally influenced by the local microenviroment. The higher dependency of UM CLL cells from extrinsic signals might be exploited to develop new therapies targeting the tumor microenvironment and to improve the outcome of more aggressive CLL.

4.50

EFFECT OF THE ABNORMAL ZAP-70 EXPRESSION IN CHRONIC LYMPHOPROLIFERATIVE DISORDERS

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Background. ZAP-70 (-associated protein) is a tyrosine kinase (PTK) of the Syk/ZAP family normally expressed in T and NK cells. Cases of chronic lymphocytic leukemia (CLL) expressing high levels of ZAP-70 have a worse clinical outcome. Mechanisms by which ZAP-70 protein expression can influence clinical outcome are not fully understood. After B-cell receptor (BCR) stimulation, activated ZAP-70 can cooperate with Syk, the tyrosine kinase of the BCR signal pathway normally expressed in B-cells, thus inducing activation of the CLL cells. It is well known that stimulation of BCR in patients with high ZAP-70 expression not only leads to the triggering of Syk protein kinase to the cytoplasmatic tails of the receptor with the consistent PI3K/Akt pathway activation, but also induces prolonged survival and proliferation. Against this background, we analyzed the functional consequences of abnormal expression of ZAP-70 protein in a heterologous system (Ramos cell line), which normally does not express ZAP-70, paying particular attention to cell metabolism and migration. Samples and Methods The Burkitt cell line Ramos was stably transfected with the ZAP-70 expression vector pEGFP-ZAP-70 as well as the control pEGFP. BCR stimulation was induced by immobilized IgM at 5 minutes and 24 hours, and phosphorylation of downstream proteins was analyzed by Western Blot. Proliferation, cell cycle, calcium flux, and cell migration were analyzed in both stable Ramos cell lines after IgM stimulation. *Results.* After IgM stimulation, phosphorylation of ZAP-70 was observed at residue Y319 and, simultaneously, phospho-Syk expression was reduced. In addition, Erk protein was strongly activated in ZAP-70 transfected cell line compared to non-transfected cells, this activation lasting more than 24 hours after stimulation. Notably, calcium flux detected by flow cytometry was higher in ZAP-70 transfected Ramos than in non-transfected. Finally, migration experiments showed that transfected ZAP-70 Ramos expressed a higher number of adhesion molecules on surface membrane and that they migrated more rapidly than cells transfected with the vector alone. *Conclusions.* Addition of ZAP-70 expression to a heterologous B-cell system enhances BCR downstream signalling, calcium mobilization, and migration. Ongoing studies are determining the influence on proliferation and on the gene expression profile dependent of ZAP-70. These data suggest that additional ZAP-70 expression in CLL and other lymphoproliferative disorders enhances tumoral activity, this explaining in part the poor clinical prognosis of patients with increased expression of ZAP-70.

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TARGETING OF CYCLING B-CLL CELLS FOR APOPTOSIS BY TREATMENT WITH P53-UPREGULATING NUTLIN AND COX-2 INHIBITOR

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P53 and COX-2 exert interrelated functions in a number of cell lineages. In B-CLL, mutations in p53 provide transformed clones with a growth advantage and UM-CLL with the worst prognosis exhibit elevated levels of COX-2. In this study, we have addressed the importance of p53 and COX-2 for the sustained viability and proliferation of B-CLL clones activated *in vitro*. To initiate B-CLL cell cycling that can occur within proliferation centers of a patient's lymphatic tissues, CFSE-labeled B-CLL cells were cultured for 7 days with stimulatory oligonucleotide ODN-2006 + IL-15. Flow cytometry revealed that, under these conditions, both p53 and COX-2 are elevated within replicating progeny. After 3 or 5 days of activation, cultures were pulsed with Nutlin (a small molecule inhibitor of the cell's negative regulator of p53, i.e. Mdm2) and/or CAY10404, a highly selective inhibitor of COX-2. Cultures were monitored for (a) number of proliferative cycles and (b) viability of cells within each division subset. In most cultures only a fraction of B-CLL cells underwent cycling. B-CLL clones fell into four subsets in terms of Nutlin-induced apoptosis: (a) greater sensitivity of divided cells vs undivided cells (9/13); (b) greater sensitivity of undivided vs divided (1/13); (c) equal sensitivity of divided vs undivided (2/13); and (d) little or no sensitivity (1/13). 11 of 13 showed reduced yield of highly divided cells in the presence of Nutlin. B-CLL clones treated with COX-2 inhibitor also fell into four subsets: (a) greater sensitivity of divided cells vs undivided cells (5/10); (b) greater sensitivity of undivided vs divided (3/10); (c) high sensitivity of both undivided and divided (1/10); and (d) little to no sensitivity (1/10). 8 of 10 showed reduced yield of highly divided cells in the presence of CAY10404. The B-CLL clone showing least susceptibility to Nutlin was also unaffected by CAY10404. Cooperation between limiting doses of Nutlin and COX-2 inhibitor in promoting B-CLL apoptosis was noted in 8/12 B-CLL clones tested. Exogenous PGE2 did not reverse apoptosis seen in Nutlin- or COX-2-inhibitor-treated cultures, unlike what has been observed in clones of normal human B cells. P53 is known to act as either a transcriptional activator or repressor of multiple genes, and initial efforts to define the mechanism of Nutlin action reveal that p53 as well as Bid and pro-cas-

pase 6 (molecules known to be transcriptionally activated by p53) are increased within the replicating and non-replicating cells in activated B-CLL cell cultures. Consistent with a role for p53 in repressing Bcl-2, the latter anti-apoptotic molecule was often downregulated within *in vitro* replicating B-CLL, and Nutlin treatment could further downregulate Bcl-2. Thus, Nutlin exerts pro-apoptotic effects preferentially on replicating B-CLL clones and appears to do this by increasing the expression of molecules known to promote intrinsic mitochondria-dependent apoptosis and by decreasing the expression of molecules that counteract it. The involvement of COX-2 in modulating the above is being investigated.

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RECURRENT CHROMOSOMAL UNBALANCE TRANSLOCATION IN TWO CASES OF CHRONIC LYMPHOCITIC LEUKEMIA WITH TP53 DELETION.

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Introduction. Chronic lymphocytic leukaemia (CLL) is the most common leukaemia in the Western world. The disease has a highly heterogeneous clinical course, with time to progression varying from months to many years. The classical clinical staging systems for CLL that were introduced 3 decades ago by Rai and Binet have been extremely useful in guiding disease management and treatment decisions. However, these staging systems have failed to predict the clinical course for individual patients at early-stage disease and to identify patients with poor prognosis. The analysis of chromosomal aberrations has provided significant prognostic information. Deletion of 17p13 (17p- CLL), the locus of the TP53 gene, identifies the most aggressive subset. Cases of 17p- CLL typically are resistant to alkylating agents and purine analogues, have a high risk of transformation and a median survival of only 36 months, with outcome independent of clinical stage or immunoglobulin heavy chain gene (IGHV) status. Genomic arrays analyses have been rule out those patients with TP53 deletion shows recurrent multiple copy number variation (CNV). Some of this CNV, like deletion 8p and gain 2p16.1-p14, seem to be relevant in clinical course as risk indicators of small free treatment time from diagnosis and overall survival. *Material and Methods.* Patients: 1. Women 46 years old, with Rai-Binet status A1 at diagnosis and overall survival of 36 months. 2. Male 67 years old, diagnosed recently with Rai-Binet status A0. Conventional cytogenetics: Cytogenetic studies were carried out in peripheral blood samples after 72-hour culture, with 12-O-tetradecanoyl-phorbol-13-acetate (TPA) as mitogen according to standard protocols, Chromosomes were classified according to the International System for Human Cytogenetic Nomenclature (ISCN). Molecular cytogenetics: FISH analyses were performed in cell suspension from the conventional cytogenetics culture, with CLL kit 32-191025 (LSIp53 / LSI ATM and LSI D13S319 / LSI 13q34 / CEP 12 Multi-colour Probe Sets), it was applied according to the manufacturer's specifications (Vysis). Two hundred nuclei were analysed for each probe. RESULTS Conventional cytogenetics Patient 1: 46,XX [12] at diagnosis, and a complex karyotype in follow-up 43-47,XX,del(1)(p36), t(2;10)(q13;q26),-3,-8,der(14)add(q32), der(17)t(8;17)(q12;p13), +der(17)t(8;17)(q12;p13),+mar1, +mar2 [cp23]/46,XX [8]. Patient 2: 45,XY,-8,der(17)t(8;17)(q12;p13)[8]/46,XY [6]. Molecular cytogenetics Both patients showed TP53 deletion at diagnosis in about 90% of nuclei (87 and 93% respectively). *Remarks*. From our knowledge it is the first time that it recurrent chromosomal abnormality is described. In the first patient is probably related with progression in a complex karyotype context, but in the second one is detected as sole anomaly in a patient with low risk clinical status (A0). We can remark the relevance to combine conventional and molecular cytogenetics in CLL to detect new recurrent clonal chromosomal aberrations that can help us to understanding the biological mechanisms related with the pathology and evolution of CLL patients. In addition, the potential relevance of 8p loss has been described in other haematological neoplasm with aggressive clinical course like mantle cell lymphoma. The clinical follow-up of this second patient can give us new data about 8p losses related with clinical course in CLL.

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CLL-TYPE MONOCLONAL B-CELL LYMPHOCYTOSIS (MBL) AND CLL SHOW DIFFERENTIAL EXPRESSION OF MOLECULES INVOLVED IN LYMPHOID TISSUE HOMING

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CLL-type Monoclonal B-cell lymphocytosis is defined as the presence of circulating B-cells with restricted immunoglobulin expression and a phenotype consistent with CLL but an absolute B-cell count below 5,000 cells/μL with no other features of a B-lymphocyte malignancy. More than 10% of individuals with a lymphocytosis have CLL-type MBL and approximately 1% per year will require treatment for progressive disease but a large proportion have a stable B-cell count over time. The aim of this study was to screen for cell surface markers that could discriminate CLL-type MBL from CLL or identify CLL cases likely to have stable disease. 6-colour flow cytometry was performed on 94 cases of CLL-type MBL at diagnosis and 387 cases of CLL at diagnosis or relapse at least 3 months after treatment, of which 39 cases were identified as having adverse-risk chromosomal abnormalities (17p and/or 11q deletion). B-cells were identified using CD19, CD20 scatter characteristics and expression of 30 markers previously shown to be informative in diagnosis and monitoring of B-lymphoproliferative disorders was analysed: CCR6, CD10, CD103, CD11c, CD138, CD200, CD22, CD23, CD24, CD25, CD27, CD31, CD38, CD39, CD43, CD49d, CD5, CD52, CD62L, CD63, CD79b, CD81, CD86, CD95, CXCR5, HLADR, IgD, IgG, IgM, LAIR1. There was no difference in expression between CLL-type MBL and CLL for the majority of markers. Most differences were only seen between MBL and CLL cases with adverse-risk chromosomal abnormalities. These differences included lower expression of known prognostic markers CD38 and CD49d which were 9.4-fold lower (p=0.007) and 3.2-fold lower (p=0.008) respectively on CLL-type MBL compared to CLL with adverse chromosomal abnormalities. Levels of the inhibitory receptor LAIR-1, which is downregulated during the normal germinal centre reaction, was 3.7-fold higher (p=0.003) in CLL-type MBL compared to adverse prognosis CLL. Also there was 1.9-fold higher (p<0.001) CCR6 expression and 1.25-fold higher (p=0.002) CXCR5 expression in CLL-type MBL compared to adverse-risk CLL. Both of these markers are normally responsible for B-cell homing to secondary lymphoid tissue. Only the expression of CD62L (L-selectin) which mediates lymphocyte homing to endothelial venules of lymphoid tissue, was expressed at a significantly different level between CLL-type MBL and CLL, with 1.3-fold lower (p=0.04) expression levels on the MBL cases. However, there was broad overlap in expression levels. CLL-type MBL is phenotypically identical to CLL for a large range of markers. Differential expression is predominantly related to known prognostic markers and proteins involved in homing to lymphoid tissue.

Prognostication

5.1

A PROGNOSTIC SCORING SYSTEM BASED ON BIOLOGICAL MARKERS IN EARLY STAGE CHRONIC LYMPHOCYTIC LEUKAEMIA

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Novel biological prognostic markers have been proposed to stratify newly diagnosed patients (pts) with early stage Chronic Lymphocytic Leukaemia (CLL) in subsets carrying a different risk of progression. When evaluated singly, ZAP70 overexpression, CD38 overexpression and unmutated IgHV status have been consistently associated with an inferior outcome. Although prognostic factors are often concordant in individual cases, a discordant pattern can be detected in up to 30% of pts. As to the hierarchy of these markers in predicting the tendency for progression, results from different studies are not univocal. We aimed to determine whether the simultaneous analysis of all three biological variables and their combination proved helpful in clinical practice, in order to plan patient monitoring and predict the time to therapeutic need. The outcome of 329 consecutive Binet stage A patients (median age: 59 yrs, range: 34-81) was reviewed. In 70% of cases, marker assessment was performed on samples collected at diagnosis. Two different cut-off values for CD38 expression (anti-CD38-PE, Becton Dickinson Lab) were evaluated: ≥30% or ≥7%; ZAP70 expression was analysed by flow cytometry (Alexa Fluor 488 Caltag Lab; cut-off ≥20%); mutational status of IgHV genes was analysed according to IMGT (2% mutation threshold). Treatment-free interval (TFI) estimates were obtained using the Kaplan-Meier method and compared using the Gehan-Wilcoxon test. *Results.* 124 pts (38%) were CD38 positive with a 7% cut-off, while 42 pts (13%) were positive with a 30% cut-off; ZAP70 overexpression was observed in 68 pts (21%). IgHV genes were unmutated in 115 case (35%). Less than 9% of cases carried unfavorable cytogenetic abnormalities (del11q or del17p) at FISH analysis. At 4 yrs median FU from diagnosis (range:1-19), 124 pts (37%) required treatment. Most of them started treatment according to NCI-WG Guidelines (1996), while some patients in the '90s were treated at clinical progression according to attending physician's decision. In multivariate analysis, mutational status was the only independent predictor of TFI (Cox multivariate regression model: HR 2.6, $p<0.00001$, 95%CI 1.7-3.8) at both CD38 cut-off levels. As to the impact of biomarker combination on TFI, similar results were achieved regardless of the cut-off point chosen for CD38 expression, but the lower cut-off level (≥7%) allowed a more stringent selection of low-risk pts. The absence of unfavorable biomarkers was predictive of the best outcome, and concordance for unfavorable parameters was associated with the shortest TFI.

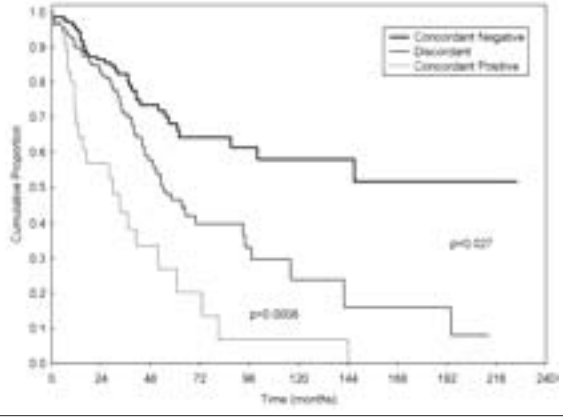

Figure 1.

The analysis of discordant cases revealed no difference in TFI curves for pts carrying one or two negative biomarkers. By combining discordant pts, we identified an intermediate risk group with significantly different outcome. TFI rate was 51% at 216 mos for the concordant favorable subset (147 pts, 44.5%), while median TFI was 53.6 mos for discordant pts (N=146, 44.5%) and 29 mos for pts with concordant unfavorable factors (N=36, 11%) (Figure 1 $p=0.00000$). The simultaneous assessment of the three biological variables proved helpful in differentiating early stage CLL pts with a concordant favorable profile and an estimated long TFI from pts with concordant adverse biomarkers who carry the highest risk of early progression and require strict clinical monitoring. To this aim, the evaluation of IgHV mutational status is essential, although laborious and time-consuming. As to the large proportion of pts with discordant factors (44.5%), reliable prognostic information is still lacking. We need to know more on disease biology and collect data from prospective and methodologically comparable studies.

5.2

BAFF AND APRIL IN CHRONIC LYMPHOCYTIC LEUKEMIA: CLINICO-BIOLOGICAL CORRELATES AND PROGNOSTIC SIGNIFICANCE

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BAFF (B-cell activating factor) and APRIL (a proliferation-inducing ligand) are TNF family proteins that upregulate anti-apoptotic genes through the NF-kB pathway. Studies *in vitro* suggest that BAFF and APRIL protect neoplastic B cells from apoptosis in chronic lymphoproliferative disorders (CLPD) including chronic lymphocytic leukemia (CLL). Serum BAFF levels have been previously shown to be lower in CLL than in other CLPD or normal subjects. To contribute to a better understanding of their role in CLL, we analyzed BAFF and APRIL at mRNA and protein serum levels and their receptors [transmembrane activator and CAML interactor (TACI), B-cell maturation antigen (BCMA) and BAFF receptor (BAFF-R)] by flow cytometry, in 82 patients with CLL, 36 with other CLPD and 35 age- and sex-matched controls. mRNA BAFF and APRIL levels were calculated as a the percentage of expression referred to an internal control and the receptor expression as the ratio between the mean fluorescence intensity (MFI) of the receptor antibody and the MFI of the isotype control. Patients with CLL showed significantly lower median BAFF and APRIL levels (0.63 g/mL and 3.18 g/mL) than those with other CLPD (1.27 g/mL and 5.51 g/mL) ($p<0.05$). Moreover, BAFF but not APRIL was lower in CLL than in healthy subjects (0.63 g/mL vs. 0.77 g/mL; $p<0.0001$). Serum BAFF levels and blood lymphocyte counts were inversely correlated. Likewise, in follicular lymphoma patients who had circulating neoplastic B cells, median BAFF levels was 0.84 g/ml vs. 1.46 g/mL in those without detectable neoplastic cells in blood ($p<0.05$). We also examined the expression of BAFF and APRIL in purified $CD19^+$ cells from 19 CLL patients and 10 healthy controls. All CLL and normal B cells expressed BAFF and APRIL although heterogeneously. Nevertheless, BAFF and APRIL were lower in CLL than in normal B cells (median: 6.24% and 12.73% in CLL vs. 11.54% and 42.26% in controls). In CLL, mRNA BAFF expression inversely correlated with BAFF serum levels. As far as BAFF and APRIL receptors are concerned, BAFF-R was the one most highly expressed in CLL and normal B cells (MFI ratios of 167.3 and 157.2, respectively). TACI and BCMA were also expressed in all CLL cells and normal B cells (MFI ratios TACI: 1.70 and 2.41; BCMA: 9.51 and 4.72, respectively), but at a significantly lower level than BAFF-R ($p<0.001$). Furthermore, whereas BCMA MFI ratio was significantly higher in CLL than in normal B cells ($p<0.05$), no differences were observed in the expression of TACI and BAFF-R. TACI expression was heterogenous in CLL cells. BAFF-R inversely correlated with BAFF and APRIL serum levels. From a clinical standpoint, there is some indication that BAFF and APRIL serum levels as well as their expression in CLL cells may correlate with clinical and biological characteristics of the disease. No significant relationship was observed between BAFF and APRIL and IGVH mutational status, ZAP-70, CD38 or cytogenetics. However, an inverse correlation was observed between BAFF serum levels and blood lymphocyte counts as well as advanced clinical stage ($p<0.05$). In contrast,

APRIL serum levels were only correlated with CD38 expression, the higher the expression of CD38 the higher the APRIL. Although blood lymphocyte counts and BAFF serum levels are correlated, a multivariate analysis showed that these two variables along with poor risk cytogenetics were independent predictors of progression (poor risk cytogenetics RR=11.699, $p<0.05$; high blood lymphocyte count RR=9.780, $p<0.05$ and low serum BAFF RR= 6.098, $p<0.05$). In summary this study confirms that BAFF and APRIL serum levels are lower in CLL than in other CLPD. In patients with CLL, BAFF serum and mRNA levels correlate with blood lymphocyte count and advanced clinical stage but not with other well known prognostic factors. Finally, although BAFF correlates with blood lymphocyte counts, our results suggest that BAFF serum levels have independent prognostic significance.

5.3

PROGNOSTIC AND THERAPEUTIC RELEVANCE OF DIPEPTIDYL PEPTIDASES IN B-CELL CHRONIC LYMPHOCYTIC LEUKAEMIA

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There is an increasing awareness of the potential therapeutic and biological relevance of dipeptidyl peptidase (DP) IV (CD26) and related enzymes in cancer. This is the first study to characterise the expression of all DPIV-like enzymes in B-CLL, and to fully investigate their potential as novel prognostic markers and therapeutic targets for this disease. We show that the mRNA expression of DPIV/CD26, DP8, DP9, DPII and PEP are constitutively expressed in B-CLL and that the expression of DP8 in B-CLL is significantly higher than in normal tonsillar B-lymphocytes. Additionally the upper and lower quartiles of DP8 expression correlated with CD38 expression, raising a potential role for DP8 in the pathogenesis of B-CLL. Contrary to other reports, surface expression of DPIV/CD26 could not be correlated with any prognostic marker for B-CLL suggesting limited prognostic application. Additionally, we show that non-selective DP inhibition using ValboroPro (VbP) or VA999043 causes non-selective cell death of both B-CLL and normal B-lymphocytes, while more selective DP inhibition fails to kill cells at all. This data would suggest that although DP8 may have a role in the biology of B-CLL, it may be inappropriate to pursue the use of VbP in clinical trials aimed at B-CLL therapy. ACKNOWLEDGEMENTS: This work was supported by PhD scholarship from the Leukaemia Foundation of Australia for M. Sulda. Dr. Kuss is a member of the CLL Australian Research Council (CLLARC).

5.4

EXPRESSION OF MUTATED IGHV3-23 GENES IN CHRONIC LYMPHOCYTIC LEUKEMIA IDENTIFIES A DISEASE SUBSET WITH PECULIAR CLINICAL AND BIOLOGICAL FEATURES

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Background. B-cell chronic lymphocytic leukemia (CLL) is a clinically heterogeneous disease whose outcome can be foreseen by investigating the mutational status of immunoglobulin heavy chain variable (IGHV) genes. Moreover, different prognosis was reported for CLL expressing specific IGHV genes in the context or not of stereotyped B-cell receptors (BCR). Here we investigated novel associations between usage of specific IGHV genes and clinical features in CLL. *Methods.* A HCDR3-driven clustering of 1,426 IG sequences (1,398 patients) was performed using ClustalX(1.83). Time to treatment (TTT) intervals, Rai staging, IGHV mutational status, CD38, ZAP-70, and karyotype abnormalities evaluated by FISH were available for 617 patients. Gene expression profiling (GEP) and quantitative real-time PCR experiments (QRT-PCR) were performed on purified CLL cells. *Results.* IGHV3-23 was totally absent in 71 identified stereotyped clusters despite being the second most frequently used IGHV gene. Although 109/134 IGHV3-23 were mutated (M), alignment of IGHV sequences revealed a high degree of conservation in the context of the 13 AA positions involved in superantigen binding by IGHV3 subgroup genes, suggesting that the majority of M IGHV3-23 cases maintained the capacity to mediate superantigen recognition and binding. Median TTT (73 months) of 43 M IGHV3-23 CLL was significantly shorter than median TTT (253 months, $p=0.0153$) of 333 M CLL, as well as of 326 M CLL in which 7 cases belonging to the bad prognosis IGHV3-21/IGLV3-21 cluster were excluded (253 months, $p=0.0082$). Multivariate Cox proportional hazard analyses selected IGHV3-23 usage ($p=0.029$), Rai stage ($p<0.0001$) and FISH group ($p<0.0001$) as independent markers of disease progression for 376 M CLL, and for the cohort in which 7 M CLL from the IGHV3-21/IGLV3-21 cluster were excluded. Comparing 5 M IGHV3-23 and 22 M non-IGHV3-23 CLL for their differential GEP, 212 genes were selected, 108 up-regulated and 104 down-regulated in M IGHV3-23 CLL. Using the "Gene-Ontology Tree Machine" platform, a set of growth/tumor suppressor genes (PDCD4, TIA1, RASSF5), all down-regulated in M IGHV3-23 CLL, were significantly enriched in several gene-ontology categories related to apoptosis. QRT-PCR confirmed a significant down-regulation of PDCD4, TIA1 and RASSF5 in 15 M IGHV3-23 compared to 35 M non-IGHV3-23 CLL. Given the notion that PDCD4 and TIA1 are among the genes under control of miR-15a and miR-16-1 a "Gene Set Enrichment Analysis" carried out on the 212 differentially expressed genes, confirmed that M IGHV3-23 samples were significantly deprived in genes whose expression is under control of miR-15a and miR-16-1. Accordingly, QRT-PCR experiments performed on 15 M IGHV3-23 and 35 M non-IGHV3-23 CLL revealed significantly higher levels of both miR-15a and miR-16-1 in M IGHV3-23 cases. No difference was found in the distribution of patients with 13q14 deletion between M IGHV3-23 CLL and M non-IGHV3-23 CLL ($p=0.19$). *Conclusion.* Expression of IGHV3-23 marks a subset of M CLL with a worse prognosis; such a peculiar clinical behaviour may be related to superantigen stimulation combined with down-regulation of specific growth/tumor suppressor genes and up-regulation of miR-15a and miR-16-1.

5.5

EVALUATION OF ZAP-70 EXPRESSION BY MEAN FLUORESCENCE INTENSITY T/B RATIO IS A MORE USEFUL PROGNOSTICATOR THAN PERCENTAGE OF POSITIVE CELLS IN CHRONIC LYMPHOCITIC LEUKEMIA

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Background. Expression of the T-cells constitutive ZAP-70 protein by CLL cells has been the focus of many studies in the last few years, due to its ability to stratify indolent and more aggressive disease subsets. Although the strength of ZAP-70 as independent negative prognosticator was demonstrated by several studies, concerns about its use derive from a lack of multicentric standardization of flow-cytometric protocols. Analyses in clinical settings are usually performed according to two methods, respectively evaluating the percentage of CLL cells expressing ZAP-70 compared to isotypic control (ISO-method), or to autologous T-cells (T-method). Of note, while the two methods yield concordant results in most patients, a fraction of cases may be discordant as for eval-

uation of ZAP-70 positivity. Moreover, either method suffers of an operator dependent variability, mainly related to subjectivity in cursor placement to determine the percentage of ZAP-70+ cells. Aims. To compare the ISO- and T-methods with the expression of ZAP-70 evaluated as Mean-Fluorescence-Intensity (MFI) Ratio between gated T and CLL cells (T/B-Ratio-method), and to assess the prognostic significance of the three approaches. *Methods*. Cytometric files relative to ZAP-70 determination according to the three readouts were retrospectively reviewed with BD-DiVa software on a cohort of 173 patients (test set), all with complete clinical and biological prognostic assessment and time-to-treatment (TTT) available. Findings were then validated in an independent cohort of 341 cases from a different institution (validation set). Notably, in the two cohorts, ZAP-70 assessment was accomplished using two different antibody combinations and instrumentations for data acquisition and analysis. *Results.* ZAP-70 expression was reviewed in the test set by applying the ISO- and T-methods. Utilizing respectively 11% for ISO-method and 20% of ZAP-70$^+$ cells for T-method, both selected as optimal cut-offs with prognostic relevance by ROC-analysis and maximally selected log-rank statistics, 66 (ISO-method) and 60 (T-method) ZAP-70$^+$ cases were defined. By applying the T/B-Ratio-method, a value of 3.0 was identified as the optimal prognostic cut-point. According to this value, 73 ZAP-70$^+$ cases (i.e. with T/B-Ratio<3.0) were identified in the test set. Univariate analyses resulted in a better separation of ZAP-70+ vs ZAP-70- CLL patients utilizing the T/B-Ratio-method (p=5.6x10^{-06}), compared to T- (p=1.27x10^{-05}) or ISO- (p=0.009) methods. In multivariate analyses with RAI stage, β-2-microglobulin, IGHV, FISH, CD38 and CD49d, ZAP-70 was selected as independent risk factor, irrespective of the readout employed for evaluation of ZAP-70 expression; however, the prognostic impact of ZAP-70 appeared stronger when the T/B-Ratio-method was applied (significative hazard ratio=2.72 vs 2.19 with the T-method, or 2.11 with ISO-method). To confirm these findings, we analyzed the 341 cases of the validation set with T-method (cut-off=20%) and T/B-Ratio-method (cut-off=3.0). Analyses yielded 180 (T-method), and 127 (T/B-Ratio-method) ZAP-70$^+$ cases. Univariate analyses also on this cohort resulted in a better separation with T/B-Ratio-method (p=7.77x10^{-16}) than with T-method (p=1.23x10^{-12}). Of note, ROC-analysis and maximally selected log-rank statistics confirmed also in this patient series, the 20% and 3.0 values as optimal cut-offs capable to separate CLL patients into two classes with different treatment probabilities. Conclusion. We suggest to evaluate ZAP-70 expression in routine settings using the T/B-Ratio-method given the operator and laboratory independent feature of this approach. We propose the 3.0 T/B-Ratio value as optimal cut-off to discriminate ZAP-70$^+$ (T/B-Ratio less than 3.0) from ZAP-70- (T/B-Ratio more/equal than 3.0) cases.

5.6

UDP GLUCURONOSYLTRANSFERASE 2B17 MRNA EXPRESSION INFLUENCES PATIENT OUTCOME IN CHRONIC LYMPHOCYTIC LEUKEMIA

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The enzyme uridine diphospho glucuronosyltransferase 2B17 (UGT2B17), localized on chromosome band 4q13, glucuronidates various endogenous compounds and xenobiotics, in particular androgens. Moreover, antileukemic drugs like anthraquinones or histone deacetylase (HDAC) inhibitors (e.g. suberoylanilide hydroxamic acid, SAHA) are subject to glucuronidation by this enzyme. The gene shows a variable polymorphism-frequency and by far the most dramatic difference in expression between the ethnic groups of Asians and Caucasians. In our previous microarray-analysis UGT2B17 was identified as differentially expressed between high and low risk CLL distinguished by high and low LPL-expression. Here, we investigated the role of UGT2B17 gene expression by real-time PCR in 152 patients with chronic lymphocytic leukemia (CLL) and the UGT2B17 polymorphism by conventional PCR in 210 CLL patients and 449 healthy donors. Treatment response to fludarabine and cyclophosphamide containing regimens was compared with mRNA expression as detected by real-time PCR in 42 patients and by microarray-analysis before and after induction of therapy in 20 patients. UGT2B17 mRNA expression ranged from 0 – 264.12 compared to normal PBMC (set as 1). High UGT2B17 (>2.285) was a poor prognostic factor for CLL and was associated with short treatment-free survival (TFS) in early stage disease (median TFS 68 months vs. 174 months; Hazard Ratio=2.68; CI 1.67- 4.31; p<0.001). Furthermore, it was significantly associated with unmutated IgVH (p<0.001), high LPL expression (p<0.001), high CD38 expression (p=0.003), and male sex (p=0.031). It was negatively associated with deletion of 13q- (p=0.002). Of note, low UGT2B17 was associated with deletion 17p- (mean expression levels 5.8 vs. 25.8; p=0.038), while there was no correlation with deletion 11q-. UGT2B17 mRNA levels up to three logs higher on CD19$^+$ cells compared to CD19$^-$ cells. Importantly, high UGT2B17 predicted poor response to therapy with fludarabine containing regimens (Odds Ratio 5.03; p=0.046). Microarray analysis showed a considerable increase of UGT2B17 mRNA expression within 48 hours of treatment only in patients with stable or progressive CLL (mean relative change=134.1%, CI 106.0%-169.7%) compared to patients who went into complete or partial remission (mean relative change=99.1%; CI 84.2-116.7%) (p=0.030). Genotype frequencies in 210 CLL patients were 39.0% for wild type (Ins/Ins), 49.0% for heterozygosity (Ins/Del), and 12.0% double deletion (Del/Del), respectively. In 449 healthy donors we found 43.0% Ins/Ins, 46.3% Ins/Del, and 10.7% Del/Del. The UGT2B17 polymorphism did not differ significantly between men and women or between CLL patients and healthy donors suggesting no decisive impact of genotype on CLL development in Caucasians. In conclusion, our data indicate that UGT2B17 is a novel prognostic and predictive marker in CLL, potentially influencing drug metabolism.

5.7

SCREENING FOR ATM DELETION BY FISH; SIZE MATTERS

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Mono allelic deletion of 11q22-q23 in CLL, detected by FISH, predicts short TFS and poor outcome following alkylator/purine analogue therapy. Currently 11q status is most frequently assessed by FISH. We report 5 cases which demonstrate an atypical and significant FISH signal pattern that has not been reported previously. Since 2003 we have undertaken prognostic ATM FISH screening, for both newly presenting CLL patients and those showing clinical progression (n=849), using the Vysis ATM probe. 84 patients have been found to have heterozygous ATM loss. In the majority of cases, analysis was of peripheral blood mononuclear cells separated by density gradient centrifugation, treated with hypotonic solution (0.75M KCl) then fixed with methanol:acetic acid (3:1) prior to co- hybridisation with Vysis ATM and CEP11 probes, according to the manufacturer's protocol. The Vysis ATM probe recognises a 733kb region of chromosome11q encompassing and centred round the ATM gene. During screening we observed 5 cases with one normal and one diminished ATM FISH signal, leading us to examine them further with a second smaller ATM probe of 158kb from Cytocell, that is also centred round the 146Kb ATM gene. Clinical details of the five patients are shown in Table 1a. Conventional karyotype analysis is available for 4/5 cases. In addition, either mutation analysis of 62 ATM coding exons or assessment by a TP53 dysfunction assay, using etopside and nutlin-3a has also been undertaken, as has SNP array analysis using Affymetrix 6.0 array. SNP arrays confirm the presence of small deletions encompassing ATM. 4/5 of these cases have either an ATM mutation or Type 2 TP53 dysfunction, which we have previously shown to correlate with the presence of an ATM mutation. Using the Cytocell and Vysis probes no further cases with microdeletions were observed in 2 further cohorts of patients. One, comprised 10 CLL patients with an ATM mutation, the other of 6 patients with Type 2 TP53 dysfunction. From our cytogenetic and FISH analysis, using other 11q BAC probes, we know that not all 11q deletions include the ATM gene and that they are heterogeneous both in size and the location of the deleted region (unpublished data). However, in this subset of patients array analysis shows small deletions with proximal breakpoints clustering within an 106 kb region proximal of ATM and extending dis-

tally according to the deletion size shown in Table1b. Deletions such as those that we describe here, are then probably rare but clinically significant; all having short treatment free and overall survival times (median = 23 and 37 months respectively). Therefore, laboratories undertaking prognostic FISH screening should be aware that the presence of discordantly sized FISH signals detected using the Vysis ATM probe, very widely used for screening, may represent ATM deletion and requires further investigation.

Table 1a. Clinical details.

Patient	Gender	Age at Diagnosis	Survival TFS	Survival OS	Treated	Alive/Dead
1	F	81,8	58	58	NO	D
2	M	49,4	23	59	YES	A
3	M	42,8	1	37	YES	A
4	M	55,3	1	21	YES	A
5	F	85	27	27	NO	D

Table 1b. Laboratory findings.

Patient	Karyotype	FISH % abnormal cells Vysis (diminished)	FISH % abnormal cells Cytocell (deletion)	ATM mutation	TP53 dysfunction	SNP array deletion size (Kb)
1	46,XX,t(3;4)(q27;q31)	23	30	mutated	NT	521,6
2	NT	69	90	mutated	NT	757,8
3	46,XY	67	87	NT	Normal	306,7
4	46,XY	78	95	NT	Type 2	571
5	46,XX	91	96	NT	Type 2	581,5

5.8

DETECTION OF CHROMOSOMAL 14Q32 ABNORMALITIES IN B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA

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Background. Conventional cytogenetic and FISH studies with the standard panel (13q14, 11q22.3, 17p13, 12, 6q23) are used to detect chromosomal abnormalities of clinical significance in patients with chronic lymphocytic leukemia (B-CLL). Recently, translocations and 5'IGH deletions involving chromosome14q32 are associated with B-CLL. Aim Our aim was to investigate the presence and the characteristics of chromosome 14q32 aberrations in a group of patients with B-CLL. *Methods.* A total of 58 patients with B-CLL were investigated by conventional cytogenetic, in addition the cultures were stimulated with CpG oligodeoxynucleotide plus IL2. FISH studies with 14q32/IGH break-apart probe designed to detect chromosomal breakage of the IGH (14q32) locus, were done for 59 patients. *Results.* Chromosomal abnormalities were detected in 60.3% of cases by cytogenetic. 14 patients showed complex cariotype while trisomy 12 was the most frequent anomaly found in 8 patients. Among the twelve other patients several chromosomal aberrations were noted: del(13)(q14), del(13)(q12q21),del(13)(q12q14), del(13)(q13q32), +21, t(11;13)(q23;q14), +12 t(1;4)(q31;p14), t(3;14)(p21;q22), del (11)(q12) +21, del(6)(q21), inv3(?p13q21), del(11)(q12). Abnormalities of chromosome 14 was found in 23.8% of patients. Deletion of 5'IGH, corresponding to the variable IGH segment, was the most frequent anomaly found in 8 patients. Interesting, a 3'IGH deletion was detected in two patients, while only one patient showed a complete deletion of chromosome 14 (47,XXY,add14q32). Three patients showed 14q32 translocations involving the IGH locus. *Summary/Conclusions*. Based on our findings, deletions of the variable region of the IGH gene (IGHv) and 14q32/IGH translocations are involved in B-CLL. As these preliminary data are based on small sample size, our goal will be to study the cytogenetic profile of a large number of CLL patients. Future studies will permit the identification of 14q32 translocations and the type and the frequency of IGH rearrangements. Finally, chromosome 14 abnormalities will be correlated to other cytogenetic and FISH abnormalities, and associations with known prognostic markers, such as IGVH mutation status and ZAP-70 expression, will be investigated.

5.9

SERUM FLC LEVELS AT PRESENTATION HAVE INDEPENDENT PROGNOSTIC SIGNIFICANCE IN CLL AND LEVELS ABOVE 50MG/L IDENTIFY PATIENTS WITH PROGRESSIVE DISEASE.

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An abnormal serum FLC ratio at presentation has been shown to be prognostic in chronic lymphocytic leukaemia (Pratt et al, 2009). Here we further analyse a retrospective cohort of 259 CLL patients (Stage: A, 209; B 23, C, 21; Male: Female ratio 1.6:1, mean age 75: Range 29-98). The levels of FLC and β2M were assessed using nephelometric immunoassays (The Binding Site, Birmingham UK) on the Siemens Dade Behring BN™II analyser. Previously recorded measurements for biological and clinical markers (age, sex, CD38, Zap70, and VH mutational status) were used to produce Kaplan Meier Survival Curves and in Cox Regression analysis. We have identified that a cut-off above 50 mg/L (in the context of an abnormal ratio to exclude renal effects) identifies a cohort of patients with progressive disease and a significantly poorer outcome. A total of 38 out of the 259 patients had serum FLC > 50 mg/L. CLL patients with serum FLC >50 mg/L had the following characteristics Stage A/B/C (26/5/5 + 2 unknown), Mutated vs Unmutated (19 vs 13, 6 unknown), Zap70 pos vs neg (19 vs 16, 3 unknown), CD38 pos vs neg (13 vs 23, 2 unknown) and 32 out of the 38 patients have progressed to treatment. Median time to first treatment for the CLL cohort with >50 mg/L serum FLC was 83 months compared to 241 months for the CLL patients with normal FLC (p=9.8x10^{-6}). Median overall survival was also significantly shorter for patients with an abnormal ratio and >50 mg/L serum FLC (p=7.6x10^{-7}). Cox regression analysis on the above population gave stage (Hazard ratio 3.9 p=<0.001), Zap 70 (Hazard ratio 1.9, p=0.001) and abnormal ratio with production above 50 mg/L (Hazard ratio 1.9 p=0.009) as the only independent prognostic variables. Importantly >50 mg/L FLC production is independent of both Zap70 and Vh mutational status as an indicator of time to first treatment. This study shows that in an unselected population of CLL patients serum FLC >50 mg/L can independently identify a group of CLL patients with progressive disease and a poorer outlook.

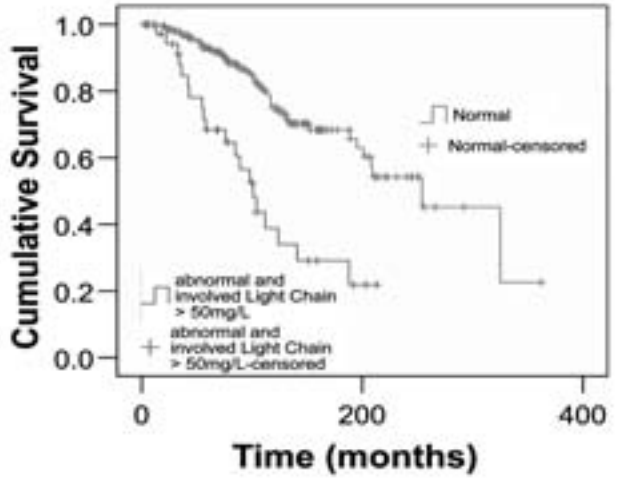

Figure 1. Overall survival for chronic lymphocytic leukaemia patients based on serum FLC > 50 mg/L (with an abnormal ratio) or < 50 mg/L.

5.10

CLINICAL OUTCOME IN PATIENTS WITH TP53 DYSFUNCTION- A SUBGROUP WITH GOOD CLINICAL PROGNOSIS

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TP53 dysfunction has been attributed to a number of different factors in CLL, including 17p deletion, 11q deletion, TP53 mutation and other, as yet undefined causes. Previous studies have demonstrated that 17p deletion is associated with poor survival, and poor response to standard chemotherapy. However, the overall effects of abnormalities of TP53 function on clinical outcome are not well defined. The presence of a TP53 mutation in CLL patients is generally associated with aggressive disease, though not all individuals with compromised p53 function have aggressive disease or require treatment. Clinical and laboratory data (including IGHV and TP53 mutational status and interphase cytogenetics) on 471 patients attending the CLL clinic at the Leicester Royal Infirmary over the past five years were assessed. Samples were collected prospectively from all patients attending the clinic. The median age at diagnosis was 62 years (range 30-92), male to female ratio was 1.8 to 1 overall with comparable age of onset for both sexes. 379 patients were Binet Stage A, 63 were stage B and 29 were stage C. Significant differences in time to progression was observed between stage A and B and also between stage A and C groups (p<0.01). Within this cohort, we identified 47 patients that had TP53 dysfunction, of which 26 were treated, but surprisingly 22 were untreated. The groups were not distinguishable by median age at diagnosis (67 vs 65) or gender (2.25:1 and 2.6:1). As expected, there were statistically significant differences in overall survival between the 47 TP53 dysfunctional patients and the whole group (p<0.0001). We found that the overall survival and progression free survival were significantly better in the untreated vs treated TP53 dysfunctional group (p=0.0019 and p<0.0001 respectively). Overall survival in the TP53 untreated was comparable with the rest of the cohort. Our data suggest that despite TP53 dysfunction, there is a substantial subgroup of patients who experience a significantly better clinical outcome than expected. The clinicopathological correlates which define these two groups remains unclear and new techniques for determining TP53 function may be required.

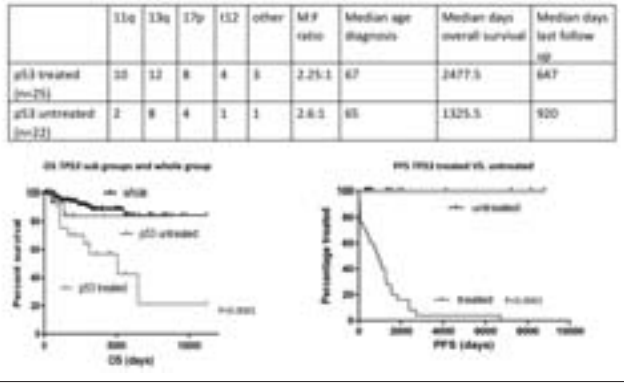

Figure 1.

5.11

HIGH RESOLUTION ARRAY-CGH IDENTIFIES NOVEL GENOMIC ALTERATIONS IN FAMILIAL AND SPORADIC CLL

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Chronic lymphocytic leukemia is a widely variable disease in which recurrent genomic abnormalities are strongly associated with prognosis. Although FISH is frequently used to identify the most common abnormalities in CLL, additional genomic abnormalities or karyotype complexity are missed with this approach. Furthermore, approximately 10% of CLL patients have a family history of the disease or a related lymphoproliferative disorder, yet the relationship of familial CLL to genomic abnormalities has not been characterized in detail. We therefore studied 75 CLL patients using high-resolution Agilent 244A array CGH in order to better define the relationship of genomic abnormalities to other prognostic factors and familial disease. We have found that the concordance between array CGH and FISH is very high when genomic abnormalities are present in more than 25-30% of cells by FISH, but that CGH usually misses abnormalities present in smaller fractions of the cell population, which may still be clinically relevant. Using CGH we have found that homozygous deletion of 13q, present in 35% of these patients with 13q deletion, is associated with mutated IgVH and low expression of ZAP-70, and a significantly longer time to first treatment compared to heterozygous deletion or lack of alteration. We have identified a region on 14q proximal to IgH near the centromere in which gains are associated both with familial CLL, and with mutated IgVH and homozygous deletion of 13q. We have found that deletion of 11q is significantly associated with sporadic disease, and with 4p loss and 21p gain. All three of these abnormalities are associated with shorter time to first treatment, as is 6q loss. 6q loss and 2p gain were primarily identified in sporadic CLL. Thus higher risk genomic abnormalities particularly 11q deletion were found more commonly in sporadic CLL in our study. Finally we assessed the number of copy number abnormalities per CLL sample; at least one abnormality was identified in every CLL. One-third of cases had two or fewer abnormalities; one-third had three abnormalities; and the final one-third of cases had four or more abnormalities. However no difference in time to first treatment was observed based on the number of abnormalities. We did find an association between 17p deletion and the presence of four or more copy number abnormalities. We conclude that high-resolution assessment of genomic abnormalities in CLL can provide new insights that will allow us to define more detailed disease subgroups for prognosis and therapy.

5.12

CONCORDANT IGVH GENE MUTATION STATUS AND CD38 EXPRESSION OFFERS THE MOST RELIABLE PROGNOSTIC INFORMATION FOR STAGE A CHRONIC LYMPHOCYTIC LEUKAEMIA PATIENTS

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Although the clinical staging systems developed by Rai and Binet are proven methods for assessing prognosis in CLL they are essentially retrospective in nature. Since most patients present at diagnosis with early stage disease there is considerable interest in accurately identifying

those patients who will develop progressive disease and require treatment in a prospective fashion. This is particularly relevant now as the therapies used are increasingly more toxic but at the same time more effective. Here we present the findings of the largest ever multi-centre study of prognostic markers in stage A CLL patients. We evaluated IgVH gene mutation status, CD38 expression, ZAP-70 expression, lymphocyte doubling time (LDT) and cytogenetics in 1153 stage A CLL patients and assessed their individual and combined ability to predict time to first treatment (TTFT) and overall survival (OS). In single parameter analysis, LDT was most predictive of TTFT (HR = 7.0) but did not retain its supremacy for overall survival. Furthermore, LDT is, by definition, a retrospective parameter so has limited value as a prospective prognostic tool. IgVH gene mutation status was more predictive of both TTFT (HR = 3.8) and OS (HR = 2.2) than CD38 (HR = 2.3 and 1.6 respectively), ZAP-70 (HR = 1.8 and 1.8 respectively) and high risk cytogenetics (11q-/17p-; HR = 3.6 and 2.0 respectively). IgVH gene mutation status in combination with CD38 was even more predictive of TTFT (HR = 4.7) and OS (HR = 2.5) as was the combination of IgVH gene mutation status and ZAP-70 (HR = 4.0 and 2.5 respectively). In multivariate analysis, lymphocyte doubling time, IgVH mutation status, CD38 status and age at diagnosis were the only parameters that retained independent prognostic significance for TTFT. The same four parameters were also included in the model for OS (see Table 1). It is worthy of note that neither high risk cytogenetics nor ZAP-70 were included in the model presumably because of the small number of high risk cytogenetic samples in this stage A cohort (n = 92) and the high degree of correlation between IgVH gene mutation status and ZAP-70 status (79% concordance). In conclusion, concordant IgVH gene mutation status and CD38 expression offers the most reliable prediction of the requirement for early treatment and overall survival in stage A CLL patients and should be considered the optimal tools for defining prognosis in this cohort of patients.

Table 1.

Covariate	*TTFT*		*OS*	
	HR (95% CI)	*p*	*HR (95% CI)*	*p*
LDT	7.0 (5.0-9.9)	<0.0001	2.0 (1.3-3.2)	0.005
IgVH gene status	3.3 (2.4-4.6)	<0.0001	2.7 (1.8-4.0)	<0.0001
CD38 status	1.6 (1.2-2.2)	0.007	1.7 (1.1-2.5)	0.009
Age (per decade)	0.85 (0.7-1.0)	0.03	2.2 (1.8-2.7)	<0.0001

5.13

CD49D IS AN INDEPENDENT PROGNOSTIC MARKER FOR TIME TO FIRST TREATMENT AND OVERALL SURVIVAL AND IMPROVES THE PROGNOSTIC POWER OF IGVH MUTATION STATUS AND ZAP-70

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CD49d/α4-integrin has variable expression in chronic lymphocytic leukaemia (CLL) and has prognostic significance in this disease. The precise biological role that this molecule plays in CLL still requires elucidation but it appears to be involved in CLL migration, activation and survival. Here we describe a multi-centre evaluation of CD49d in 432 CLL samples. We assessed the relationship between CD49d and CD38, ZAP-70, IgVH gene mutation status and CXCR-4. In addition, we determined its prognostic value both alone and in combination with other prognostic markers. In agreement with previous reports, we found a correlation between CD49d and CD38 (r2 = 0.13; $p<0.0001$) and ZAP-70 ($r^2 = 0.22$; $p<0.0001$) but not IgVH mutation status ($r^2 = 0.003$; $p=0.31$). However, the strongest correlation was found between CD49d and the chemokine receptor, CXCR4 ($r^2 = 0.31$; $p<0.0001$) suggesting that these molecules may be functionally linked or up regulated in a co-ordinated fashion. We next evaluated the prognostic relevance of CD49d in terms of time to first treatment (TTFT) and overall survival (OS) in the same series of CLL patients. We used a cut-off of 30% expression to define two cohorts of CD49dlo and CD49dhi CLL cases. In univariate analysis, CD49d was prognostic for both TTFT (HR = 2.2) and OS (HR = 2.7); the only superior marker in this context was IgVH gene mutation status (see Table 1). When the parameters were combined, concordance between CD49d and IgVH gene mutation status was most prognostic for TTFT (HR = 4.5) and concordance between CD49d and ZAP-70 was most prognostic for OS (HR = 10.6). In multivariate analysis, only CD49d ($p<0.0001$), IgVH gene mutation status ($p<0.0001$) and CD38 status ($p=0.02$) retained prognostic significance for TTFT in the model. In terms of OS, Age at diagnosis ($p<0.0001$), CD49d ($p<0.0001$) and IgVH gene mutation status ($p<0.0001$) were the only independent variables. Taken together, these findings support the introduction of flow cytometric evaluation of CD49d for the prognostic assessment of CLL patients and also suggest that CD49d represents a promising therapeutic target in this disease.

Table 1.

Parameter	*TTFT*		*OS*	
	HR (95% CI)	*p*	*HR (95% CI)*	*p*
CD49d	2.2 (1.7-3.4)	<0.0001	2.7 (2.0-5.2)	<0.0001
IgVH gene status	2.7 (2.4-5.0)	<0.0001	3.3 (0.5-6.5)	<0.0001
CD38	2.1 (1.6-3.1)	<0.0001	1.9 (1.3-3.2)	0.002
ZAP-70	1.8 (1.1-3.2)	0.02	2.9 (1.5-5.9)	0.002
CD49d + IgVH	4.5 (4.9-14.6)	<0.0001	6.0 (6.5-26.6)	<0.0001
CD49d + CD38	3.0 (2.4-6.3)	<0.0001	3.9 (3.2-12.6)	<0.0001
CD49d + ZAP-70	3.5 (1.9-6.6)	0.0001	10.6 (2.9-20.5)	<0.0001

5.14

ZAP-70 EXPRESSION AND IGVH MUTATIONAL STATUS AS MARKERS FOR GENE EXPRESSION SIGNATURE IN B-CLL PATIENTS FOR PROGNOSTIC CLASSIFICATION

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Background. Chronic lymphocytic leukemia (CLL) is a heterogeneous disease; ZAP-70 protein expression and IgVH mutational status have shown to be strong associated and to offer important prognostic information. Aim Our aim was to determine gene expression profiles of 46 CLL patients divided into three classes: group one (n=26) with mutated IgVH and ZAP-70-, group two(n=12)with unmutated IgVH and ZAP-70+, and group three (n=8) included CLL patients with unmutated IgVH and ZAP-70$^-$, or mutated IgVH and ZAP-70$^+$ respectively. Afterwards 16 patients were investigated. Finally, the purpose was to define prognostic biomarkers and the biological pathways related to CLL. Methods We determined gene expression profiles using Affymetrix HG U133 Plus 2.0 in CD19$^+$ leukemic cells. Differentially expressed genes were detected using ANOVA and t-test adapted for microarray data analysis and corrected for multiple testing using false discovery rate p-values. Subjects were clustered in groups with similar expression signature using cluster analysis (K-means, Euclidean distance. Results Statistical analysis revealed 154 differentially expressed probe-sets in the first (mutated IgVH and ZAP-70$^-$) vs the second (unmutated IgVH and ZAP-70$^+$) group, corresponding to 88 genes annotated in public databases. Interestingly, six genes were associated to the following biological pathways: MAPKsignaling (heat shock 70kD protein 8 HSPA8), B cell receptor signaling (ZAP-70, CKLF-like MARVEL transmembrane domain containing 3 CMTM3, dual adaptor of phosphotyrosine and 3-phosphoinositides DAPP1), Matrix Metalloproteinasis (transcription factor 20, TCF20), Apoptosis (X-linked inhibitor of apoptosis XIAP) and T cell receptor signaling (ZAP-70). In particular, ZAP-70, HSPA8, CMTM3 were significatively underexpressed while XIAP, TCF20 and DAPP1 were overexpressed in the first class of patients in comparison to the second class, respectively. Based on the expression of the 88 genes identified in the comparison between the first and the second group of patients, the 8 patients of the third class were divided in two clusters: 5 subjects were more similar to the first class, while 3 subjects appeared to be more similar to the second one. In particular, cluster analysis revealed that the 46 patients were better partitioned in two rather than in three classes, based on their expression profiles. 16 additional subjects were independently analyzed in a second phase of the project. Based only on the expression of the 88 genes previously identified, all of them were correctly classi-

fied in group one and group two. Further analysis was carried on the total of 62 subjects, dividing them in group A (n=17), showing deletion of 17p13 region and group B (n=45) without the deletion. Statistical analysis showed no correlation between groups A and B with respect to the previously defined group one, two and three. Moreover, no genes were identified as significantly differentially expressed in group A vs B. Conclusions Our preliminary data revealed that MAPK signaling, B cell receptor signaling, apoptosis and T cell receptor signaling may ultimately influence CLL biology. GEP studies are in progress on larger series of CLL patients in order to assess the association of the molecular signature, based on the identified genes and their pathways, with respect to prognostic information.

5.15

EXTENSIVE CELL SURFACE IMMUNOPHENOTYPE PROFILES TO IDENTIFY STABLE VERSUS PROGRESSIVE CHRONIC LYMPHOCYTIC LEUKAEMIA

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Background. Chronic lymphocytic leukaemia (CLL) may be divided into two basic biological groups, i.e. stable versus progressive disease. Approximately 20-30% of CLL patients ultimately develop disease progression and require treatment. The DotScan microarray containing 147 CD antibodies (Medsaic Pty Ltd, Eveleigh, NSW, Australia) enables classification of leukaemias based on an extensive surface expression profile (i.e., immunophenotype). Correlation of extensive immunophenotypes with clinical characteristics and known prognostic factors (e.g. stage and progression, ZAP-70 expression and IgVH gene mutational status) could enable early identification of progressive CLL soley from the extensive surface immunophenotype. *Methods.* The DotScan microarray containing 147 CD antibodies was utilized. Two surface chemistries for CD antibody microarrays, FAST (nitrocellulose) and Hydrogel (carboxylated dextran) slides were used to minimise background binding of CLL cells to microarrays. Frozen, Ficoll-purified mononuclear leukocytes from 59 CLL patients with known prognostic factors were profiled using the microarrays. Live cells were suspended in PBS and incubated on microarrays for 10 min, then unbound cells were gently washed off. Digital images for the dot patterns of captured cells were recorded and analyzed for binding intensities using DotScan software. *Results.* Analysis of the surface expression profiles from FAST and Hydrogel microarrays showed that the following CD antigens were differentially abundant. The results are shown in Table 1.

Table 1.

CLL category	*Increased expression*	*Decreased expression*
Progressive CLL with unmutated IgVH gene (UN IgVH) and ZAP70 positive vs stable CLL with mutate IgVH gene (MU IgVH) and ZAP 70 negative	CD38, CD123	CD57, CD62L, CD71, CD185
UN IgVH gene and ZAP70 positive vs MU IgVH gene and ZAP 70 negative	CD38, CD49d, CD123, CD125, CD218a, CD252, CD281, CD283	CD57, CD71, CD184, CD185
UN IgVH gene vs MU IgVH	CD38, CD69, CD123, CD191, CD192, CD195, CD256, CD266, CD267, CD281, CD282, CD283	CD57, CD71, CD184, CD185
ZAP 70 positive vs ZAP 70 negative	CD38, CD49d, CD69, CD79b, CD123, CD191, CD192, CD254, CD256, CD266, CD267, CD281, CD282, CD283	CD57, CD71, CD185
Clinically progressive vs stable CLL	CD38, sIg	CD57, CD71, CD81, CD185

Analysis of the surface profiles of CLL cells from 59 patients showed that for progressive CLL, there was increased CD38, CD123 and decreased CD57, CD71 and CD185. Acquisition of data from a larger number of CLL patients will be presented with a statistical analysis. *Conclusions.* Extensive surface phenotype profiles of CD antigens obtained with DotScan microarrays show differential antigen expression between clinically stable versus progressive disease and with known prognostic factors. Further analysis should enable distinction between stable and progressive CLL based on surface phenotype, enabling triaging of those patients who have progressive disease and require treatment.

5.16

PROGNOSTIC SIGNIFICANCE OF ZAP-70 EXPRESSION BY IMMUNOHISTOCHEMISTRY ON FORMALIN-FIXED BONE MARROW BIOPSIES IN PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKEMIA

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Introduction. Somatic hypermutational status (SHM) of the immunoglobulin heavy-chain variable-region is the most powerful prognostic factor in the patients with chronic lymphocytic leukemia (CLL). SHM analysis is very expensive, complex and not suitable for routine diagnostic use in clinical laboratory. According to some studies it appears that ZAP-70 expression could be a potential surrogate marker for SHM and become very important as a prognostic factor. ZAP-70 expression can be analyzed by several methods: flow cytometry (FCM), immunohistochemistry (IHC), Western-Blot and PCR technique. *Aim.* The aim of our study was to analyse ZAP-70 expression and its correlation with classical and other new prognostic factors in CLL. *Materials and methods.* We assessed the expression of the ZAP-70 by IHC on formalin-fixed bone marrow (BM) biopsies in 40 newly diagnosied B-CLL patients. ZAP-70 was detected using goat policlonal anti-human antibody and StreptaAvidin Biotinoylated peroxidase method. Samples were scored as ZAP-70 positive if the staining were observed in >20% of the B-cells and for CD38 antigen positivity if the staning were observed in >30% of the B-cells. We analysed B-cells with the clearly cytoplasmatic staining of ZAP-70. *Results.* Thirteen specimens (35%) were negative for ZAP-70 expresssion and 27 (65%) were positive for ZAP-70 expression. We found that expression of CD38 antigen and expression of ZAP-70 were associated with a unfavourable clinical course. Both new markers were shown to predict the clinical course of the disease. When we analysed the expression of ZAP-70, we found that this factor has the greatest prediction of disease progression in patients with Binet A stage. CD38 and ZAP-70 positivity significantly correlate with classical parameters such as advanced Binet stage (C), diffuse BM infiltration, lymphocyte doubling time <12 months, increased lactate-dehydrogenase and β-2 microglobulin serum levels. *Conclusions.* Although the number of patients in our study was limited, we showed that ZAP-70 expression have prognostic value in CLL patients, especially in patients with Binet A stage and that finding is comparable with previous studies in which various methodologies were used for ZAP-70 analysis. The IHC analysis of formalin-fixed BM biopsies for ZAP-70 expression could be used successfuly in a routine clinical laboratory assessment of patients with CLL.

5.17

PATIENTS WITH B-CHRONIC LYMPHATIC LEUKEMIA AND SUBGENE V3-21 HAVE VARIABLE CLINICAL COURSE, DEPENDENT ON MUTATION STATUS IGVH AND CYTOGENETIC CHANGES

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Backround. Mutation status IgVH is independent prognostic factor in patients with B - chronic lymphatic leukemia (B-CLL). A lot of clinical trials documented that patients with infrequent subgen V3-21 have clinically unfavourable prognosis independent on mutation status IgVH. *Aim*. To analyze group of B-CLL patients with subgene V3-21 dispensarized and treated in Department of Hemato - oncology, Faculty Hospital, Olomouc and describe their clinical course and survival in dependence on other prognostic factors. *Methods.* Mutation status IgVH was analyzed in 352 patients with B-CLL, which were diagnosed according to NCI-WG criteria. Subgene V3-21 was found in 20 of all 352 tested patients, i.e. 5.6%. 10 patients had mutated status IgVH, i.e. 50%. Flu-

orescence *in situ* hybridisation (FISH) was performed in 19 of 20 patients, high risk abnormalities 17p- or 11q- were detected in 8 of 19 patients, i.e. 42%. Median of age at diagnosis was 66 years, in range 36-76 years. Distribution to clinical stadium was equable, Binet A 35%, Binet B 35%, Binet C 30%. 17 from 20 patients (85%) were treated because of activity of disease. Median of observation was 54 months, 9 patients (45%) died. *Findings.* 7- years overall survival (OS) was 61% (95% CI 0.35-0.87). Survival without treatment after 2 years was 39% (95% CI 0.16-0.61). Prolongation of interval without treatment is associated with mutated status IgVH and with absence of unfavourable cytogenetics changes. Patients without 17 p- or 11q- have longer survival interval (OS in 3 years is 90%) compared to patients with 17 p- or 11q - (OS in 3 years is 57%). Patients with mutated status of IgVH tended to have longer interval of overall survival (OS in 7 years 72% compared with 40%, log rank 0.09). *Summary*. Patients with subgene V3 -21 had heterogenous clinical course of disease. Survival and beginning of treatment were affected by unfavourable cytogenetic changes and mutation status of IgVH. Currently used FISH tests and tests of mutation status in B-CLL patients with subgene V3 21 do not loose their predictive role. *Supported by Grant MSM 6198959205 and Grant IGA NR 9484.*

5.18

A COMPARISON OF THE PROGNOSTIC VALUE OF SERUM MARKERS IN THE UK CLL4 TRIAL DEMONSTRATES THE SUPERIORITY OF BETA 2 MICROGLOBULIN OVER THYMIDINE KINASE, FREE LIGHT CHAIN RATIOS AND SOLUBLE CD23.

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Serum Beta 2 Microglobulin (β2M) measured by the referring centre, was an independent prognostic marker for both PFS and OS in the UK CLL4 trial. Other serum markers including serum thymidine kinase (sTK), soluble CD23 (sCD23) and more recently serum free light chains (sFLC) have been shown to have prognostic value in CLL. We therefore compared the prognostic value of the above four markers measured centrally in a cohort of 289 patients, in whom samples were available, from the UK CLL4 trial. This cohort was older (p=0.0004) but otherwise comparable to the trial overall. The median follow up was 56 months (45-81). Cut-offs at the median value and other previously defined values were considered. β2M results were comparable to those measured locally and using the median cut off at 4 mg/L only 21 (10%) would have been categorised differently. There was a significantly poorer OS and PFS with high β2M levels (Table 1). β2M retained independent significance for PFS after allowing for treatment allocation and VH gene mutational status and for OS when poor risk genomic abnormalities were also included in the model. The median sTK level was 19.8 u/L (1.6-259.0). High sTK levels were associated with advanced stage, unmutated VH genes, high CD38 and ZAP 70 expression, trisomy 12, p53 loss, no 13q loss, and with poorer PFS and OS (Table 1). Using a cut off of 8.5 u/L, previously shown to be the optimal cut off for predicting time to first treatment, there was poorer OS and PFS with high sTK (p=0.03 and p=0.002 respectively). sTK did not retain independent significance for OS, and only retained borderline significance for PFS in models which did not include FISH markers. An abnormal sFLC ratio (<0.30 or >1.60 mg/l) was found in 210 patients (73%), of whom 143 had clonal Kappa sFLC, and was associated with unmutated VH genes, p53 loss and 13q loss. There was a poorer overall response rate in those with an abnormal compared to normal sFLC ratio (71% vs. 91%, p= 0.0008), and a suggestion of poorer PFS and OS (table 1). After adjustment for other known poor risk variables sFLC was no longer significant. Using a higher cut off of 4 mg/L, 146 (51%) of patients had an abnormal ratio. PFS was significantly poorer in this subgroup (p=0.007) but there was no difference in OS. High sCD23 levels were associated with advanced stage, unmutated VH genes and CD38 positivity. Using a median cut off of 167.8 u/mL there was a significant difference in OS, but not in PFS, between the two groups of patients (Table 1). sCD23 did not retain significance in multivariate analysis. In conclusion, of the 4 serum markers evaluated, β2M was the most significant predictor of both PFS and OS independent of treatment allocation, age and the standard panel of biomarkers. Table 1 5 year PFS (se) Log rank p-value 5 year OS (se) Log rank p-value β2M Low 24.9% (3.6) 0.001 72.8% (3.7) <0.00001 High 12.0% (2.8) 38.8% (4.4) sTK Low 24.5% (3.6) 0.004 62.5% (4.2) 0.01 High 12.6% (2.8) 50.6% (4.2) sFLC ratio Normal 27.5% (5.1) 0.03 66.2% (5.5) 0.04 Abnormal 15.5% (2.6) 53.2% (3.6) sCD23 Low 23.7% (3.6) 0.1 61.7% (4.1) 0.04 High 13.6% (2.9) 50.4% (4.4)

Table 1.

		5 year PFS (se)	Log rank p	5 year OS (se)	Log rank p
β 2M	Low	24.9% (3.6)	0.001	72.8% (3.7)	<0.00001
	High	12.0% (2.8)		38.8% (4.4)	
sTK	Low	24.5% (3.6)	0.004	62.5% (4.2)	0.01
	High	12.6% (2.8)		50.6% (4.2)	
sFLC ratio	Normal	27.5% (5.1)	0.03	66.2% (5.5)	0.04
	Abnormal	15.5% (2.6)		53.2% (3.6)	
sCD23	Low	23.7% (3.6)	0.1	61.7% (4.1)	0.04
	High	13.6% (2.9)		50.4% (4.4)	

5.19

CLLU1 IS A PROGNOSTIC INDICATOR PRIOR TO INITIATION OF THERAPY IN CLL PATIENTS WITH MUTATED IGVH GENES: RESULTS FROM THE LRF CLL4 TRIAL

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Background and Introduction. CLLU1 is a novel transcript, located at chromosome 12q22 which has been recently identified as a CLL-specific marker with potential prognostic value. In particular, it has been shown that high CLLU1 expression is associated with shorter overall survival (OS) and shorter time to first treatment in CLL patients, especially in younger patients (<70 years of age). We analysed a large series of samples from the LRF CLL4 trial in order to assess the value of CLLU1 expression as a predictive and prognostic marker in the setting of a randomised controlled trial. *Patients and methods*. 319 CLL samples, collected immediately prior to the start of treatment, were available for this study. After RNA extraction and cDNA synthesis, CLLU1 expression was assessed by qPCR and the Ct method using β2M as the endogenous control gene and RNA extracted from a B-cell pool as the reference control. Low and high CLLU1 expression values were defined as RQ values <9 or >9, respectively (corresponding to a cut-off Ct of 11). CLLU1 expression values were correlated with response, OS, and other relevant clinical and biological variables.

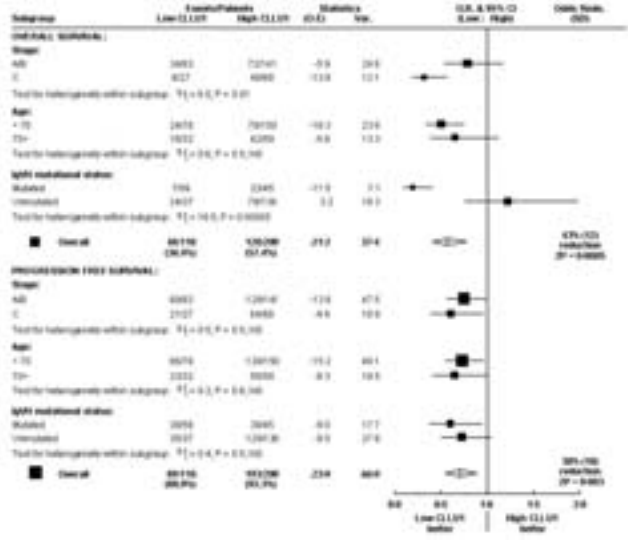

Figure 1.

Results. A total of 110 and 209 samples were identified as having low and high CLLU1 expression, respectively. High CLLU1 expression was significantly associated (p<0.0001) with unmutated IGVH genes, ZAP-70 positivity and absence of 13q deletion. Furthermore, trisomy 12 (the chromosome containing the CLLU1 locus) was significantly over-represented in cases with very high CLLU1 expression (RQ values >150;

p<0.003). Patients with high CLLU1 expression had significantly shorter OS from randomisation than patients with low expression (51% vs 69% alive at 5 years; OR=1.76, 95% CI: 1.28-2.43; p=0.0004) as well as a lower rate of response to treatment (74% vs 87%; p=0.01). CLLU1 expression was an independent predictor of OS in bi-variate analyses with every variable except IGVH mutational status. Strikingly, subgroup analysis showed that CLLU1 expression had marked prognostic value in IGVH-mutated cases (87% of patients with low CLLU1 expression were alive at 5 years versus 50% with high CLLU1 expression; p<0.0001), but not in unmutated cases (44% versus 50% respectively, p=0.5) (p(heterogeneity)=0.00005). Similarly, low CLLU1 expression was significantly associated with longer OS in patients younger than 70 years, as well as in patients with Binet stage C disease. *Conclusion.* We have confirmed that the level of CLLU1 expression provides prognostic information in CLL patients. In particular, we recommend the inclusion of CLLU1 expression in the algorithms for prognostication in CLL patients with mutated IGVH genes prior to initiation of therapy.

5.20

SURFACE IMMUNOGLOBULIN ISOTYPE AS A MARKER OF DISEASE BIOLOGY AND CLINICAL OUTCOME IN CLL

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Background. During the germinal center reaction, B cells undergo somatic modifications to increase affinity for a target antigen, specifically mutation of the immunoglobulin heavy and light chains and immunoglobulin isotype switching. Several lines of evidence have shown that CLL B cells are antigen-experienced. Despite this, approximately half of all CLL cases have an unmutated (<2% deviation from germline) immunoglobulin heavy chain, and these patients follow a more aggressive disease course. The mechanism by which this subset of CLL escapes somatic mutation is not definitively known, though it has been proposed that these cases mature through a T cell independent pathway. Because T cell help is also required for immunoglobulin isotype switching and because the surface immunoglobulin isotype in CLL is variable, we hypothesized that the immunoglobulin isotype would correlate with the immunoglobulin heavy chain mutation status and clinical outcomes. *Methods.* We tested this hypothesis by performing flow cytometric analysis of fresh and cryopreserved patient samples from our CLL specimen repository that includes detailed clinical outcomes and biologic characteristics. Viable CLL cells were identified using forward and side scatter characteristics, exclusion of VitalDye, and expression of cell surface markers CD5, CD19, and CD20. The surface immunophenotype was then determined using anti-IgM (clone G20-127), anti-IgD (IA6-2), and anti-IgG (G18-145) fluorescently labeled antibodies. Surface expression used to define positivity were >40% for IgM and IgD and >25% for IgG.

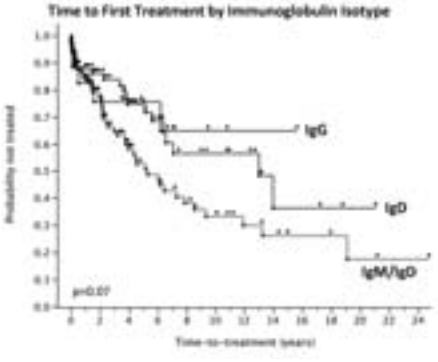

Figure 1.

Results. 187 patient samples were analyzed. 93 were IgM$^+$IgD$^+$ (50%), 69 were IgD$^+$ (37%), 17 were IgG$^+$ (9%), and 8 were negative for all markers (4%). We observed a significant association between immunoglobulin isotype and immunoglobulin mutation status: among unmutated samples 75% were IgM$^+$IgD$^+$ and only 21% were IgD$^+$ (p<0.0001). This association extended to other prognostic markers: IgM$^+$IgD$^+$ samples showed a significantly greater frequency of CD38 expression (both >30% and as a continuous variable; p<0.005) and Zap70 expression >20% (p<0.01) than IgD$^+$ samples. Interestingly, although only 1 of 17 IgG+ samples was unmutated, the frequency of CD38 expression >30% was significantly higher (33%) than observed in IgD$^+$ samples (8%, p<0.01). The immunoglobulin isotype did not correlate with age at diagnosis, gender, or Rai stage at diagnosis. IgM$^+$IgD$^+$ cases showed shorter lymphocyte doubling times than IgD$^+$ or IgG$^+$ cases (p<0.05). There was a trend toward shortened median time to first treatment (IgM$^+$IgD$^+$ 5.3 yrs vs. IgD$^+$ 13.0 yrs; Figure) and shortened overall survival (median time not reached for any group), but these comparisons were not significant using the Rank-sum test (p=0.07 for both TTT and OS). *Conclusions.* CLL cases that retain an "unswitched" surface immunoglobulin isotype with co-expression of IgM and IgD are associated with an unmutated immunoglobulin heavy chain, increased surface expression of CD38, increased intracellular expression of Zap70, and shorter lymphocyte doubling times. This association extended to the clinical endpoints of time to first treatment and overall survival, but these comparisons do not meet statistical significance. Current efforts are focused on characterization of signaling pathways downstream of the B cell receptor to elucidate the biologic basis of this observed association.

5.21

SERUM BAFF (B-CELL ACTIVATING FACTOR OF THE TNF FAMILY) PREDICTS TIME TO FIRST TREATMENT IN EARLY B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA

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We analyzed the correlation between well-established biological parameters of prognostic relevance in B-cell chronic lymphocytic leukemia [CLL] (i.e, mutational status of the immunoglobulin heavy chain variable region [IgVH], ZAP-70$^-$ and CD38$^-$ expression) and serum levels of BAFF (B-cell activating factor of the TNF family) by evaluating the impact of these variables on the time to first treatment [TFT] in a series of 176 previously untreated Binet stage A B-cell CLL patients. By using a commercial ELISA (R & D Systems, USA) we found that higher levels of BAFF characterized more frequently females (p=0.01), patients with Rai stage 0 (p<0.0001), mutated IgVH disease (p=0.002) and low ZAP-70 (p<0.0001) or CD38-expression (p=0.02) and higher occurrence of normal cytogenetic profile or presence of 13q deletion (p=0.02). We used an optimal cut-point search to determine how to best split soluble BAFF data. Maximally selected log-rank statistics plots identified a BAFF serum concentration of 313 ng/mL as the best cut-off (p<0.0001). Accordingly, patients who had BAFF levels higher than 313 ng/mL experienced a longer TFT (median 108 months) in comparison to patients whose BAFF levels were lower than 313 ng/mL (median 30 months; p<0.0001). Along with serum concentration of BAFF, the univariate Cox proportional hazard model identified Rai substage I–II (p<0.0001), lower PLT count (p<0.0001), higher peripheral blood lymphocytosis (p=0.001), increased LDH (p=0.001), ZAP-70 expression > 20% (p=0.001) ,absence of mutation of IgVH (p<0.0001) and CD38-expression > 30% (0.005) as predictor of shorter TFT. In multivariate analysis only soluble BAFF (Hazard ratio [HR], 8.23; CI 95%,3.0-22.6, p<0.0001) and mutational status of IgVH, (HR= 2.60; CI 95% 1.10-6.14, p=0.03) maintained their discriminating power. Taking into account variables significant in multivariate analysis a recursive partitioning (RPART) model was used to build a classification tree. Three groups were identified: 1. Low-risk group (n=117), patients with high levels of soluble BAFF whatever mutational status of IgVH; 2. Intermediate-risk group (n=32), patients with low levels of soluble BAFF and IgVH mutated; 3. High-risk-group, patients (n=27), patients with low levels of soluble BAFF and IgVH unmutated. Interestingly, the probability of remaining free from therapy of these patients' subgroups at 3 years were respectively 91.8%, 51.3% and 27.5% (p<0.0001). Our results indicate that in early B-cell CLL biological profile including among other parameters soluble BAFF may provide a useful insight into the complex interrelationship of prognostic variables. Furthermore, BAFF along with mutational status of IgVH can be adequately used to predict clinical behaviour of patients with low biological risk.

5.22

ZAP-70 EXPRESSION MAY CHANGE SIGNIFICANTLY DURING THE COURSE OF CHRONIC LYMPHOCYTIC LEUKEMIA

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Background. ZAP-70 is one of the most frequently discussed prognostic factors in chronic lymphocytic leukemia (CLL). It is widely accepted that ZAP-70 expression (in contrast to CD38) remains stable during the course of the disease; however, data supporting this opinion are suspris-ingly scarce. Therefore, the aim of our study was to assess expression of ZAP-70 in sequential samples taken during the course of CLL. *Patients and Methods*. Between May 2005 and April 2009, ZAP-70 was measured sequentially in 44 patients (34 males, 10 females, median age, 62 years. Furthemore, three samples were available in 8 a four samples in 5 patients. A total of 104 samples were analyzed. Median time between the collection of first and second blood sample was 13 months (range, 2-36). ZAP-70 expression was detected by flow cytometry (cytometer Epics XL, Beckman Coulter) using heparinized whole blood without cell selection. Peripheral blood mononuclear cells were fixed and permeabilized with IntraPrep Permeabilization Reagent (Beckman Coulter). ZAP-70 expression was determined by using phycoerythrin-conjugated monoclonal antibody clone 1E7.2 (Caltag,Laboratories, USA). The expression of ZAP-70 was estimated using negative isotype control. Threshold of positivity was set as usual to 20%. All measurements were performed on fresh cells and within 6 hours from blood collection. *Results.* Significant change of ZAP-70 expression (i.e., from positivity to negativity or vice versa) was detected in 15/44 patients (8/26 became positive, 7/18 negative). Interestingly, 7/8 patients in whom ZAP-70 became positive carried unmutated IgVH genes. The shift to positivity was accompanied by clinical progression/relapse in all but one patient. On the contrary, 5/7 patients whose ZAP-70 dropped below 20% had stable clinical course. In one case there was a loss of ZAP-70 expression during treatment with steroids. *Conclusions.* The significant change in the expression of ZAP-70 was found in approximately one third of our CLL cohort. Whereas the loss of ZAP-70 expression was found mainly in patients with stable disease, the shift to positivity was typically seen in patients with unmutated IgVH genes at the time of progression or relapse of the disease. We conclude that in contradiction to commonly accepted opinion, expression of ZAP-70 may change markedly in a significant proportion of patients. Based on our pilot results, repeated assessment of ZAP-70 expression might be particularly useful at the time of progression or relapse in patients who were initially ZAP-70 negative. *Acknowledgments: this work was supported by research project MZO 00179906 from Ministry of Health, Czech Republic.*

5.23

COMPLEX IMMUNOPHENOTYPE DETECTING BY FLOWCYTOMETRY AS PROGNOSIS PANEL IN CHRONIC LYMPHOCYTIC LEUKAEMIA

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Background. The most frequent chronic lymphoproliferative disorder, chronic lymphocytic leukaemia (CLL), assessed mainly with clinical systems Rai and Binet in practice, has many other prognostic markers established last years, related to disease and to the patient status, and the most important seem to be now immunophenotypical, genetics and molecular. CD38 and ZAP-70 are used now in a strong prognosis panel of markers for CLL. *Aims.* The present study proposes to asses the prognosis with consecrated markers CD38 and the new ZAP-70 related to the various patterns of CLL immunophenotype. *Methods.* We have analyzed 187 patients diagnosed with CLL in order to find correlations between clinical stage, immunophenotype, and outcome. The immunophenotyic diagnosis was made by flowcytometry on a BD FACS Flow Calibur, first step with classical association of markers CD19, CD5, CD23, CD79b. In these patients we have analyzed other surface markers, like CD20, FMC7, CD27, CD43, CD38, and intracellular markers cyclin D1, BCL-2 and ZAP-70 (Crespo protocol). *Results.* The patients with CLL were stratified by clinical stage as follows: 22.34% stage 0, 30.85% stage I, 26,6% stage II, 5.32% stage III, and 14,89% stage IV. We found correlations between expression of CD38 related to clinical outcome, (dr: 0.541, $p<0.05$.), and between ZAP-70 and CD38 (dr:0.666; $p=0.018$). The expression of BCL-2 was correlated to outcome (dr:0.533, $p<0.01$) and response to treatment (dr:0.420, $p<0.01$). Cyclin D1 expression was found in correlation with outcome (p:0.014) and bcl-2 expression (p:0.034). The threshold of CD23 was established at 50% for statistical significance, and the lower expression was associated with poor outcome and expression of CD38 and ZAP-70 (p-value<0.05; dr:-0.117). A particular pattern was found in patients groups with diferrent expression of cyclin D1. We found in patients with cyclin D1 positive comparative to those cyclin D1 negative the association of high intensity $CD20^{+}$ (28/3%), $FMC7^{+}$ (33/8%) lower CD23 (30-60%/>60%). Also, CD38 was positive in 44 vs 10% and ZAP-70 in 66 vs 5%. This association defines a "lymphoma"-like immunophenotype for cyclin D1 positive cases. Treatment was used with Chlorambucil, FC or FCR related to patient status in progressive cases. Conclusion. Flowcytometry is the most practical method used in CLL for diagnosis and prognosis evaluation. The immunophenotypical markers surrogate for IgVH mutation status, as CD38 and ZAP-70 have a strong correlation with outcome in CLL and our results found that analysis by flowcytometry of both CD38 and ZAP-70 could be used in evaluation of CLL patients as strong prognosis markers. The complex immunophenotype in CLL could be used to define two main prognosis patterns: (1) cyclin $D1^{+}$ - $CD38^{+}$ - $CD20^{high}$ - $FMC7^{+}$ - CD23 weak with poor prognosis and (2) cyclin $D1^{-}$ - $CD38^{-}$ - $CD20^{low}$ - $FMC7^{-}$ - $CD23^{high}$ with good prognosis. These patterns have strong association with expression of ZAP-70 and could be used as prognosis assesement of CLL patients.

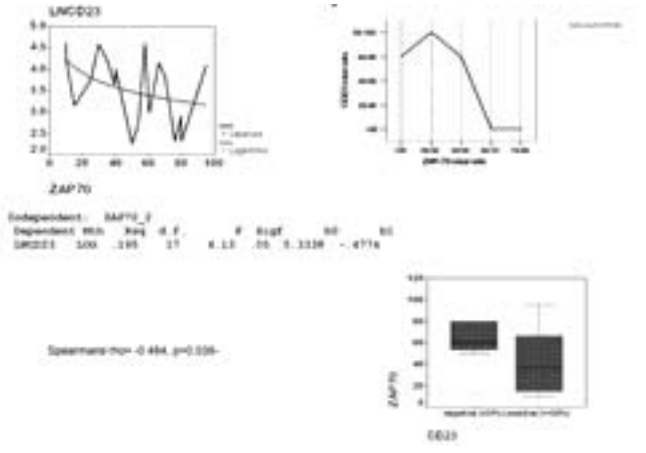

Figure 1. Mathematical expression of CD23 ZAP70 interdependence.

5.24

INTERNATIONAL EFFORT FOR HARMONIZATION OF ZAP-70 TECHNIQUE: CONTRIBUTION OF THE EXCHANGE OF ELECTRONIC FLOW CYTOMETRY RAW-DATA AND MULTICENTRIC EVALUATION OF A STANDARDIZED PROCEDURE

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Chronic lymphocytic leukemia (CLL) follows a highly variable clinical course. As the treatment options have evolved, there is a crucial need for reliable predictors of disease progression. Heavy-chain variable-region mutational status is a major prognostic factor, however, its determination being complex, time consuming and costly, surrogate markers would be of interest. Of these, 70-kD zeta-associated protein (ZAP-70) expression has been identified through transcriptomal studies. Biomarkers need to fulfil technical validation and standardization before proceeding to clinical validation for widespread use and it has proven challenging for ZAP-70. As several factors were evidenced as introducing

result variability, an international effort for ZAP-70 harmonization (Clinical Cytometry 2006) was organized. However, consensus was not reached on issues such as optimal technique and mode of expression of the results. Therefore, the issues of gating strategy and interpretation of results were addressed through an electronic trial (e-trial) consisting in exchange of raw data, allowing comparison of techniques and interpretation without exchange of samples. Fifteen laboratories participated in this study that was carried out on four blood samples. Antibody combinations used for gating lymphocyte subsets were equally effective in segregating CLL, T and NK cells. Inter-centre variability was low for CLL cells or T-lymphocytes and increased when the cell subset corresponded to less than 2%. First, we considered the results expressed as percentages of ZAP-70-positive CLL cells according to isotype control or ZAP-70 expression in T-cells. We observed false positive results for negative samples, mainly when isotype control was used as reference. Among positive CLL samples, when using T-lymphocytes as reference, half of the conditions lead to false negative results. Then we considered mean fluorescence intensity (MFI) ratios (ratio of ZAP-70 MFI in CLL to ZAP-70 MFI in patients' normal B-lymphocytes or T-lymphocytes). Expression as MFI ratios did not yield false positive or negative results when using T-lymphocytes as reference population. A threshold was easily set for positivity of CLL cells in all conditions and SBZAP clone showed good segregation between positive and negative samples. Expression as MFI ratios according to normal B-cells was more heterogeneous. Finally, based upon these results, a new procedure was tested and standardized on a series of normal and CLL samples and underwent multicentric validation through testing of two fresh CLL samples in five centres. The procedure included a step of standardization for instrument set-up. Analysis of lymphocyte subsets targeting showed homogeneous results from one centre to another. Analysis of ZAP-70 results showed a 100% concordance with the actual status in both samples. When considering the values of percentages and MFI ratios, the variability was not clearly different suggesting both methods were applicable. In conclusion, considering that new reagents will be available and that many laboratories have already adopted a technique they are familiar with, the important consensus likely resides in harmonizing the procedure of interpretation rather than reaching consensus on a definite technique. This has been achieved through the e-trial that allowed resolution of technical issues and validation of a technique proposed here as a standardized method.

5.25

IMPACT OF MONOALLELIC TP53 DEFECTS ON SURVIVAL AND DNA DAMAGE RESPONSE IN CHRONIC LYMPHOCYTIC LEUKEMIA

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Presence of 17p deletions is currently considered to be the most important negative prognostic factor in CLL patients. The importance of mutations in the gene TP53, localized in the 17p locus, is recently under investigation. Our aim was to asses the frequency of separate mutations without 17p deletions and to study their effect on patients' survival and the DNA damage response. We used methodologies with similar sensitivity for detection of deletions (I-FISH) and mutations (FASAY - functional analysis in yeast). We analyzed a large consecutive series of 400 CLL patients and collected the cohort of 70 patients with TP53 defects. As expected, the most common type of TP53 abnormality was complete inactivation, i.e. deletion of one allele accompanied with mutation on the other allele (42 patients; 10.5%). However, relatively large proportion of patients had isolated mutations (20 patients; 5%), or two mutations on separate alleles (5 patients; 1.3%). On the other hand, sole deletions were very rare (3 patients; 0.8%). All the three isolated deletions were detected only in fraction of CLL cells (20, 21 and 24 %) suggesting that TP53 deletions are not preferentially selected in CLL patients and that mutations represent more severe defect in comparison to the deletions. All TP53 defects had negative impact on patients' survival. There was no significant difference in survival between groups with biallelic and monoallelic TP53 abnormalities (p=0.167). We compared the survival of patients harboring different TP53 abnormalities with the group of patients with unmutated IgVH, which had been described as a group with bad prognosis. The biallelic TP53 defects showed a clear association with shorter survival (p<0.001), while the monoallelic changes manifested much weaker, but still significant impact (p=0.021). Furthermore, we tested the differences in *in vitro* response to fludarabine by viability measurment. The cells with both biallelic and monoallelic TP53 inactivation were significantly (p<0.001) more resistant to the treatment than cells with intact TP53 gene. At the clinically relevant fludarabine concentration, there was no difference between the biallelic and monoallelic TP53 defect. However, when the higher concentration of fludarabine was used, the cells with monoallelic defect showed intermediate viability. Finally, the separate mutations occur quite frequently in CLL patients, they have negative impact on survival and DNA damage response and therefore we suggest, that mutation analysis of TP53 should be added to FISH screening in CLL patients. *Supported by grants IGA MH CR NR9305-3, NR9858-4, and NS10439-3.*

5.26

THE USE OF TETRADECANOYLPHORBOL ACETATE (TPA)-STIMULATED PERIPHERAL BLOOD CELLS ENHANCES THE PROGNOSTIC VALUE OF THE PRESENCE OF 13Q DELETION BY INTERPHASE FLUORESCENCE *IN SITU* HYBRIDISATION IN PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKAEMIA

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Background. Genomic aberrations, as identified by interphase fluorescence in-situ hybridisation (I-FISH), have a remarkable prognostic value in patients with chronic lymphocytic leukaemia (CLL). I-FISH studies performed on tetradecanoylphorbol acetate stimulated peripheral blood cells (I-FISH-TPA) can identify abnormalities that might be overlooked when unstimulated peripheral blood mononuclear cells (I-FISH-PBMC) are used. *Aims.* The aim of the study was to evaluate whether this finding was clinically relevant in a group of 235 consecutive CLL patients. *Methods.* Fifty-six patients had both I-FISH-TPA and I-FISH-PBMC results, which were compared directly (head-to-head comparison). Then, I-FISH-PBMC results from 93 historical patients were compared with 86 different patients with I-FISH-TPA results (historical comparison). *Results.* Head-to-head comparison: compared to uncultured cells, the cytogenetic detection rate rose from 57% to 80% with the use of TPA-stimulated cells. Fourteen (25%) patients had their I-FISH result changed. In five patients with normal results by I-FISH-PBMC we could identify the presence of trisomy 12 (3 cases) and 11q deletion (2 cases) by I-FISH-TPA. I-FISH-TPA provided a better prediction of treatment-free survival (TFS) compared to I-FISH-PBMC (p=0.031 vs 0.116). Historical comparison: Genomic aberrations were detected in 46% and 67% of patients from the I-FISH-PBMC and I-FISH-TPA cohorts, respectively. The detection rate of 13q deletion as the only aberration increased from 10% to 37% when blood cells were previously cultured and stimulated with TPA. In the I-FISH-PBMC cohort, patients with 13q deletion had a significantly shorter TFS than patients with no abnormalities (18 vs. 107 months, p=0.037). In the I-FISH-TPA cohort, patients with and without del(13q) had similar TFS (132 months vs. not reached, p=0.352), more in keeping with the published literature (Döhner *et al.*, 2000). *Conclusion.* I-FISH-TPA increased the detection rate of 13q deletion over a normal karyotype and had an improved prognostic value compared to I-FISH-PBMC. Further studies with larger numbers of patients are warranted.

5.27

PROGNOSTIC FACTORS FOR BINET STAGE A CHRONIC LYMPHOCYTIC LEUKEMIA PATIENTS. A PROSPECTIVE COHORT OF 339 PATIENTS

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Within the past decade, the treatment options in CLL have moved from being palliative in intent to potentially curative. Therefore, reliable prognostic factors that may help in predicting disease progression in Binet stage A are needed. However, the identification of high-risk (or low risk) patients remains challenging. Moreover, publications on large cohort of patients are usually not conducted exclusively on stage A patients at the time of diagnosis, therefore making the interpretation of disease progression difficult. We studied, in a multicentric prospective cohort of 339 stage A patients at diagnosis, the most widely used prognostic factors: IgVH mutational status, ZAP-70 and CD38 expression, serum thymidine kinase level (sTK), lymphocytosis, β2M and presence of the recurrent cytogenetical abnormalities (13q14 deletion, trisomy 12, 17p deletion, 11q deletion). All cases were $CD5^+$ $CD23^+$, with low surface Ig, and a Matutes score > 4. Progression free survival was defined as the time from diagnosis to progression to stage B or C. Comparisons were based on the two-sided log-rank test. Multivariate Cox regression modelling was used to identify a set of prognostic variables. All analyses were performed with S-Plus 2000 (MathSoft Inc, Seattle, WA) software and all tests were two-sided, with a significance level of 0.05. Sex ratio was 1.5. Median age was 64.8 years (range, 29-87 years). Median follow-up time was 53.8 months. The mean lymphocyte count was 13x103/mm^3 (min: 4; max: 223). sTK level > 10 UI/L was observed in 29% of the patients. Median β2M was 2.4 (min: 0.9; max: 11.2). In 30 % of the patients, CLL cells had IgVH unmutated genes. ZAP-70 overexpression was observed in 34%, and CD38 expression (considering the cut-off of 7%) in 32% of the cases. FISH analysis revealed the expected frequency of the 4 abnormalities in stage A cases: less than 5% del 17p, 6.5% del 11q, 11% trisomy12 and more than half (56%) del 13q. Median PFS was 112 months. In univariate analysis, parameters predictive of PFS were: Lymphocytosis (p<10-4), CD38 expression (p<10-4), β2 microglobulin (p<10-4), sTK level (p<10-4), mutational status (p<10-4), ZAP70 expression (p<10-4), presence of a del 11q (p<10-4), Tri 12 (p=0.01), del 17p (p=0.01). Age, sex and del 13q were not predictive of PFS. In multivariate analysis, 4 factors were independent predictors of the PFS: sTK (HR = 2.75, p=0.0002), del 11q (HR=2.48, p=0.005), lymphocytosis over 30 G/L (HR=1.71, p=0.04), and elevated β2 microglobulin (HR=1.79, p<10-4). Several conclusions may be drawn from this study. The multivariate analysis points out to the crucial importance of proliferation markers in predicting disease progression. These results do not hamper the significance of other prognostic factors such as IgVH status for predicting response to therapy or overall survival.

5.28

CLONAL EVOLUTION DURING LONG-TERM FOLLOW-UP USING FLUORESCENT IN-SITU HYBRIDISATION (FISH) IN CHRONIC LYMPHOCYTIC LEUKEMIA

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Background. The prognostic importance of certain chromosomal abnormalities in CLL is well-known. It is also established knowledge that deletions of the short arm of chromosome 17, del(17p), is more commonly found in chemoresistant patients. However, there is still uncertainty on how findings of small clones should be interpreted and only scarce sequential data of existing clones and emergence of new aberrations. *Aim.* To evaluate the chromosomal aberrations del(13q), del(11q), del(17p and trisomy 12 (+12) during the course of the disease with fluorescent in-situ hybridisation (FISH), with focus on the emergence of new clones and increase/decrease of existing clones. *Patients and methods.* All patients from Karolinska University Hospital, Huddinge, diagnosed with CLL between 1983-2007 and with at least two FISH analyses on blood or bone marrow at different time points during the course of the disease were selected for further prospective samples (also from lymph nodes and spleen). In total 168 samples from 51 patients have been analysed (≥3 samples in 32 patients). Median age at diagnosis was 59 (range 35-77) years. The first FISH sample was taken at diagnosis in 25 patients, before any therapy in further 17 patients, and in the remaining 9 patients after therapy. Median time between the first and last FISH sample was 44 (range 4-300) months. Before the last FISH analysis totally 34 patients had been treated with purine analogs (n=13), alemtuzumab (n=5) and both these drugs (n=10). The remaining six patients were treated with alkylator-based therapy only. *Results.* At the first FISH analysis, all but two patients showed an aberration (96%) using a 5% cut-off; 23 with a single abnormality, del(13q) in 13 cases, del(17p) in 4 cases and +12 and del(11q) in 3 cases each. A combination of two or three aberrations was found in 26 patients. In total the most common aberration was del(13q) (n=32). Del (17p) was found in 24 patients and del(11q) and +12 in 18 and 7 patients, respectively. Clones affecting more than 50% of the cells were mostly stable during follow-up, which was also the case for small clones (≤20% of the cells) with del (17p) in 17 out of 19 cases. However, a small del(13q) clone progressed in 3 out of 9 such cases. New clones appeared during the course of the disease in 15 patients (29%); five new del(17p), two +12 and four cases each with del(13q) and (11q). All patients except four had been treated before the new clone emerged. New aberrations in these four untreated patients were del (17p) (n=2), del (13p) (n=1) and +12 (n=1). New clones did affect > 20% of the cells in only four cases; 2 del(13q) and 2 del(11q). *Summary.* Sequential analyses of chromosomal aberrations with FISH during long-term follow-up of CLL patients show a low likelihood of progression of small del(17p) clones, but a clonal evolution in a third of patients including all the analysed aberrations.

5.29

PHENOTYPIC FEATURES OF ATM MUTANT TUMOURS AND THE IMPACT ON SURVIVAL IN PATIENTS TREATED ON CLL4 TRIAL

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Ataxia Telangiectasia Mutated (ATM) gene plays a central role in signalling cellular responses to DNA damage in the form of DNA double strand breaks (DSBs) and encodes for a serine threonine-kinase that phosphorylates a range of downstream targets involved in DSB repair, regulation of cell cycle and induction of apoptosis. ATM gene inactivation represents the single most frequent genetic event in CLL. We have previously shown that ATM mutations confer poorer overall and treatment free survival in an unselected CLL cohort as well as in CLL patients selected for 11q deletions. However, the impact of ATM mutations on disease phenotype and survival has not been addressed in the context of a CLL clinical trial. We have analysed the entire ATM coding region consisting of 62 exons and flanking introns in 237 patients enrolled in UK national CLL4 trial by using a high-performance liquid chromatography (HPLC) and sequencing approach. We classified Identified sequence changes into three categories. Sequence changes predicted to cause truncation of the protein were designated as truncating pathogenic mutation. Missense changes previously described in individuals with constitutional biallelic ATM inactivation (A-T) were classified as non-truncating pathogenic mutations, whereas novel single base pair changes predicted to lead to the significant change in protein structure, conserved between mouse and man and not present in 400 alleles in healthy controls were defined as non-truncating possibly pathogenic mutations. We have identified 28 mutations that fulfilled these criteria. Compared to previously analysed CLL cohorts, we observed an enrichment for truncating mutations, primarily clustered in the 5' end of the gene and localised in the p53 binding region. Apart from a high frequency of 11q deletions and an increased DAT positivity ATM mutant tumours were clinically indistinguishable from the rest of the cohort. Notably, however, ATM mutant tumours exhibited a distinctive profile of VDJ recombinations and compared to the rest of the cohort showed a clear enrichment for stereotypic VDJ recombinations involving V1-69 family. Interestingly, when all three categories of ATM mutations were considered together no impact was observed on either overall or progression free survival. In contrast, when putative pathogenic mutations (truncating or described in A-T families) were considered separately, their presence rendered significantly poorer both overall and progression free survival (p=0.04 and p=0.008 respectively). We conclude that putative pathogenic ATM mutations affect patients' responses to DNA damaging agents included in CLL4 treatment and that therefore should be considered as a separate cohort that might benefit from treatments that for their activity do not require activation of DNA damage induced apoptosis.

5.30

A COMPARATIVE STUDY OF RNA-BASED MARKERS IN CHRONIC LYMPHOCYTIC LEUKEMIA REVEALS LPL AS A POWERFUL PREDICTOR OF CLINICAL OUTCOME

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Recent studies have proposed the RNA expression levels of certain genes, i.e. LPL, CLLU1, TCL1, MCL1 and ZAP70, to be novel predictors of clinical outcome in chronic lymphocytic leukemia (CLL). However, a comprehensive assessment of these RNA-based markers is still lacking. The current study aimed to investigate the potential of these markers in CLL prognostication, either as a single marker or in combination with established markers. By applying real-time quantitative PCR, we measured the RNA expression levels of LPL, CLLU1, TCL1, MCL1 and ZAP70 in 256 newly diagnosed CLL patients from a Scandinavian population-based cohort and correlated with clinical outcome. The expression cut-offs for each RNA marker was determined by constructing ROC curves. Additionally, Binet stage, IGHV mutation status, CD38 expression (cut-off 7%) and the presence of recurrent genomic aberrations (i.e. 11q-, 17p-, 13q- and +12) were evaluated for all cases. High expression of all RNA-based markers except MCL1 predicted significantly shorter overall survival (OS) and time to treatment (TTT), with LPL being the most significant prognostic marker in both log-rank (Table 1) and Cox's univariate regression analyses. In multivariate analysis, only LPL expression and the established markers remained independent prognostic factors for OS and TTT. Interestingly, all of the RNA-based markers could add further prognostic information to established markers in subgroups of patients, with LPL expression status giving the most significant results. Notably, LPL expression could subdivide good prognostic subgroups such as patients with Binet stage A, CD38 negativity or favorable genomic aberrations (Table 2). Altogether, we conclude that LPL expression is the strongest among the RNA-based markers for prediction of clinical outcome in CLL and thus could be applied in the clinical laboratory to predict outcome, particularly in combination with established markers.

Table 1. Prognostic impact of RNA-based markers.

Variable	Overall Survival			Time to Treatment		
	N	Median (months)	p	N	Median (months)	p
LPL	256			223		
High	112	89		96	28	
Low	144	NR	<.001	124	NR	<.001
TCL1	256			223		
High	128	NR		106	41	
Low	128	NR	.01	117	NR	.007
ZAP70	256			223		
High	125	NR		112	47	
Low	131	NR	.003	111	NR	.02
CLLU1	228			197		
High	130	NR		113	23	
Low	98	NR	.001	84	NR	<.001

N.R. Not reached

Table 2. LPL expression in subgroups of CLL.

Variable	Overall Survival			Time to Treatment		
	N	Median (months)	p	N	Median (months)	p
Binet A	186			172		
High LPL	77	98		72	34	
Low LPL	109	NR	<.001	100	NR	<.001
<7% CD38	177			153		
High LPL	53	NR		49	48	
Low LPL	124	NR	<.001	104	NR	<.001
11q-/+12 aberration	194			167		
High LPL	64	94		56	23	
Low LPL	130	NR	<.001	111	NR	<.001

NR: Not reached

N.R. Not reached

5.31

PROMINENT PROLIFERATION CENTERS (PC) IDENTIFY A HISTOLOGICAL SUBTYPE OF CHRONIC LYMPHOCYTIC LEUKEMIA (CLL) WITH ADVERSE CLINICAL FEATURES

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Introduction. The proliferation compartment in lymphoid tissues involved by CLL is essentially represented by large-cells that form proliferation centers (PC) whose biological and clinical significance is a matter of investigation. *Methods.* Tissue biopsies consecutively obtained from 100 patients diagnosed with CLL were analyzed. Histological review was independently performed by two investigators (AM and DM) in a BX51 Olympus microscope. Each biopsy was analyzed for the presence and size of proliferation centers (PC), and for the proliferation rate inside the PCs as assessed by means of mitosis count, p27 and Ki-67 immunostains. PCs were delineated by p27 negative staining and quantified using a computerized image analysis system. Histological characteristics were correlated with the main clinical characteristics and survival from the time of biopsy. To define the best cut-offs for histological variables in predicting survival, the Maximally Selected Rank Statistics was applied. The Fisher's exact test, Chi2-tests or T test were applied to correlate different clinical and biological parameters with histological patterns. *Results.* CLL/SLL diagnosis was established in 78 cases, whereas DLBCL transformation was observed in 22 cases. Upon variables selection by the Maximally Selected Rank Statistics, CLL/SLL cases with enlarged and confluent PC (broader than 20x field) (p=0.001), >2.4 mitosis per PC (p=0.019), or >30% Ki-67 per PC (p=0.0024) were considered as having "accelerated" CLL (n= 23). Median survival of "accelerated" CLLs from the time of biopsy was 34 months, whereas it was of 75 months (HR 2.2, 95%CI HR 1.21-3.86; p=0.008) for cases without adverse features in the tissue biopsy. Patients diagnosed with DLBCL transformation (n=22) had a median survival of 4 months. Notably, only one patient with an accelerated CLL/SLL pattern transformed into DLBCL after a median follow-up of 5 years. "Accelerated" CLL/SLL cases were detected early in the course of the disease (median time from diagnosis: 16 months; 39% of cases at diagnosis), as opposite to DLBCL transformed cases (median time form diagnosis: 45 months; 14% at diagnosis). Positive p53 immunostaining was observed in 24% "accelerated" CLLs compared to 5% CLLs without adverse features in the biopsy (p=0.02). The presence of p53 positivity was also frequently observed in DLBCL transformed cases (67%). *Conclusions.* In lymph node biopsies from CLL patients, enlarged and confluent PC and/or an increased proliferation activity in the PC ("accelerated" CLL) correlated with an inferior survival in spite of the fact that they do not seem to transform into DLBCL. Whether these patients require specific therapeutic approaches warrants investigation.

5.32

BONE MARROW ANGIOGENESIS IN CHRONIC LYMPHOCYTIC LEUKEMIA - A STUDY OF CD34 AND VWF EXPRESSION IN BONE MARROW BIOPSIES -

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Introduction. Angiogenesis is a physiologic process of new blood vessels formation mediated by various cytokines called angiogenic and angiostatic factors. Enhancement of angiogenesis in chronic lymphocytic leukemia (CLL) has been recognized more recently. Our study assesses CD34 and vWf expression and microvessel density (MVD) in the bone marrow of patients with CLL. *Aims*. 1. To assess bone marrow MVD in CLL using two different monoclonal antibodies CD34 and vWf and a reproducible method of MVD quantification; 2. To examine the possible association of marrow MVD and clinical course, pattern of marrow infiltration, Rai stage, CD 38 positivity and cytogenetic abnormalities detected by fluorescence in situ hybridization (FISH). Materials and method: Bone marrow specimens from 33 patients with CLL and 10 controls were studied. MVD was calculated on sections stained immunohistochemically for CD 34 and FVIII. A total of 10 microscopic fields were examined for the number of events (immunoreactive elements identified as cells). The number of events per field and thus the MVD were determined. *Results.* There was a significant difference between MVD counts according to the antibody used. MVD was higher using CD34 vs vWF (CD34, mean±standard deviation [SD], 35.91±15.7, 95% confidence interval of mean [CI], 30.34-41.48 vessels/field vs vWF, 8.15±4.65, 95% CI, 4.11-12.44 vessels/fileld, p<0.0001). Bone marrow MVD detected by CD34 was significantly higher in patinents with CD38 expression More than 30% (p=0.006). However, no significant MVD differences were detected between CLL subgroups with regard to clinical course, pattern of marrow infiltration, Rai stage and FISH abnormalities. Bone marrow MVD in patients with CLL was significantly higher than that in controls (p<0.0001). *Conclusion.* MVD assessment using anti-CD34 resulted in higher MVD counts than when using anti-vWF antibody. However, no MVD differences were detected between CLL subgroups subdivided according to the above-mentioned prognostic factors except CD38 expression.

5.33

THE PROGNOSIS OF CLINICAL MONOCLONAL B CELL LYMPHOCYTOSIS DIFFERS FROM PROGNOSIS OF RAI 0 CHRONIC LYMPHOCYTIC LEUKAEMIA AND IS RECAPITULATED BY BIOLOGICAL RISK FACTORS

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Monoclonal B-cell lymphocytosis (MBL) is an asymptomatic monoclonal expansion of <5.0x10^9/L circulating CLL-phenotype B-cells. Since the introduction of the new IWCLL guidelines, it has been a matter of debate whether the 5.0x10^9/L CLL-phenotype cell cut-off for CLL diagnosis has a clinical rationale for the definition of MBL. The relationship between MBL and Rai 0 CLL, as well as the impact of biological risk factors on MBL prognosis, are unknown. Out of 460 B-cell expansions with CLL-phenotype, 123 clinical MBL (cMBL) were compared to 146 Rai 0 CLL for clinical and biological profile and outcome in order to: i) to define whether clinical features at diagnosis, biological profile, and outcome of cMBL are distinguishable from Rai 0 CLL; ii) to identify clinical or biological variables at diagnosis that best predict the risk of evolution from cMBL to CLL requiring treatment. cMBL had better humoral immune capacity and lower infection risk, lower prevalence of del11q22-q23/del17p13 and TP53 mutations, slower lymphocyte doubling time, and longer treatment-free survival. Also, cMBL diagnosis was a protective factor for treatment risk. Despite these favourable features, all cMBL were projected to progress, and lymphocytes <1.2x10^9/L and >3.7x10^9/L were the best thresholds predicting the lowest and highest risk of progression to CLL. Although IGHV status, CD38 and CD49d expression, and FISH karyotype individually predicted treatment-free survival, multivariate analysis identified the presence of +12 or del17p13 as the sole independent predictor of treatment requirement in cMBL (HR: 5.39, 95% CI 1.98-14.44, p=.001). In this study we confirm that the 5.0x10^9/L cell cut-off can be used to distinguish cMBL from CLL. More importantly, however, we document that: i) cMBL and Rai 0 CLL differ in some biological features and outcome; and ii) cMBL who are destined to progress to symptomatic CLL/SLL requiring treatment can be identified by biological risk factors. Overall, these data show that cMBL has a more favourable clinical course than Rai 0 CLL. Since the biological profile can predict treatment requirement, stratification based on biological prognosticators may be helpful for cMBL management.

5.34

CHARACTERIZATION OF CD38+ VS. CD38- CLL CELLS IN A NOD/SCID XENOGRAFT MODEL

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Chronic lymphocytic leukaemia (CLL) is a heterogeneous disease with a highly variable clinical course. The absence of mutations in the IgV genes, together with presence of ZAP-70 and CD38, are reliable negative prognostic markers, that allow clinicians to split patients into different risk groups. Further investigation of the functional role of these molecular markers for CLL pathogenesis *in vivo* represents an important aim of several studies. The introduction of a novel model for CLL generated by infusion of human primary CLL cells into immunodeficient nonobese/severe combined immunodeficient (NOD/SCID) mice by our group was an initial step towards this issue. In the current study we confirm the validity of the NOD/SCID mice model and focus on the role of CD38 in homing of freshly purified PBMC from 56 clinically and molecularly well characterized CLL patients. Combined i.v. and i.p. injections in a total of 277 irradiated NOD/SCID mice resulted in highly reproducible splenic engraftment after 4 weeks. The engraftment correlated significantly with markers reflecting disease activity, such as Binet stage at time of transplantation (A vs. C, p<0.0001), levels of serum lactate dehydrogenase (LDH) (p=0.007) and lymphocyte doubling time (LDT) (p=0.004). Grouping the cells on the basis of molecular markers allowed further distinction of the engraftment pattern. Stratification on the basis of the presence of mutations in the IgV genes or the expression of Zap-70 showed a clear trend but lacked statistical significance (p=0.15 and p=0.06, respectively). However, after transplantation of CD38$^+$ cells a higher number of human CD19$^+$ cells was detected in the murine spleens than after injecting their CD38- counterparts (p=0.002). Interestingly, the combination of CD38 with Zap-70 expression displayed the highest difference in engraftment, when confronting CD38$^+$/Zap-70$^+$ with CD38$^-$/Zap-70$^-$ cells (p=0.001). Subsequent immunohistochemical staining for CD20 in combination with Ki-67 of the respective murine spleens demonstrated that engraftment of human CD38$^+$ CLL cells was associated with enhanced proliferation compared to engraftment of their CD38$^-$ counterparts. In conclusion these results confirm that this NOD/SCID mouse model reflects the heterogeneity and important clinical characteristics of the disease, and thus may serve as a tool for preclinical drug testing and investigation of the pathophysiology of the disease. Further, it underlines the importance of CD38 in disease progression permitting its consideration as a possible therapeutic target in a subgroup of CLL patients.

5.35

EPIGENETIC MARKERS FOR PROGNOSIS IN CHRONIC LYMPHOCYTIC LEUKAEMIA

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Aberrant epigenetic changes, such as hypermethylation of gene associated CpG islands, likely plays a key role in the development of CLL and indeed all types of cancer. In addition to identifying key genes in cancer development analysis of altered DNA methylation may prove to be a rich source of molecular markers which can be used for prognosis or prediction of response to therapy. DNA methylation makes an ideal molecular marker due to its high stability, low levels of normal background and technological ease of detection. We have recently examined the potential utility of methylation of a number of genes (CD38, HOXA4, BTG4) in CLL prognosis. CD38 expression has already been shown to predict outcome in CLL, although the level of expression used is still controversial. We investigated the potential role of methylation in CD38 expression. This analysis found that CD38 gene methylation was frequent in CLL, with slightly over half of CLL patients (52/103) exhibiting hypermethylation. Furthermore methylation of the gene strongly correlated with reduced CD38 expression (p=5.50x10^{-17}). Both CD38 methylation status and CD38 expression (>30%) correlated with reduced survival in CLL patients. Indeed, in this sample set, CD38 methylation status (p=0.000915, hazard ratio 3.04) was a better predictor of outcome than CD38 expression (p=0.0212, hazard ratio 2.03). However, numerous previous studies have shown that methylation of genes at distal loci is often not an independent event and that increased methylation of CpG islands throughout the genome may correlate with poor outcome in patients. This suggests that the optimal methylation based marker may consist of a number of physically separated loci. To this end we have extended these studies to include two other potentially useful epigenetic markers. We have previously shown that hypermethylation of the HOXA4 gene is a frequent event in all leukaemia types and correlates with poor prognosis patients in CLL, AML and CML. In CLL, hypermethylation of HOXA4 is significantly more common in patients with unmutated IgVH genes (poor prognosis) and it predicts for shorter time to requiring treatment. We have also investigated the methylation status of the BTG4/miR34b/c locus, which maps to the commonly deleted 11q23 region, a poor prognostic feature in CLL. Methylation of this region is negatively correlated with the 11q23 deletion and with advanced stage disease (Binet stage B and C). To assess the possibility of combining these methylation markers we are assessing the methylation status of the CD38 and BTG4 loci in the original 130 samples used for the analysis of HOXA4 methylation. This will be used as a test set to define the optimal algorithm for combing all 3 epigenetic markers for the prediction of outcome in CLL. All three markers have also been analysed in a second confirmatory set of 105 CLL samples and this second set will be used to assess the validity of the combined epigenetic marker algorithm.

5.36

CHROMOSOME 14 ALTERATIONS IN CHRONIC LYMPHOCYTIC LEUKEMIA. CORRELATION WITH CLINICOPATHOLOGIC CHARACTERISTICS

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Cytogenetic analysis allows the detection of specific structural abnormalities, new chromosome markers or complex changes which may not be detected by FISH (Fluorescence in Situ Hybridization). The analysis of chromosome aberrations represents an important prognostic indicator in chronic lymphocytic leukemia (CLL). In this study, we have evaluated cytogenetic, FISH and clinicopathological characteristics of CLL patients with cytogenetic alterations involving chromosome 14 (C14) detected by conventional cytogenetics or FISH. All patients gave their informed consent and the study was approved by the local Ethics Committee. Chromosome analysis were performed on stimulated peripheral blood lymphocytes, cultured for 72-96 hours at 37°C in F-10 medium supplemented with 15% fetal calf serum. G-banding technique was used. Karyotypic abnormalities were described using the International System for Human Cytogenetic Nomenclature. For FISH analysis, slides were hybridized with the LSI p53 (17p13), LSI ATM (11q22), LSI D13S319 (13q14), CEP12, LSI p53/ATM/13q14/13q34/CEP12 and LSI Dual color Dual fusion BCL-2/IGH DNA probes (Vysis-Abbott), according to manufacturer's protocol. Four hundred interphase nuclei were analyzed for each probe. The cut-off for positive values (mean of normal control + 3SD), determined from samples of 10 cytogenetically normal donors, were: 3.02%, 10.2%, 7.7%, 5.1% and 1% for trisomy 12, monosomies of D13S319, ATM and p53, and BCL2/IGH fusion, respectively. Different painting probes were also used. Statistical analysis was performed with the Fisher exact test and the Student t test. Survival curves were plotted by the Kaplan-Meier method and compared by the log-rank test. A total of 49 CLL patients cytogenetically studied in our laboratory showed structural chromosome alterations. Among them, 12 (24.5%) cases presented abnormalities involving the C14 (7 males; median age: 67 years; range: 38-79 years; Rai stages: 0: 2, I-II: 5, III-IV: 5). A total of 14 chromosomal structural alterations were found. Six deletions: del(14)(q22) [2] and del(14)(q24) [4], three of them as a single clonal alteration and three as a part of complex karyotypes, two of them with translocations involving the other C14: der(14)t(10;14)(q11;p11) (not previously described) and t(14;18)(q32;q21). The other alterations were: der(14)t(11;14)(q13;q24), t(10;14)(q22;q32), t(2;14)(p11;q32) and two more cases with t(14;18)(q32;q21), one of these only detected by FISH, and one inversion: inv(14)(q22q32). Breakpoints at 14q32 [6], 14q24 [5] and 14q22 [3] were the most frequently observed. A comparison of clinical and hematological data between patients with C14 alterations and those of the favorable risk group (absence of detectable abnormalities by FISH or cytogenetics or 13q- as single abnormality) (54 patients; 25 males; median age: 67 years, range: 41-89; Rai stages: 0: 18, I-II: 21, III-IV: 15) was performed. No significant differences between both groups in age (p=0.92), sex (p=1.00), Rai stage distribution (p=0.49), LDH (p=0.33), β2 microglobulin (p=0.38), median lymphocyte count (p=0.50), LDT (p=1.00), haemoglobin (p=0.73), platelet count (p=0.39) and CD38 (p=0.56) were found. However, a significant difference in lymphocyte count (p=0.03) was observed. Treatment-free survival (TFS) of the C14 group was significantly shorter than those observed in CLL patients from the favorable risk group (24 months vs. 93 months, p=0.0007). These findings show the prognostic value of structural C14 alterations in CLL patients with different breakpoints. They may involve molecular events of importance in disease progression and should have implications in clinical management.

5.37

ASSOCIATIONS BETWEEN IMMUNOGLOBULIN GENE STEREOTYPY, CYTOGENETIC ABNORMALITIES AND CLINICAL OUTCOME

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We have analysed the immunoglobulin heavy chain gene usage and mutational status of 1072 well characterised CLL cases. 496 cases registered in the UK CLL4 trial, all with progressive stage A, stage B or stage C disease and 576 archived, present or referred cases from the Royal Bournemouth Hospital, which consist predominantly of stage A0/A disease 436/576 (75.7%). Age at diagnosis ranged from 27-93 years (median 64 years) and the M:F ratio was 1.9:1 (non-stereotypic) and 2.7:1 (stereotypic) (p=0.0334). Of the 1072 IGHV-D-J sequences analysed 47% were mutated (<98% homology to germline). 25.4% (272/1072) of cases were assigned to 74 subsets with stereotypic HCDR3 sequences; confirming 16 previously published potential subsets (13 - Murray et al 2008 and 3 - Messmer et al 2008) and identifying 28 novel subsets (8 confirmed, 20 potential). 71.3% (194/272) of stereotypic cases were unmutated and subset 2 (Stamatopoulos et al 2007) was the largest subset (n=46). Stereotypic IGHV1-69, IGHV3-21 and IGHV4-34 genes accounted for 47.2% of all stereotypes (60/61 IGHV1-69, 24/47 IGHV3-21 and 2/20 IGHV4-34 unmutated). Time to first treatment (TFT) was available in 969 cases, stereotypic subsets containing sufficient numbers of cases were analysed individually; subset 1 showed no significant difference in TFT when compared with non-stereotypic cases utilising the same IGHV genes (IGHV1-2, 1-3, 1-8, 1-18, 1-46 and 5-a). Subset 2 had a sig-

nificantly shorter TFT than cases utilising non-stereotypic IGHV3-21 (p=0.005), confirming previously published data. There was no significant difference between the individual stereotypic subsets utilising IGHV1-69 (subsets 3, 5, 6, 7 and 59) and non-stereotypic unmutated IGHV1-69 cases. Interestingly we found that a novel mutated IGHV4-34 stereotype (subset 902) had a significantly shorter TFT (median <2 months) than other mutated stereotypic (median 125 months) and non-stereotypic (months 133 months) IGHV4-34 cases (p=0.001). Novel subset 901 has mixed homology (3/5 mutated), all diagnosed at stage B or C and all requiring treatment within 26 months of diagnosis (median 0.4 months), however, there was no significant difference in TFT between this subset and non-stereotypic cases utilising the same IGHV gene. When analysing stereotypes, found only in cases diagnosed at stage A0, novel subsets 912 and 917 were identified. For subset 912 both cases had greater than 10 years treatment free survival. Cases assigned to 917 required treatment within 60 months and had greater than 7 years overall survival. IGHV gene usage was also compared with cytogenetic abnormalities commonly assessed in CLL (del 11q, del 13q, del 17p and trisomy 12). Of note, case utilising the IGHV3-11, IGHV3-21 and IGHV3-48 genes (111/968 cases), all members of a distinct evolutionary branch of the IGHV3 subgroup genes, never showed del 17p (p=0.0011). Del 11q was shown to be associated with cases utilising IGHV1-69 (p=0.0001) and specifically with stereotypic IGHV1-69 cases (p=0.0314). Trisomy 12, as previously reported was associated with IGHV4-39 in all cases (p=0.0065) and more specifically within stereotypic cases (p=0.0005). Del 13q was associated with mutated, non-stereotypic cases (p=0.0001) and when all IGHV genes were assessed, del 13q was significantly associated with IGHV3-7 (p=0.0040). Larger studies will be required to determine whether rarer stereotypes have biological significance.

Table.

Novel subset	*No. of cases*	*IGHV*	*IGHD*	*IGHJ*	*Representative CDR3*
901	5	1-18		1/4/2	CARASGGSSLDW
902	4	4-34	6-19	3	CARRREQWLLSEGDGYDIW
922	4	2-5	2-2	6	CAHSSRLGYCSSTSCYYYYYGMDVW
929	4	4-61/3-11/3-48	3-22	6	CARDHGGEGYYDSSGYYYVGYYYGMDVW
921	3	1-69	3-9	6	CARDLGNFDILTGYYWDYYYGMDVW
917	3	1-69/4-4	2-2	5	CAVGGDIVVVPAAMESFWNEFDPW
903	2	4-39	6-19	6	CARHGQWLVSHYYYGMDVW
904	2	3-48	6-13	4	CAIPPQQLVEDYW
905	2	1-18	3-10	4	CARVGWYGEYYFDYW
907	2	4-34		4	CARRDGYNFVYW
908	2	3-33		4	CARDGPGRDLDYW
910	2	3-23	3-3	4	CAKHYDFWSGYFDYW
911	2	3-23	4-23	4	CAKTDGGDSGAFDYW
912	2	3-23	3-10	4	CAKDYLLDGSGSSLDYW
913	2	1-2	3-22	4	CARSYDSSGYYCYFDYW
914	2	3-11	3-9	4	CARERRYFDWLLHLLDYW
915	2	2-5	1-7	4	CAHRRQLNGNWDGGHFDYW
916	2	3-11	3-10	4	CARDKLWFGELLSHYFDYG
918	2	1-69	3-3	6	CARDPREGVVNLYYYYGMDVW
919	2	1-69	3-3	6	CARRLIFGVVITPPNYYGMDVW
923	2	1-69	3-3	6	CARSTPNYDFWSGYTVGYYYYGMDVW
925	2	1-69/3-30	3-3	6	CARRKTYDFWSGYYAMGGYYYYGMDVW
926	2	3-9	3-3	6	CAKDTIEGYYDFWSGYPYYYYGMDVW
927	2	4-31/4-61	3-3	6	CARGVYYDFWSGYYKPYYYYMDVW
928	2	1-69	2-2	6	CASSLGGYCSSTSCYRPLYYYYGMDVW
803	3	3-9	3-10	6	CAKDRSLNYYGSGSPYYYYYGMDVW
813	4	1-69	2-15	6	CARVGCSGGSCYVLGWSRYYYYMDVW
930	2	5-51		4	CARLPGASGYYSNLDYW

5.38

EXPRESSION AND PROGNOSTIC SIGNIFICANCE OF LIPOPROTEIN LIPASE GENE IN CHRONIC LYMPHOCYTIC LEUKEMIA.

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Introduction. Chronic lymphocytic leukemia (CLL) is characterized by high individual variability in clinical course and for therapy requirement. IgVH mutational status is currently the best predictor of CLL outcome independent of clinical stage. Unfortunately, DNA sequencing is not available in most medical facilities. Alternatively, microarray studies uncovered an overexpression of lipoprotein lipase (LPL) gene in progressive unmutated (UM) IgVH cases. Purposes: 1) to evaluate LPL profile in CLL patients; 2) to compare results with clinical and biological prognostic factors: Binet staging, IgVH status, expression of CD38 and Zap-70; 3) to correlate LPL expression with progression free survival (PFS). *Materials and methods*. sixty four patients with CLL were studied between March 2005 and December 2009. Flow cytometry analyses for CLL diagnosis (score system of Moreau *et al.*), CD38 and Zap-70 expression were performed at patient admission with fresh (whole) peripheral blood cells. IgVH and LPL studies were simultaneously performed after mononuclear cells separation by Ficoll Hypaque gradient. LPL and IgVH expressions were evaluated by qualitative RT-PCR, using the housekeeping gene GAPDH as internal control. Then, IgVH mutational status was classified by direct sequencing of PCR products. The Kaplan-Meier method was employed to estimate PFS. The log-rank test and the Cox proportional-hazards model were used to analyze the correlation between LPL and the clinical/biological prognostic factors in univariate and multivariate analyses, respectively. *Results.* Advanced Binet clinical stage, high expression of LPL and CD38 (but not Zap-70), and UM IgVH status were associated with lower PFS. Moreover, the impact of these markers on disease progression/prognosis was also observed among Binet early stage patients. In multivariate analysis, Binet staging (p=0.04), LPL expression (p=0.007) and IgVH mutational status (p=0.003) were identified as independent prognostic risk factors for lower PFS. *Conclusions*. LPL gene expression demonstrated strong prognostic prediction in CLL, even among Binet early stage patients, and was comparable to IgVH mutation status using a much easier and feasible technique. LPL analysis could be an attractive alternative for IgVH mutational studies.

5.39

EXPRESSION AND PROGNOSTIC SIGNIFICANCE OF THE VASCULAR ENDOTHELIAL GROWTH FACTOR RECEPTOR (VEGFR) IN CHRONIC LYMPHOCYTIC LEUKEMIA.

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Introduction. In recent years, significant progress in understanding the role of angiogenesis in Chronic Lymphocytic Leukemia (CLL) has been achieved. Further evidence for the importance of angiogenesis in CLL came from the finding that CLL cells express and release considerable amounts of pro-angiogenic molecules, such as Vascular Endothelial Growth Factor (VEGF). Serum levels of this growth factor have been associated with advanced clinical stage and disease progression. Purposes: 1) to evaluate VEGF receptor (VEGFR) profile in CLL patients; 2) to compare results with clinical and biological prognostic factors: Binet staging, IgVH status, expression of CD38 and Zap-70; 3) to correlate VEGFR expression with progression free survival (PFS). *Materials and methods.* sixty four patients with CLL were studied during March 2005 and December 2009. Flow cytometry analyses for CLL diagnosis (score system of Moreau et al.), CD38 and Zap-70 expression (fresh whole peripheral blood) and VEGFR (mononuclear cells by Ficoll Hypaque gradient method) were performed at patient admission. The IgVH genes mutational status was evaluated in mononuclear cells by RT-PCR followed by sequencing analysis. The Kaplan-Meier method was employed

to estimate PFS. The log-rank test and the Cox proportional-hazards model were used to analyze the correlation between VEGFR and the clinical/biological prognostic factors in univariate and multivariate analyses, respectively. *Results.* Advanced clinical stage, expression of CD38, VEGFR and unmutated IgVH genes were associated with lower PFS. Moreover, the impact of these markers to the disease prognosis was also observed among Binet A patients, showing their importance as strong predictors of disease progression even in the early stage patients. In multivariate analysis the Binet's staging (p=0.04), VEGFR (p=0.007) and mutational status of IgVH (p=0.003) were identified as independent risk factors for lower PFS. *Conclusions.* VEGFR expression demonstrated strong prognostic prediction in CLL, even among Binet early stage patients, and was comparable to IgVH mutation status. VEGFR evaluation by flow cytometry is a fast and easy technique, representing an advantage when compared to the time consuming molecular method of IgVH mutational status determination.

5.40

TYPE C P53 PATHWAY DYSFUNCTION RESULTING FROM THE PRESENCE OF AN EMERGING TP53 MUTATED SUBCLONE IN CHRONIC LYMPHOCYTIC LEUKAEMIA

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Both p53 and p21 are integral to the normal DNA damage response to double stranded breaks. By measuring expression of p53 and p21 in CLL cells before and after exposure to ionising radiation, Carter et al described p53 pathway dysfunction as a failure to up regulate both p53 and p21 expression. Dysfunctional cases were subdivided into those with increased basal levels of p53 (type A) and those with low baseline p53 expression (type B). Type A dysfunction was confined to cases with TP53 loss in >50% of cells, while type B dysfunction was found in cases with TP53, ATM and/or 13q14 deletions. Johnson et al recently described a third type of response (type C) whereby p21 is not up regulated despite a normal p53 response. They went on to suggest that, in around one third of cases, this phenomenon may be related to single nucleotide polymorphisms (SNP) in the p21 gene, showing that heterozygosity at the codon-31 SNP (rs1801270) was associated with this novel response and that this was abrogated by heterozygosity at the 3'UTR SNP (rs1059234). In our own analyses of 513 CLL cases, using the radio-mimetic drug etoposide, a type C response was found in 23 cases (4.5%). Genotyping of rs1801270 and rs1059234 on all 23 type C cases plus 20 abnormal and 80 normal response cases was performed. Consistent with Johnson *et al.*, 7/23(30%) were heterozygous at codon-31 and this was associated with type C response (p=0.006). However, in our heterozygous codon-31 cases, the association between normal p21 response and heterozygosity at the 3' UTR did not reach significance (p=0.08). In longitudinal analysis of samples from 49/513 cases, 3/49 were observed to change response during disease course, one from normal to type C and two from type C to to type B. In each of these three cases, a mutation of p53 was detectable by direct sequencing. We therefore hypothesised that the type C response may be due to an emerging p53 abnormal clone. Direct sequencing of TP53 exons 5-9 detected mutations in 9/21 type C cases compared to 1/43 normal function cases, showing a clear association between TP53 mutation and type C response (p=0.0009). The incidence of TP53 mutations in type C cases did not differ between codon 31 heterozygous and homozygous cases, suggesting that the two mechanisms are not exclusive (p=0.642). To replicate the effect of an emerging p53 abnormal clone, samples comprised of tumour cells from normal cases, spiked with tumour cells from type B cases with p53 mutations were analysed. Using this technique we were consistently able to emulate a type C response. These data suggest that the type C phenotype may result from differing genotypes and that due to the potential clinical relevance, it will be essential to identify cases where type C response results from an emerging p53 dysfunctional clone.

5.41

IMPACT OF IGVH GENE MUTATIONAL STATUS AND VH FAMILY USAGE ON CHROMOSOMAL ABERRATIONS DETECTED BY HIGH-RESOLUTION ARRAY-CGH IN B-CLL

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B-cell chronic lymphocytic leukemia (B-CLL) is characterized by a variable clinical course. Genomic aberrations as detected by FISH analysis and the mutational status of the IgVH genes have been found to have prognostic clinical significance with unmutated IgVH genes associated with clinically more aggressive disease. Previous studies have suggested that unmutated IgVH B-CLL is associated with increased chromosomal instability and increased chromosomal aberrations leading to accelerated disease progression. In early 2009 we began a project to performed high-resolution array-based comparative genomic hybridization (HR-CGH) using the Agilent one million probe array in B-CLL. To date 58 previously untreated patients with B-CLL have been analyzed. Our hypothesis was that we would see increased genomic aberrations and instability in the unmutated IgVH group. Variations in chromosomal aberrations in various VH families were also evaluated. The total number of copy number aberrations and total megabases (Mb) deleted or gained were used as a surrogate marker for genomic instability. CLL with unmutated IgVH genes utilized members of the VH1, VH3, VH4 and VH5 families with VH1-69 the most prevalent. CLL with mutated IgVH genes utilized VH families 1, 2, 3, 4, and 6. Considerable variation was seen in the number of genomic abnormalities between cases. Median number of chromosomal aberrations in mutated group was 5 (range 1-17) and unmutated group was 4 (range 0-20). Copy number deletions were significantly more common than chromosomal gains for the total group and in both the unmutated and mutated IgVH gene groups. Mean number of megabases (Mb) of DNA deleted in the unmutated IgVH group was 17.80Mb (standard deviation 24.17 Mb, median 6.63 Mb, range 0 - 90.55Mb) and 40.65 Mb in the mutated IgVH group (standard deviation 86.04 Mb, median 15.35 Mb, range 0-307.57 Mb). Mean number of Mb gained in the unmutated group was 37.74 Mb (standard deviation 57.58 Mb, median 0.44Mb, range 0-132.47Mb) and 32.99 Mb (standard deviation 59.76 Mb, median 0.14Mb, range 0-172.50Mb) in the mutated group. Nine patients had B-CLL with VH1-69 genes and 8 of them were unmutated. Median number of chromosomal deletions was 2 and gains also 2 (ranges 0-8 deletions and 0-4 gains) for this group. Similar findings were seen for the other VH families. These results initially suggest that total copy number variations do not account for the observed clinical differences between mutated and unmutated IgVH B-CLL. The catalogue of interesting and potentially clinically relevant chromosomal abnormalities found in this HR-CGH study is being currently evaluated.

5.42

SINGLE-CELL PROFILES OF B-CELL RECEPTOR PHOSPHO-PROTEIN NETWORKS ASSOCIATED WITH PROGNOSIS AND PROGRESSION IN CHRONIC LYMPHOCYTIC LEUKEMIA

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B-cell chronic lymphocytic leukemia (B-CLL) patients exhibit a variable clinical course. Several biological parameters have been shown to be associated with clinical outcome in CLL. Among them, the most reliable markers are represented by the absence of somatic mutations within the immunoglobulin variable heavy chain genes (IGHV), the expression of CD38 antigen, the presence of the ZAP-70 tyrosine kinase. These parameters of poor clinical outcome are structurally and/or functionally linked to B-cell Receptor (BCR) expressed by CLL cells, thereby strengthening the hypothesis that antigenic stimulation mediated by the BCR represents a driving event in the onset and progression of the malignant B cells. To investigate whether different BCR signaling networks may distinguish clinical-biological groups of CLL patients, we applied a "network level" analysis of BCR signaling by measuring single-cell profiles of phosphoprotein networks by flow cytometry. We

evaluated the response to BCR engagement in primary cells isolated from 27 CLL patients by analyzing the phosphorylation states of 5 phosphoproteins on the route of BCR signaling, including p-Syk, p-NF-kappaB, p-Erk1/2, p-p38 and p-JNK. BCR was cross-linked by incubating cells with anti-IgM antibodies. The unsupervised clustering analysis distinguished BCR response profiles of phospho-proteins that differentiated cases of CLL with mutated IGHV from those with unmutated IGHV (p=0.0003), cases with low levels of CD38 expression from those with high levels (p=0.0004) and cases with ZAP-70-negative leukemic cells from cases that were ZAP-70-positive (p=0.001). Furthermore, the same BCR response profiles were also associated with time to progression (p=0.0014) and with overall survival (p=0.049), as assessed by Kaplan-Meier curves and the log-rank test. This study shows that single-cell profiles of BCR phospho-protein networks are associated with prognostic parameters, disease progression and overall survival in CLL. (This study was supported by: Regione Veneto "Ricerca Sanitaria Finalizzata"; "Fondazione G. Berlucchi per la Ricerca sul Cancro"; AIRC - Associazione Italiana Ricerca sul Cancro; Fondazione CARIVERONA and Fondazione CARIPARO).

5.43

VH3-21 SUBGENE USAGE DOES NOT INFER WORSE PROGNOSIS OF CLL IN THE CZECH POPULATION

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The mutational status of IgVH genes is one of the most important prognostic factors in early stages of CLL. Moreover, the restricted pattern of usage of certain IgVH subgenes in chronic lymphoproliferative diseases (LPD) has shed light on their etiology and pathophysiology. Previous studies, especially the Swedish one (Tobin *et al.*), have postulated that usage of the VH3-21 is overrepresented in CLL patients (making up to 25% all cases in their earlier and up to 9% in later papers). In contrast to patients using other VH gene segments, a poorer prognosis was noted even in patients with mutated VH3-21 genes. We have successfully amplified 1724 IgVH genes in altogether 1528 patients with a suspected LPD. Among these, 58 cases (3.4%) with VH3-21 usage were detected (24 mutated and 34 unmutated on the 2% cut-off level). In the data files of a single Prague hospital, of 353 patients with a confirmed diagnosis of CLL (according to NCI guidelines) with a detected IgVH gene, 27 individuals (7.6%) used the VH3-21 subgene (15 mutated and 12 unmutated on the 2% cut-off level). The remaining patients' IgVH subgenes were mutated in 167 cases and unmutated in 159 cases, respectively. The survival without leukemic death (SWLD; i.e. cases not succumbing to CLL due to progression or complications of the disease and its therapy) of patients with VH3-21 did not differ from patients with other subgenes (median survivals, 174 mos and 282 mos, respectively, p=0.33). Notably, all 15 patients with mutated VH3-21 genes are alive. However, even out of the 12 patients with unmutated genes, only 2 died of CLL, so that the survival curves of the mutated/unmutated cases were not statistically different (p=0.37). (In contrast, the IgVH gene mutation status was the strongest predictor of SWLD in the total CLL cohort, p<0.0001). 21 patients with VH3-21 had also a FISH analysis performed. Of interest, none of the patients with VH3-21 had a del(17p). According to the Döhner's hierarchical classification, 2 patients had a 11q deletion, 4 had trisomy 12, 8 had the favorable del(13q) and 6 had no aberration detected. This result may be the background of the overall favorable SWLD results of patients with VH3-21. The results of the current study of VH3-21 genes from Czechia in the Western Slavic population resemble more the later Swedish results (in the Northern Germanic population) in respect to overrepresentation of VH3-21 subgenes in CLL, than the results of a small Chinese study. However, in contrast to Tobin et al, we were unable to prove a survival disadvantage of patients utilizing VH3-21 subgenes. In our hands, even patients with mutated VH3-21 genes survived extremely well. It is tempting to study further the differences of the impact of VH3-21 usage in different populations. However, the current study has demonstrated a huge difference in the proportions of VH3-21 usage in patients' samples arriving to the PCR laboratory (3.4%) and in patients receiving the hospital care (7.6%). The study was supported by grant MSM 0021620808.

Immune disturbances

6.2

CXCL12-INDUCED CHEMOTAXIS IS IMPAIRED IN T CELLS FROM ZAP-70- CHRONIC LYMPHOCYTIC LEUKEMIA PATIENTS

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T-cells from chronic lymphocytic leukemia (CLL) patients may actually play an important role in contributing to the onset, sustenance, and exacerbation of the disease by providing survival and proliferative signals to the leukemic clone within lymph nodes and bone marrow. By performing chemotaxis assays towards CXCL12 (1 ug/mL), CCL21 (1 ug/mL) and CCL19 (5 ug/mL), we sought to evaluate the migratory potential of T cells from CLL patients towards the main lymphoid organ chemokines. We found that T cells from CLL patients are less responsive to these chemokines than T cells from healthy adults (p<0.01, n=28 CLL samples and n=14 healthy donors), despite similar CXCR4 and CCR7 expression. When we discriminated CLL samples in high or low risk groups according to CD38 expression in leukemic cells, we observed that, although not statistically significant, T cells from $CD38^-$ patients were less responsive to CXCL12 and CCL19, while a comparable response was found for CCL21 compared to $CD38^+$ samples. Of note, when we analyzed T cell migration in $ZAP\text{-}70^+$ and ZAP-70- subsets, a lower migratory capacity towards CXCL12 in ZAP-70- samples (n=16) compared to $ZAP\text{-}70^+$ samples (n=12) was found (p=0.009) despite T cells from both groups of CLL patients expressed similar CXCR4 surface levels. Once we compared the migration indexes of T cells towards CXCL12 from healthy donors and CLL groups segregated by ZAP-70, we surprisingly found that only T cells from $ZAP\text{-}70^-$ samples showed a significant defective migratory capacity compared to healthy donors (p<0.001). By contrast, no statistically significant differences were found between T cells from $ZAP\text{-}70^+$ CLL patients and healthy donors. Given that CXCL12-induced migration seems to be regulated by intricated mechanisms besides CXCR4 expression, we further investigated other molecules and mechanisms capable to regulate CXCL12 responsiveness of T cells from CLL patients. We found that CXCL12 induces similar time- and dose-dependent CXCR4 endocytosis in T cells from healthy donors, $ZAP\text{-}70^+$ and $ZAP\text{-}70^-$ CLL patients. Moreover, since it was previously reported that ZAP-70, CD38 and CD45 expression was associated with an enhanced response through CXCR4, we evaluated their expression in T cells from CLL patients without finding any significant difference between $ZAP\text{-}70^+$ and $ZAP\text{-}70^-$ groups. Finally, given that previous work has demonstrated that the presence of leukemic cells can induce specific changes in T cells from CLL patients, we decided to evaluate whether CLL cells from ZAP-70- and $ZAP\text{-}70^+$ samples can modulate autologous T cell responses towards CXCL12. To this aim, purified T cells were cultured alone or with autologous purified CLL cells in a 1:4 ratio. T cell chemotaxis towards CXCL12 was evaluated with freshly purified cells and 48hs cultured cells. A comparable migratory capacity towards CXCL12 were found between freshly purified T cells alone (pT) or with autologous CLL cells (pT+pCLL) in both $ZAP\text{-}70^-$ and $ZAP\text{-}70^+$ samples. Similar results were obtained with T cells from $ZAP\text{-}70^+$ patients after 48hs of culture (n=9). By contrast, in all of the $ZAP\text{-}70^-$ patients evaluated (n=10), pT cells cultured for 48hs alone displayed a higher migratory capacity towards CXCL12 (p=0.001) compared to T cells cultured with autologous CLL cells (pT+pCLL), although they both showed similar CXCR4 surface expression. In summary, the results presented in this study demonstrate that T cells from $ZAP\text{-}70^-$ patients have a defective migratory response to CXCL12 which might be caused by inhibitory signals provided by the leukemic clone itself. We propose that leukemic cells from $ZAP\text{-}70^-$ patients would impair T cell migratory responses towards CXCL12, restricting the entry of circulating T cells to lymphoid organs. Since T cells in proliferation centers may help CLL cells to survive and proliferate, the low migratory response towards CXCL12 in T cells from ZAP-70- CLL patients might favor the indolent clinical course of the diseases in these patients.

6.3

T CELL SUBSET DYNAMICS IN THE TCL1A TRANSGENIC MURINE MODEL FOR HUMAN B CELL CHRONIC LYMPHOCYTIC LEUKAEMIA

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Numerous T cell dysfunctions have been described in B cell chronic lymphocytic leukaemia (B-CLL) that not only account for the inability to mount an effective anti-tumor response, but are also believed to contribute to tumor clone maintenance. These changes in the T cell compartment in B-CLL include an abnormal increase in absolute $CD4^+$ and $CD8^+$ T-cell numbers, inversion of the CD4/CD8 ratio, loss of co-stimulatory molecules on the T cell surface, abnormal cytokine profile and/or aberrant cytokine receptor expression profiles, unresponsiveness to mitogenic stimulation, and defects in synapse formation. We have recently demonstrated a difference in the relative distribution of naive and memory T-cell subsets in the peripheral blood from patients with mutated and unmutated IgVH genes. Unmutated CLL (UM-CLL) cases had a significant increase in relative numbers of central and effector memory $CD4^+$ T cells as compared to mutated CLL (M-CLL) cases. Here we extend our findings of T cell dysfunction in B-CLL to include the TCL1a transgenic (tg) mouse model of this disease. Similar to human B-CLL, leukaemic TCL1a tg mice have increased absolute numbers of T cells, owing to an expanded $CD8^+$ population. We also observed an increase in the relative numbers of memory and effector T cells predominantly in the CD8+ subset. The shift to more differentiated T cells parallels the incidence of CD19/CD5 hyperplasia in the TCL1a tg mice. Furthermore, transplantation of tumor cells into congenic recipients could direct similar changes in T cell subset distribution in recipient mice. Because memory and effector T cells represent antigen-experienced cells, we analysed the complementarity determining region 3 of the T cell receptor of purified $CD4^+$ and $CD8^+$ populations to determine the degree of clonality of the various T cell families. More oligo- and monoclonal T cells were observed in the infiltrated organs of leukaemic mice as well as the transplanted recipients. Thus, in many aspects, the TCL1a tg mouse recapitulates many of the T cell abnormalities observed in CLL patients, particularly those with the unmutated subtype. Our results demonstrate a crosstalk between B-CLL-like cells and the microenvironment T cells in this murine model that support the significance of this model in the study of the immune dysfunction in CLL.

6.4

IMMUNOGLOBULIN G SUBCLASS DEFICIENCY IS MORE COMMON THAN HYPOGAMMAGLOBULINAEMIA IN CHRONIC LYMPHOCYTIC LEUKAEMIA AND IS ALSO ASSOCIATED WITH AN INCREASED INCIDENCE OF RECURRENT INFECTION

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Hypogammaglobulinaemia is a common complication of Chronic Lymphocytic Leukaemia (CLL) with an incidence that varies considerably in the reported literature. Immunoglobulin replacement therapy has documented benefit for patients with hypogammaglobulinaemia, CLL and recurrent episodes of infection. Very little data exists on IgG subclasses in CLL. We measured IgG subclasses together with immunoglobulins G, A and M, protein EPG and immunofixation in a cohort of patients (both treated and untreated) with CLL representing all disease stages and with a variable history of infection, to analyse the implications of IgG subclass deficiency in the disease. There were 155 patients analysed with 89 males and 66 females with mean age 67.4 (range 21-95) years. Distribution by Binet Stage was A - 107, B - 38, C - 10. There were 111 untreated patients and 44 patients who had received chemotherapy, 21 received fludarabine based therapy and 11 received therapy which included rituximab (all FCR, 2 with lumiliximab). Five patients had received prior intravenous gammaglobulin (IVIg) and were excluded from further analysis. In the remaining 150 patients low immunoglobulin levels were as follows: IgG <6.0 g/L - 46 (30.6%); IgA <0.69 g/L - 46 (30.6%); IgM <0.5g/L - 87 (58%). IgG subclass deficiency was as follows: IgG1 <4.0 g/L - 39; IgG2<1.3 g/L - 28; IgG3 <0.4 g/L - 79; IgG4 <0.05 g/L - 35, with a total of 97/150 patients (64.6%) having a deficiency of at least one IgG subclass. IgG subclass deficiency was seen in 52 (35%) patients who had a normal total IgG level >6.0g/L. IgG subclass deficiency with recurrent episodes of infection was seen in 24 patients (16%) of whom 13 had hypogammaglobulinaemia with total IgG <6.0 g/L. Eleven patients had IgG subclass deficiency with recurrent infections but a normal total IgG level (>6.0 g/L). Entirely normal immunoglobulin levels (IgG, A, M, IgG subclasses, and no paraprotein) were seen in only 26 (17%) patients, and none of these patients had recurrent infections. The incidence by clinical stage, infection and IgG subclass deficiency are shown in the table. Chemotherapy exposure correlated strongly with clinical stage and disease progression and a separate treatment effect could not be discriminated in this series. Paraproteins were seen in 24 (16%) patients, 14 IgG, (3 IgG1, 2 IgG4, others indeterminate), 6 IgA and 4 IgM. Polyclonal hypergammaglobulinaemia was present in 5 patients, 3 of whom had predominantly an increased IgG2. Analysis of immunoglobulin levels and IgG subclasses in a cohort of patients with variety of clinical stage shows IgG subclass deficiency is common, especially in advanced Stage B and C disease. All combinations of IgG subclass deficiency may be seen in CLL. IgG subclass deficiency (64.6%) is more common than low total IgG (30.6%) and the rate of infection (16%) but is also associated with an increased risk of infection. All recurrent infection patients had an IgG subclass deficiency. In this cohort, the risk of recurrent infection was absent with normal Ig and subclass levels, but with hypogammaglobulinaemia was 1:2 and with IgG subclass deficiency was 1:4.

Table.

Stage	*No. patients*	*Infection*	*% infection*	*No. with IgG subclass deficiency*
A	106	10 yes 96 no	9.4%	All (100%) 50 (52%)
B	37	11 yes 26 no	29.7%	All (100%) 19 (73%)
C	7	3 yes 4 no	43%	All (100%) All (100%)

6.5

CHRONIC LYMPHOCYTIC LEUKAEMIA DRIVES THE PRODUCTION OF T REGULATORY CELLS FROM THE CD4+ CD25- COMPARTMENT WITH A CD27HIGH/CD127 LOW PHENOTYPE

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Patients with chronic lymphocytic leukaemia (CLL) have defects in both cellular and humoral immunity including changes in the numbers and function of T regulatory cells (Tregs). The identification of Tregs is an ever-evolving field and in this study we readdressed the phenotype using the markers CD25, FoxP3 and CD127-/lo and confirmed function by classical suppressor assays in CLL patients on and off treatment. Using the combination CD4 and FoxP3 we observed increased Treg frequencies in CLL patients, in particular with advanced disease, supporting previous studies showing an increase in Tregs in CLL. However in contrast to previous studies, there was no increase in the $CD25^+FoxP3^+$ population in CLL patients rather the increase in FoxP3 expression occurred in the $CD25^-$ compartment of CLL patients. Interestingly CLL induced a 7-fold increase in the expression of FoxP3 in $CD4^+CD25^-$ T cells following short-term co-culture. The T regulatory cells in CLL patients had a significantly higher expression of CD27 compared to healthy controls and although CD127 expression was low in both healthy and CLL patients it was significantly lower in CLL patients. Fludarabine treatment initially induced increased expression of FoxP3 in the $CD4^+$ T cell compartment but this declined gradually to reach levels below that pre-treatment. Here we propose that CLL drives the pro-

duction of Tregs from the CD4+CD25$^-$ compartment as has been shown recently in Non-Hodgkins Lymphoma (NHL) and the mechanism of induction could provide alternative avenues for treatment.

6.6

CHRONIC LYMPHOCYTIC LEUKEMIA -ASSOCIATED PURE RED CELL APLASIA AND RITUXIMAB

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PRCA is a rare and often life threatening complication of CLL. Corticosteroids, recombinant-erythropoietin (r-Epo) and packed red cell transfusions are frequently given. We report 6 patients (mean age 66 yr; range 40-78 yr) affected by acquired PRCA complicating CLL, recently seen at our Institutions. Two patients developed PRCA after rituximab, fludarabine and cyclophosphamide (R-Flu-Cy) therapy (first and fourth cycle, respectively), and treated with corticosteroids and r-Epo. Of them, one patient responded to therapy, one other died after 3 months without response. The remaining 4 patients were treated with rituximab. Five out of 6 patients were given packed red cell transfusions. Steroids and r-Epo were also administered as first-line therapy and only 1 patient showed a response. After a mean time of 57 days (range 23-62 days) from PRCA diagnosis, 4 non responding patients received rituximab at a dosage of 375 mg/m^2/week for 4 consecutive weeks. First injection side effects of rituximab were minimal. All patients showed an increase in hemoglobin levels in response to rituximab, in 1 patient just after the first dose, in another patient after the second and in 2 other patients after the third dose. Three patients (75%) were considered in complete remission (CR) and one patient (25%) in partial remission 4 weeks after the last rituximab infusion, despite a CR was obtained later (16 weeks following the beginning of the therapy). At the last follow-up (mean 18.5 months, range 2-60 months), all patients were alive and in continue CR. Despite very limited in number, these results suggest that rituximab is very effective in the treatment of PRCA complicating CLL. Why in some cases rituximab have not a protective role for the onset of a PRCA in CLL (2 cases developing the disease after R-Flu-Cy) need to be better elucidated.

6.7

THE PROGNOSTIC SIGNIFICANCE OF AUTOIMMUNE CYTOPENIA IN PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKEMIA (CLL)

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Clinical staging systems are the backbone for assessing prognosis in patients with CLL. Clinical stages, however, are assigned without taking into consideration the mechanisms of the disease. In this regard, the prognosis of patients with advanced (Binet C, Rai III, IV) stage disease due to immune cytopenia is controversial. The case for considering stage C "immune"as having a better prognosis than stage C due to bone marrow infiltration was first raised by M.M. Hansen more than 20 years ago (Scand J Haematol, 27: 279, 1986). To address the prognosis of patients with CLL in advanced clinical stage due to immune mechanisms (stage C "immune"), we studied two cohorts with and without autoimmune cytopenia. The first cohort consisted of 52 patients (36 men, median age 64 yrs; range 33-89) with advanced stage due to autoimmune cytopenia (C "immune") (37 positive direct antiglobulin test (DAT) hemolytic anemia (AIHA) with hemoglobin <10 g/dL and 16 with immune thrombocytopenia (ITP), <100.000/mm^3 platelets with normal megakaryocytes in bone marrow or no reticulocytopenia). The second cohort included 55 patients (36 men, median age 65 yrs; range 37-90) with stage C and negative DAT. Demographics, clinical characteristics and follow-up were similar in both groups. When considered from time of diagnosis, patients who present with stage C "immune" had a significant better survival than those in stage C due to bone marrow infiltration (median survivals, 109 months vs. 44 months ; p=0.05). In contrast, the prognosis of 12 patients who developed immune cytopenia during the course of the disease was not different to that of 25 patients who had progressed to stage C with no evidence of autoimmunity (median survivals, 110 months vs. 129 months, p=0.39). When the analysis was restricted to the 52 patients with autoimmune cytopenia, no significant differences in survival were observed according to the time at which the autoimmune disorder was detected, i.e. at diagnosis or during the course of the disease (median survivals, 108 months vs.110 months, p=0.41). In summary, this study shows that the outcome of patients who present with advanced clinical stage differs according to the origin of the cytopenia, i.e., immune or infiltrative. The most likely reason for this is that many patients with autoimmune cytopenia are down-staged as a result of the treatment with corticosteroids. These results also emphasize the importance of determining the origin of cytopenia when evaluating patients with CLL and "advanced" clinical stage.

6.8

SEQUENTIAL ANALYSIS OF DISEASE PROGRESSION AND IMMUNE SUPPRESSION IN CLL-TYPE MONOCLONAL B-CELL LYMPHOCYTOSIS

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Individuals with CLL-type MBL are at risk of developing progressive CLL that requires treatment but most are elderly and have additional healthcare issues. In order to provide safe monitoring with a minimal impact on lifestyle we developed an outreach service. The aim of this study was to assess the safety and efficacy of an outreach monitoring service and investigate the incidence of immune suppression in CLL-type MBL cases. Service users were sent a pack containing information sheets, a symptom self-assessment questionnaire and blood tubes. Participants completed the questionnaire and had blood taken in their local community. Laboratory analysis, including flow cytometry to monitor B-cell and circulating plasma cell levels, was performed centrally and the results and symptoms reviewed. Any evidence of disease progression resulted in a recall to clinic. Outcome was reviewed for 141 individuals initially diagnosed with CLL-type MBL (n=85) or stage A CLL (n=56). The median number of follow-up assessments is 5 (range 1-10), with a median inter-assessment period of 6 months (range 1-12). During follow-up, 8/141 were returned to clinic monitoring: 7 developed progressive CLL, of which 5/7 were originally diagnosed with MBL, and 1/8 developed hairy cell leukaemia in addition to CLL. Six remain on clinic watchful waiting, one required treatment for progressive CLL and one developed metastatic pancreatic cancer. Six of 56 cases originally diagnosed with CLL showed progressively decreasing CLL counts and were reclassified as MBL. One individual died after developing rapidly progressive cerebral lymphoma and two individuals have died from unrelated causes. Overall, only two individuals had an interim referral, neither of which would have been detected more rapidly by clinic follow-up indicating that the outreach approach is safe. Nine of 85 CLL-type MBL cases progressed to CLL after a median 26.1 months from diagnosis (range 14.7-42.7 months) and a median 7.6 months from enrolment on Outreach program (range 0-21 months). None of these individuals have yet required treatment for progressive disease; 2 remain on outreach monitoring, 5 are seen in clinic; 2 died of unrelated causes. Normal B-cells were depleted in 41/85 MBL cases: in 3/85 at all time points from enrolment, and 25/85 cases during follow-up at a median 9 months from enrolment (range 1-43 months). In the remaining cases the normal B-cell numbers was sub-normal at some but not all time points. Normal numbers of polyclonal B-cells persisted in 44/85 CLL-type MBL cases. Higher CLL counts were associated with lower levels of normal B-cells. Hypogammoglobulinaemia occurred in 18/85 cases: in 9/85 at all time points from enrolment, and 7/85 cases during follow-up at a median 13 months from enrolment (range 2-43 months). In the remaining cases hypogammoglobulinaemia occurred at some but not all time points. Immunoglobulin levels were normal at all time point in 67/85 CLL-type MBL cases. Outreach monitoring is safe, reliable and convenient and is greatly preferred by patients. In CLL-type MBL, suppression of normal B-cells is a frequent occurrence which develops over time and may be a significant cause of morbidity in this patient group.

Treatment (excluding Phase I/II)

7.1

TREATMENT OF AUTOIMMUNE CYTOPENIA COMPLICATING PROGRESSIVE CHRONIC LYMPHOCYTIC LEUKEMIA/SMALL LYMPHOCYTIC LYMPHOMA WITH RITUXIMAB, CYCLOPHOSPHAMIDE, VINCRISTINE, AND PREDNISONE

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Abstract. Autoimmune cytopenia is a potentially serious complication in 5-10% of patients with CLL. The choice of therapy can be difficult in patients who require concomitant treatment for autoimmune cytopenia and progressive CLL. Patients with autoimmune cytopenia caused by increased blood red blood cell destruction (autoimmune hemolytic anemia (AIHA)) and increased platelet destruction (immune thrombocytopenia (ITP)) often have partially compensated for the blood cell loss by increasing blood cell production and thus do not tolerate myelosuppression. In addition, use of purine analogue therapy, especially as monotherapy, can exacerbate autoimmune cytopenia. This retrospective study was conducted at Mayo Clinic Rochester with the approval of the Institutional Review Board and included all patients treated for both autoimmune cytopenia and progressive CLL from the beginning of 2002 to the end of 2008 with a rituximab, cyclophosphamide, vincristine, and prednisone (R-CVP) based therapy. Eighteen patients had been previously treated for autoimmune cytopenia with a median interval from their first treatment for AID to their first treatment with R-CVP of 20 months (range 1-40 months) and 15 patients had been previously treated for progressive CLL. Autoimmune cytopenia responded to treatment in 19 patients (14 CR and 5 PR). The median time to progression (TTP) was 13.6 months (range 5-29). Six responding patients required maintenance corticosteroid therapy after completing R-CVP. Five patients remain in sustained remission at last follow up (range 15-30 months) and 4 patients have had recurrent autoimmune cytopenia requiring re-treatment. Progressive CLL responded to treatment in 17 patients (9 CR/CCR, 8 PR). The time to progression for responding patients was 6.3 months (range 1-40 months) and 14 patients have required additional treatment for CLL at a median of 5.4 months (range 4-22 months). Five patients have not required additional treatment for either CLL or autoimmune cytopenia at 15-30 months after completing treatment. Four patients did have significant infections (pneumonia, grade 3, n =2 and C. difficile diarrhea, grade 2, n = 1). No patient required red blood cell or platelet transfusions after the completion of cycle 1 of therapy. Vincristine was not given to 4 elderly patients and one patient with diabetic neuropathy. Vincristine was stopped in 2 patients due to the development of peripheral neuropathy. Our experience shows that many patients with progressive CLL complicated by autoimmune cytopenia can benefit from treatment with R-CVP. However, the duration of response to this treatment remains suboptimal and additional interventions should be developed to improve management of this problem.

7.2

CLADRIBINE PLUS CYCLOPHOSPHAMIDE VERSUS FLUDARABINE PLUS CYCLOPHOSPHAMIDE IN PREVIOUSLY UNTREATED CHRONIC LYMPHOCYTIC LEUKEMIA: FINAL REPORT OF PHASE III RANDOMIZED STUDY (PALG-CLL3 STUDY)

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In the PALG (Polish Adult Leukemia Group) CLL3 prospective randomized trial we compared the efficacy and toxicity of cladribine (2-CdA) and cyclophosphamide (CC) with fludarabine and cyclophosphamide (FC) in untreated progressive CLL. CC protocol consisted of 2-CdA given at a dose 0.12 mg/kg in 30 min i.v. infusion for days 1 - 3 and cyclophosphamide at a dose of 250 mg/m^2 i.v. in days 1 - 3. In FC protocol FA was administered at a dose of 25 mg/m^2 in 30 min i.v. infusion in days 1 - 3 with cyclophosphamide at a dose 250 mg/m^2 i.v. in days 1 - 3. Both regimens were repeated every 28 days for up to six courses. No routine, prophylactic antibiotics, antiviral agents or growth factor administration was planned. Study end points were complete response (CR), overall response (OR), minimal residual disease (MRD), progression-free survival (PFS) , overall survival (OS) and toxicity. From January 2004 to May 2007, 423 patients were enrolled, and 395 patients were evaluated in the final analysis. At the time of this report, the median follow-up among the censored patients was 3 years 2 months. We found comparable responses to CC and FC. The complete response rates reached 47% and 46% (p=0.25), and overall response rates were 88% and 82% (p=0.11) in CC and FC arm, respectively. The flow cytometry assessment of MRD was performed in 109 patients out of 183 patients who obtained CR, and was negative in 77 (71%) patients. MRD eradication was associated with longer PFS as compared to patients in CR with detectable MRD. Progression-free survival (PFS), overall survival (OS) and grade 3/4 treatment-related toxicity did not differ between study arms.

Table.

Characteristic	*CC arm*	*FC arm*	*p*
Pts enrolled	211	212	-
Pts evaluated	192	203	-
Median age (range), years	58 (37-81)	59 (27-81)	0.40
Time since diagnosis, months	1.1 (0-208)	1.6 (0-263)	-
Rai stage, no (%)			-
0-progressive	5 (2.6)	6 (3.0)	-
I	39 (20.3)	45 (22.2)	-
II	88 (45.8)	84 (41.4)	-
III	23 (12.0)	25 (12.3)	-
IV	37 (19.3)	43 (21.2)-	
CR	47%	46%	0.25
OR	88%	82%	0.11
PFS (median, years)	2.34 (95%CI = 2.03-2.64),	2.27 (95%CI = 1.99-2.54	0.51
Four-year OS	62.4% (95%CI = 53.2%-70.8%)	60.6% (95%CI = 52.9%-67.8%)	
Gr3/4 Neutropenia	39 (20%)	43 (21%)	0.81
Gr3/4Thrombocytopenia	24 (12%)	22 (11%)	0.62
Gr3/4 Anemia	21 (11%)	18 (9%)	0.50
Gr3/4 Infections	53 (28%)	54 (27%)	0.84
AIHA	19 (10%)	14 (7%)	0.30
ITP	14 (7%)	8 (4%)	0.16
Secondary neoplasms	6 (3%)	11 (5%)	0.26

The activity of CC and FC regimens was compared within patient subgroups characterized by clinical and laboratory parameters with the potential to influence CLL prognosis. No significant differences concerning proportions of responders to CC and FC were found in patients stratified according to age, sex, clinical stage, Döhner's hierarchical cytogenetic group, CD38 expression, β-2 microglobulin plasma level and pattern of bone marrow infiltration. The prognostic factors that were

associated with short PFS in univariate analyses included presence of deletion 17p13 or 11q22 (p=0.016), elevated β-2 microglobulin (p=0.013), Rai III-IV clinical stage (p=0.001), CD38 expression ≥30% (p=0.004) and a diffuse bone marrow infiltration pattern (p=0.008). Most importantly, the activity of both regimens was unsatisfactory in patients with deletion 17p13 (TP53 gene) who had significantly shortened survival in both study arms. In conclusions, no important differences in efficacy and toxicity were found between CC and FC regimens. Supported in part by Grants 4P05B0619 and 2P05B01828 from The Ministry of Science, Warsaw, Poland and a grant from The Medical University of Lodz, Poland (No 503-1093-1) .

7.3

RITUXIMAB IN COMBINATION WITH DEXAMETHASONE OR HIGH-DOSE METHYLPREDNISOLONE IN REFRACTORY CHRONIC LYMPHOCYTIC LEUKEMIA

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Background. The optimal salvage treatment for patients with refractory CLL remains undefined. Currently, regimens based on high-dose steroids seem to be a promising treatment option for these patients. High-dose methylprenisolone plus rituximab (R-HDMP) is the most often used regimen; first reports have been published for dexamethasone plus rituximab (R-Dex) regimen, which should be less toxic and immunosuppressive than R-HDMP. Very important consideration in relation to R-HDMP and R-Dex regimens is that these should be effective in CLL patients with p53 defects. *Aims and Methods*. In order to assess the efficacy of R-HDMP (rituximab 375-500 mg/m^2, day 1; HDMP 1 g, day 1-5) and R-Dex (rituximab 500 mg/m^2, day 1, 8, 15, 22; dexamethasone 40 mg day 1-4 ± day 15-18) regimens in refractory CLL, we performed a retrospective analysis of all patients treated using these regimens at the University Hospital Brno and University Hospital Hradec Králové since April 2006. *Results.* These regimens were indicated in 22 patients (R-HDMP n=8, R-Dex n=14; 8 women, 14 men) with a median age of 65 years (44-81). Rai stage at the start of therapy was as follows: 0, n=1; I, n=1; II, n=2; III, n=7; IV, n=11. Autoimmune hemolytic anemia was indication for treatment in 4 patients; other signs of progression according to NCI-WG criteria were indication for treatment in the others. IgVH genes were mutated in 5 patients (VH3-21, n=3), unmutated in 15 patients, in 2 patients not available; cytogenetic aberrations detected by FISH revealed del 17p in 4 patients; del 11q in 7 patients; del 13q in 8 patients; trisomy 12 in 5 patients. Median number of previous therapies was 2 (0-9). Median of administered R-Dex or R-HDMP cycles was 2 (1-6). The effect of R-Dex in patients without hemolysis (7 evaluated patients) was as follows: complete remission (CR), n=0, partial remission (PR), n=4 (57%); stable disease (SD) n=3 (43%). All 4 patients receiving R-Dex for hemolysis achieved complete resolution of hemolysis (100%). The effect of treatment in R-HDMP patients (6 evaluated patients) was as follows: complete remission (CR), n=0, partial remission (PR), n=2 (33%); stable disease (SD) n=1 (17%); progression n=3 (50%). Four patients died during the course of treatment (infection, n=2; CLL progression, n=1; sudden death, n=1). Median time to progression for patients reaching CR or PR was 5 months. Progression was not registered only in patients treated for hemolysis (median follow-up, 5 months). None of the patients with del p53 achieved CR or PR. Grade III or IV infections were seen in 7 patients (32%), steroid diabetes in 4 patients (18%), rituximab infusion-related side effects in 2 patients (9%) and tumor lysis syndrome in 1 patient (5%). Complications were observed equally for both treatment regimens. *Conclusions.* Our pilot study shows that R-Dex and R-HDMP regimens are feasible in patients with refractory CLL. However, treatment toxicity, especially opportunistic infections, may represent a serious issue. In addition, significant and long-term disease control could be expected in a minority of patients only. Therefore, in younger and fit patients, they should be used as a bridge towards allogeneic stem cell transplantation. On the other hand, these protocols appear to be highly effective in CLL with autoimmune hemolysis. *Acknowledgements*. Supported by research project MZO 00179906 from Ministry of Health, Czech Republic, by research grant MSM 0021620808 and by the Czech Leukemia Study Group for Life.

7.5

IMMUNOCHEMOTHERAPY WITH FLUDARABINE (F), CYCLOPHOSPHAMIDE (C), AND RITUXIMAB (R) (FCR) VERSUS FLUDARABINE (F), CYCLOPHOSPHAMIDE (C) AND MABCAMPATH (CAM) (FCCAM) IN PREVIOUSLY UNTREATED PATIENTS (PTS) WITH ADVANCED B-CHRONIC LYMPHOCYTIC LEUKEMIA (B-CLL) : EXPERIENCE ON SAFETY AND EFFICACY WITHIN A RANDOMISED MULTICENTER PHASE III TRIAL OF THE FRENCH COOPERATIVE GROUP ON CLL AND WM (FCGCLL/MW) AND THE "GROUPE OUEST-EST D'ETUDES DES LEUCÉMIES AIGÜES ET AUTRES MALADIES DU SANG" (GOELAMS): CLL2007FMP (FOR FIT MEDICALLY PATIENTS)

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Introduction. Recent data suggest that FCR immunochemotherapy improves response rates and Progression-Free Survival (PFS) of previously untreated CLL pts. The monoclonal antibody alemtuzumab, a humanized anti-CD52 antibody (Campath), has shown activity alone and in combination in CLL pts. In order to validate the place of Campath in combination with the synergic association FC, the FCGCLL/MW and the GOELAMS conducted a multicenter French and Belgian phase III trial, CLL2007FMP, to evaluate the efficacy and toxicity of FCCam versus FCR in previously untreated patients with advanced CLL. PFS was the primary-end-point of this trial. *Methods and Patients.* A cohort of 178 fit medically pts (cumulative illness rating scale (CIRS) score of up to 6), less than 65 years old, without 17p deletion, were enrolled between November 2007 and January 2009. Pts were randomly assigned to receive 6 oral courses of FC (F 40 mg/m^2 d1-3 and C 250 mg/m^2 d1-3; q 28 days) in combination with either R (N=83; 375 mg/m^2 i.v. d 0 at first cycle and 500 mg/m^2 d1 all subsequent cycles; q 28 days) or Cam (N=82; 30 mg s/cut d1-3; q 28 days). Patients were stratified according to IgVH mutational status and 11q deletion. Anti-infective prophylaxis included trimethoprim-sulfamethoxazole and valaciclovir during immunochemotherapy and until the CD4$^+$ lymphocyte count reached 0,2x10^9/L. In the FCCAm arm, CMV monitoring was monthly performed by either PCR or antigenemia. The use of GSCF was recommended. The trial's recruitment was stopped in January 2009 after 165 pts had been randomized (83 and 82 respectively in the FCR and FCCam arms) because of an excess of mortality in the FCCam arm, while 13 patients were enrolled but not randomized because of this decision. *Results.* We reported here a preliminary analysis including baseline characteristics and response rates in the first 100 included pts but safety analysis of the whole cohort ; among the first 100 pts, 81% were Binet B, 19 % Binet C; median age was 56.8 years (range 52.8 to 60.6). Percentages of pts with 11q deletion, unmutated IgVH status, and elevated 2m, were 18.5%, 48.4%, and 77.6%, respectively. A number of 76.5% (FCR arm) and 71.4% (FCCam arm) of pts received 6 cycles. Reasons for discontinuation were mainly related to persistent grade 3-4 neutropenia. Safety analysis data were available for 165 pts. Number of patients reported with Common Toxicity Criteria (CTC) grade 3-4 adverse events were observed in 87.8% (FCCam arm) versus 90.2% (FCR arm) (p= 0.76). Grade 3-4 neutropenia was the most frequently reported adverse event (79.6% with FCCam and 74.6% with FCR arm). Interestingly, percentage of observed grade 4 neutropenia was stable during FCR treatment (17.6% for cycle 1 and 17.9% for cycle 6) but increased during FCCam treatment (28.4% for cycle 1 and 45.5% for cycle 6). A total of 63 Serious Adverse events (SAE) were declared (19 with 18 pts in FCR arm, and 44 with 35 pts in FCCam arm) consisting mostly of the cases in febrile neutropenia (13 with FCR arm, 27 with FCCam arm); 7 patients died, all in the FCCam arm : 3 of B diffuse large B-cell lymphoma (one of them EBV positive), 1 of mucormycosis, 1 of septic shock due to P.aeruginosae and 2 of heart failure during neutropenia. The Overall Response Rate (ORR) in the first 100 patients was 96% in the FCR arm compared to 85% in the FCCam arm (p=0.086). The Complete Response rate was 78% (FCR arm) versus 58% (FCCam arm) (p=0.072). PFS and OS are not yet evaluable. *Conclusion.* the FCCam regimen for the treatment of advanced CLL appeared to be associated with an unfavourable safety profile representing a significant limitation of its use in this indication. Other combinations with Alemtuzumab may be studied.

7.6

NO CLINICAL RELEVANCE OF IMAGING TECHNIQUES IN TREATMENT OF PATIENTS WITH ADVANCED CHRONIC LYMPHOCYTIC LEUKEMIA (CLL): RESULTS OF A METAANALYSIS OF THE GERMAN CLL STUDY GROUP (GCLLSG)

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Introduction. Both, the Rai and Binet staging systems, are based on physical examination and peripheral blood count. According to the recently updated IWCLL guidelines for CLL (Hallek *et al.,* Blood, 2008) no radiological examinations are needed for staging and response evaluation outside clinical trials. However, it is still unclear, how much additional information can be gained by using CT scans by routine at pretherapeutic evaluation and during follow-up phase. The GCLLSG initiated a metaphase analysis based on the results of three phase III studies (CLL4, CLL5 and CLL8 protocol of the GCLLSG), in order to evaluate the clinical relevance of CT scans on disease outcome. *Patients.* A total of 1372 patients (pts) receiving first line therapy for CLL within a phase III trial of GCLLSG were included in this analysis. 362 pts younger than 65 years were included in the CLL4 trial, 193 pts older than 64 years in the CLL5 trial and 817 pts with normal renal function and a comorbidity score < 6 were included in the CLL8 trial. 100 pts received chlorambucil, 257 fludarabine (F), 590 F plus cyclophosphamide (FC) and 408 FC plus rituximab. Response as well as progression during later follow-up was assessed according to the NCI-WG criteria (Cheson *et al.*, Blood 1996). CT scan or other radiological examinations were not performed by routine, but were recommended at pretherapeutic staging, interim and final staging and during the follow-up phase, if clinically indicated. *Results.* The median overall follow up time for all pts alive was 31 months. During follow-up CT scans were performed in 569 pts, ultrasound examinations in 936 pts and chest x-rays in 606 pts, overall any imaging technique in 1089 pts. In a first step we evaluated the number of pts for whom the radiological examination was crucial in order to reconcile progressive disease (PD) according to the NCI criteria. A total of 491 pts were considered as progressive during treatment or follow-up. In 100 pts the definition of PD by the treating physician could not be confirmed by a strict, independent application of the NCI-WG criteria and were therefore excluded from the analysis. In the remaining 391 pts PD was clinical apparent in 326 (83%), while CT scans were relevant for the decision of PD in 39 pts (10%) only and ultrasound in 26 pts (7%). In a next step we assessed the number of pts for whom CT scan or ultrasound had a clinical consequence by initiating relapse treatment because of bulky disease (defined as a lymph node of > 5 cm) in the thorax or abdomen. Out of 176 pts receiving relapse treatment due to PD, in only 2 pts (1%) the retreatment decision was based on the result of the CT scan or ultrasound: in these pts a new bulk was detected by radiological examination, while physical examination and blood count were normal. Moreover, an initially detected bulky disease by radiological examination had no prognostic value as well. In 721 of 1371 pts (52%) a CT scan was performed initially. Additionally or alternatively an ultrasound was performed in 996 pts (73%) at pretherapeutic staging. Interestingly, no significant difference in overall response rates (86% versus 89%, p=0.2), as well as progression free survival (36 versus 39 months, p=0.2) and overall survival (OS) (median OS not reached vs. 85 months, p=0.08) was assessed. *Conclusion.* These data from 1089 pts included in three prospective phase III trials show that the use of imaging techniques has no apparent clinical benefit for the evaluation of PD or the decision to initiate relapse treatment in the follow-up phase of CLL. Our data suggest that physical examination and blood count remain the methods of choice for clinical follow-up of patients with CLL.

7.8

THE SAFETY AND TOLERABILITY OF ORAL FLUDARABINE, ± ORAL CYCLOPHOSPHAMIDE AND IV RITUXIMAB THERAPY IN PREVIOUSLY UNTREATED PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKAEMIA (CLL) AGED ?65 YEARS – PRELIMINARY PROGRESS REPORT FROM THE AUSTRALASIAN LEUKAEMIA AND LYMPHOMA GROUP (ALLG) AND CLL AUSTRALIAN RESEARCH CONSORTIUM (CLLARC) STUDY

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Introduction. Recent trials show that combination therapy with fludarabine (F) and cyclophosphamide (C) gives superior progression free survival (PFS) compared to F or chlorambucil alone (UK CLL4). More recently the CLL8 Study showed that adding rituximab (FCR) improved PFS compared to FC. The median age of patients in these studies was 65 and 61 years respectively while the median age of patients with CLL is 72 years. There is ongoing debate regarding the tolerability and safety of FCR therapy in elderly patients. Arbitrary dose reduction appears to be common practice in older patients. Oral FC therapy is more convenient for many older patients. *Methods.* Previously untreated patients with progressive CLL aged ≥65 were randomised to one of three treatment regimens: (i) F 24 mg/m^2 po D1-5 + rituximab (R) 500 mg/m^2 iv D1 (FR5), (ii) F 24 mg/m^2 po and C 150 mg/m^2 po D1-3 + R iv D1 (FCR3) or (iii) F 24 mg/m^2 po + C 150 mg/m^2 po D1-5 + R iv D1 (FCR5), all given at monthly intervals for 6 cycles. The total per cycle dose of F and C with FCR5 are identical to the doses of those drugs in UK CLL4 (which did not include R). In order to assess safety and tolerability, patients were administered the dosage for their arm of the study with no dose reduction. Therapy was delayed by 2 weeks if there was any grade 3 or 4 toxicity. If grade 3 or 4 toxicity was unresolved after 2 weeks, patients are taken off study. If the toxicity resolves to grade 2 or less, therapy proceeded. *Results.* The study has been open to accrual for 8 months and 29 patients of a planned 120 have been enrolled. The median age is 72.1 (range 65-84 years). Randomisations are FR5 – 9, FCR3 – 11 and FCR5 – 9. Of 12 patients who commenced treatment ≥6 months ago, 4 have completed all 6 cycles of therapy, 4 ceased early due to toxicity and 4 remain on therapy with delays due to toxicity. A total of 11 of 28 (39%) evaluable patients had toxicity related treatment delays, 9 haematological with neutropenia and / or thrombocytopenia. One patient was taken off study for treatment associated episodes of amnesia. There have been no deaths on study, but one patient died 2 months after cessation of therapy from infection. Of 17 patients evaluable for response (some with therapy not yet completed), there are 6 clinical CR and 11 PR. CONCLUSION In general, administration of oral F(C)R therapy appears tolerable in patients requiring treatment aged ≥65 years. Delay in therapy due to grade 3 or 4 toxicity for <2 weeks has been seen in 39% and 4 of 12 evaluable patients required cessation of therapy for grade 3 or 4 toxicity lasting >2 weeks. Responses rates are high with all treated patients having obtained an objective response. Accrual is ongoing.

7.9

FLUDARABINE, CYCLOPHOSPHAMIDE, AND ALEMTUZUMAB(FCC)COMBINATION IN PREVIOUSLY TREATED PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKEMIA (CLL)

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Fludarabine (F) in combination with cyclophosphamide (C) seems to have a relevant advantage over single-agent F in patients with relapsed CLL who had previously received alkylators or alkylators plus F, but are still F sensitive. Although minimal residual disease (MRD) remains detectable either by flow cytometry or by PCR for IgVH gene mutations or rearrangements in many patients achieving a CR, the combination of F and C seems to reduce MRD more efficiently. Still, patients in CR eventually relapse and require treatment, demonstrating the need for improved treatments able to further reduce or eliminate MRD and

induce "better quality" and thus more durable responses. Monoclonal antibody alemtuzumab (CAM), directed towards CD52 antigen expressed on the surface of B-CLL cells acts synergistically with F *in vitro* and appears to have synergistic activity *in vivo*. Additionally, CAM is highly effective at clearing disease from bone marrow, the usual site of residual disease following purine analogue-based. Therefore, we designed a phase II study to determine the efficacy and safety of a 4-weekly combination regimen consisting of F, C, and CAM (FCC). The study population is represented by patients with CD52-positive B-CLL with relapsed or refractory disease after at least one line of treatment. Since subcutaneous CAM administration has a better side effect profile, greatly reducing the discomfort for the patients seen with intravenous administration we adopted the subcutaneous route of administration of CAM in this trial . Objectives of this study were to evaluate the feasibility, overall response rate (ORR), duration of response (DR), and the presence of MRD following treatment with FCC. MRD was measured by 4-color flow cytometry in the bone marrow. Patients received FCC therapy after a short period of CAM dose escalation on 2 consecutive days. The FCC regimen consisted of FAMP 40 mg/m^2/day os (Days 1–3), CTX 250 mg/m^2/day os (Days 1-3) immediately followed by alemtuzumab 10 mg sc (Days 1-3). This combination was repeated on Day 29 for up to 6 cycles. According to the safety profile of the schedule described above the dose of CAM was increased after the first cohort of 10 treated patients from 10 mg to 20 mg. Currently, 39 patients have been enrolled in this trial. The median age of the patients was 59.0 years (range, 42-79), 23/16 (59%) were male, 37/39 (94%) had Binet stage B and C disease, and the median number of prior treatment regimens was 2 (range, 1-5). Twelve patients received monoclonal antibodies as previous treatment: Rituximab, in 7 cases and CAM in 5 cases. In 10/31 (32%) patients 17p deletion was detected. IgVH unmutated was observed in 23/31 (74%) patients. At the moment of writing 31 patients are eligible for evaluation of toxicity and response. The ORR was 71%, with 11 (35.5%) patients achieving a complete response (CR) and 11 (35.5%) patients a partial response (PR). Four patients had stable disease (SD), while 5 showed progression of the disease (PD). MRD negativity was achieved in the bone marrow of 6/22 (27%) patients. Grade III-IV neutropenia episodes were observed in 37% of the administered courses while grade III-IV thrombocytopenia episodes were detected only in 9.0 % of cycles. Six major infections were recorded: two sustained by Staphylococci (sepsis), one Mycobacterium tuberculosis (lung), one by Nocardia (lung) one by E. coli (sepsis) and one by Pseudomonas aeruginosa. One patient reactivated HBV while treated by Lamivudine. The patient with pneumonia due to M. tuberculosis died because of respiratory failure. CMV reactivation occurred in 6 patients: no CMV disease was recorded. After a median follow up of 16.5 months 25% of patients didn't progressed. Median time to progression was 14 months (2-39). In conclusion, results from the interim analysis of this new, 4-weekly dosing FCC regimen suggest that combination therapy with F, C and CAM is feasible, safe, and effective in patients with adverse prognostic factors who received multiple lines of treatment.

7.10

A COST-EFFECTIVENESS ANALYSIS OF THE TREATMENT OF RELAPSED/REFRACTORY CHRONIC LYMPHOCYTIC LEUKAEMIA (CLL) WITH RITUXIMAB IN COMBINATION WITH FLUDARABINE AND CYCLOPHOSPHAMIDE (R-FC) VERSUS FLUDARABINE AND CYCLOPHOSPHAMIDE ALONE (FC) IN THE UNITED KINGDOM

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Introduction. Decision problem analysis is utilised in many countries like the UK to determine what treatment options are not only clinically effective but also a cost-effective use of finite healthcare resources. This requires economic modelling to infer the potential lifetime costs and benefits associated with different treatments. This analysis assesses whether R-FC compared to FC offers a cost-effective treatment option for relapsed/refractory CLL patients in the UK, using evidence from the phase III RCT, BO17072 (REACH). *Methods.* The REACH trial demonstrated a statistically significant and clinically meaningful benefit for R-FC compared to FC in patients with relapsed/refractory CLL, prolonging median progression-free survival (PFS) by 10 months (20.6 months for FC; 30.6 months for R-FC). This important benefit was achieved with a manageable tolerability burden and no unexpected safety signals. A cost-effectiveness analysis was conducted with patients modelled to be in one of three health states; PFS, Progressed or Death. The best parametric fit (Weibull) was used to extrapolate PFS beyond the end of the REACH trial follow-up period (2.1 years) to a life-time horizon. Because median overall survival was not reached in REACH, a Markov process was used to model the transition from the progressed health state to death. Predicted time in each health state was weighted using CLL utility scores based on expert opinion to account for patient quality of life. Adverse events, blood transfusions, bone marrow transplants, and subsequent CLL treatments collected prospectively in REACH were included in monitoring costs. *Results.* The model predicted that R-FC compared to FC improves mean life expectancy by 0.670 years (95%CI=0.21-1.04 years) and quality-adjusted life years (QALYs) by 0.585 years (95%CI=0.21-0.92 years). Improvements in health outcomes were attributed to an increase in the time R-FC patients spent in the PFS health state (0.915 years, 95%CI=0.42-1.44 years). Total direct costs were higher for R-FC by £8,226(~9,500) per patient. The incremental cost-effectiveness ratio (ICER) was estimated to be £14,240(~16,500) per QALY, and ranged between £11,886(~13,700) and £21,589(~24,900) per QALY depending on the assumptions used, all below the UK willingness to pay threshold of £30,000(~34,500) per QALY. Although there is uncertainty associated with parameters used to describe the subsequent progression of CLL, the ICER was considered robust. The probability of R-FC not surpassing the £30,000(~34,500) per QALY threshold compared to FC was 94.4%. *Conclusions.* With the cost of healthcare continually rising, it is increasingly important for payors to consider the value of the products they reimburse. This study presents the results of an economic evaluation based on the standard methods utilised in the UK to inform such decisions. Based on the significant prolongation of PFS demonstrated in REACH, R-FC increased quality-adjusted life expectancy for relapsed/refractory CLL patients and is considered cost-effective by UK standards. Results based on the REACH trial are quite comparable to other indications previously evaluated in the UK for the use of rituximab in diffuse large B-cell lymphoma, follicular lymphoma, and first-line CLL which all resulted in cost-effectiveness ratios less than £20,000(~23,000) per QALY gained.

7.11

CYTOMEGALOVIRUS (CMV) REACTIVATION IN PATIENTS TREATED WITH ALEMTUZUMAB – QUANTITATIVE MONITORING IS IMPORTANT

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Background. Viral infections are the most frequent complications in pts. treated with alemtuzumab (CAM). Vast majority of these infections is represented by CMV reactivation. The standard of care during treatment with CAM includes CMV prophylaxis or preemptive approach with regular monitoring of virus reactivation (PCR or antigenemia) and early start of antiviral treatment. *Methods.* We performed retrospective analysis of CMV reactivations during CAM administration in pts. treated in our dept. between 2003-2008. Qualitative PCR was used for CMV monitoring until 2005 and then was replaced by quantitative real-time PCR (available samples from the period of qualitative monitoring were retested by real-time PCR). CMV reactivation was defined as follows: 2 consecutive PCR positive samples (when qualitative method was used) or one sample with more then 500 copies of CMV DNA per microgram of DNA (for quantitative PCR). *Results.* 98 pts were treated with CAM in the study period. In 73% (n=72) of cases CAM was used as the second/third line treatment. CMV reactivation occurred in 21 (22%) pts. There was not significant difference in frequency of CMV reactivation between pts. treated with CAM as the first versus the second/ third line therapy (23% versus 21%). However, only 50% of pts. treated with CAM as the first line therapy had quantitative PCR monitoring of viral load during their treatment compared to 86% of pretreated pts. Furthermore, median of CMV copies per positive sample was 208 in CAM first line treated pts. (retrospective analysis of PCR positive samples obtained before start of antiviral treatment in qualitative method) compared to 1006 in CAM second/third line treated pts. Frequency of CMV reactivation followed by antiviral treatment was higher between 2003-2005 (when qualitative criterion for CMV reactivation was used) compared to period of 2006-2008 (quantitative monitoring) – 48% versus 15%. Retrospective analysis of samples positive in qualitative PCR showed significantly lower viral load before start of antiviral treatment in the peri-

od of 2003-2005 compared to 2006-2008 when quantitative monitoring was used – 228 versus 1157 copies/microgram of DNA. CMV reactivation occurred with median of 4 weeks from the start of CAM therapy and after median dose of CAM 283 mg. 71% of CMV reactivations were asymptomatic, 19% were accompanied by fever. Only one case of probable CMV disease (colitis) and one episode of CMV primoinfection occurred in our study group. 40% of pts were treated with ganciclovir, 24% with valganciclovirem, 24% with ganciclovir followed by foscarnet and 12% only with foscarnet. Efficacy of antiviral therapy given with median of 15 days were prompt and in all pts. viral load rapidly decreased to zero. *Conclusion.* Even though CMV reactivation is the most frequent infection during CAM treatment, its course and severity is different from pts. after allogeneic HSCT. Quantification of CMV viral load is essential for correct decision about the start of antiviral treatment and/or CAM discontinuation. Interlaboratory comparison of CMV PCR quantitative methods and consensus in cut-off level of viral load for start of preemptive antiviral treatment could decreased the risk of CMV over treatment in pts. on CAM therapy.

7.12

FLUDARABINE, CYCLOPHOSPHAMIDE AND RITUXIMAB IN FIRST-LINE TREATMENT OF PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKEMIA: RETROSPECTIVE ANALYSIS OF CZECH CLL STUDY GROUP

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Background. Combination of fludarabine, cyclophosphamide and rituximab (FCR) is currently considered the first-line treatment of choice in physically fit patients (pts) with chronic lymphocytic leukemia (CLL) based on the results of CLL-8 study (Hallek *et al.*, 2008). Although higher dose of rituximab (500 mg/m^2 from 2nd cycle) was used in CLL-8 as well as landmark phase II study (Keating *et al.*, 2005), it is purely empirical and ideal rituximab dosing remains unknown. Furthemore, there is a very limited amount of data regarding the use of FCR in real practice. Aims: to perform a retrospective efficacy and safety analysis of FCR regimen used as routine first-line treatment in CLL. *Patients and Methods*. Between October 2002 and May 2008, we treated 107 pts with active CLL (68% males, median age, 60 years [range, 27-75]) by FCR regimen as first-line therapy at five centers cooperating within Czech CLL Study Group. Diagnosis of CLL, indication for treatment and assessment of response to therapy followed NCI-WG criteria. Patients received standard doses of fludarabine (25 mg/m^2 i.v. or 40 mg/m^2 p.o. d1-3) and cyclophosphamide (250 mg/m^2 i.v. or p.o. d1-3). Rituximab was administered i.v. on day 1 of each cycle at the dose of 375 mg/m^2 in all cycles (n=87) or 500 mg/m^2 from 2nd cycle (n=20). Treatment was repeated every 4 weeks. Antimicrobial prophylaxis and growth factors were not routinely used. Low/intermediate/high risk according to modified Rai staging was present in 1/72/27%. IgVH mutation status and FISH aberrations were available in 85% and 79% of pts. IgVH genes were unmutated in 74%; according to hierarchical model, del 13q was present in 31%, trisomy 12 in 9%, del11q in 26% and del17p in 8%. *Results.* At the time of analysis (February 2009), the median observation time was 22.3 months (mo). Median number of FCR cycles was 5. The overall response rate/complete response rates were 92/47%. Median PFS was 30 mo; median overall survival (OS) was not reached. Patients with unmutated IgVH genes had significantly shorter PFS (p=0.0051). Small numbers of pts in each FISH aberration group precluded a meaningful statistical analysis. Four out of 7 pts with del 17p responded to FCR (1x CR, 3x PR). Patients treated with lower dose of rituximab (375mg/m^2) did not have significantly different ORR, CR and PFS from those treated with 500 mg/m^2 (p=0.79, p=0.24 and p=0.65). Grade III/IV neutropenia occured in 22/14% of cycles and thrombocytopenia grade III/IV in 5/3% of cycles. Serious infections occured in 1% of cycles only. G-CSF was administered in 54% and recombinant erythropoietin in 13% of pts. *Conclusions.* Treatment of CLL patiens in first line with fludarabine, cyclophosphamide and rituximab resulted in high number of overall and complete responses despite unfavourable prognostic factors present in the majority of pts. Toxicity was acceptable and manageable. Further studies are needed to address the question whether lower dose of rituximab (375mg/m^2) in FCR yields the same therapeutical efficacy as 500 mg/m^2. *Supported by grant MSM 0021620808 from Ministry of Education and research project MZO 00179906 from Ministry of Health, Czech Republic.*

7.13

TREATMENT-RELATED MYELODYSPLASIA AND AML FOLLOWING FLUDARABINE COMBINATION CHEMOTHERAPY

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Background. Fludarabine combination chemotherapy achieves high response rates in CLL and indolent lymphoma. Fludarabine inhibits DNA repair and augments the cytotoxic effect of DNA damaging agents such as cyclophosphamide and mitoxantrone. This mechanism may also affect marrow progenitor cells to increase the risk of myelodysplasia and acute myeloid leukaemia (t-MDS/AML). *Aims*. To investigate the incidence and characteristics of t-MDS/AML after treatment with fludarabine in combination (F$^+$) for lymphoproliferative disorders and identify risk factors for its development. *Methods*. Review of the Peter MacCallum Cancer Centre Pharmacy database from 1996-2008 identified 187 patients with indolent lymphoproliferative disorders treated with fludarabine (F) combined with cyclophosphamide (C) and/or mitoxantrone (M) +/- rituximab (R) who have at least 6 months follow-up since starting treatment. Kaplan-Meier analysis was used to estimate MDS-free survival (MDSFS), defined as the time from first exposure to F in combination to onset of t-MDS/AML, censored at date of last follow-up or by death. The Mantel-Cox log-rank test was used to estimate the effects of patient characteristics on MDSFS, including age, gender, disease type, treatment with alkylators, anthracyclines or radiotherapy at other times, treatment with high dose chemotherapy and autologous stem cell transplantation, number of F containing treatment episodes, number of other cytotoxic therapies, and addition of M to the first F combination therapy. *Results*. 187 patients treated with F combination were followed for a median of 38 months (range 0-125). 122 patients (65.2%) were male and the median age was 60 years (range 26-85). 19 cases of t-MDS/AML have been identified for an overall rate of 10.2% (13 refractory cytopenia with multilineage dysplasia, 2 chronic myelomonocytic leukaemia, 1 refractory anaemia with excess blasts and 3 AML with multilineage dysplasia). Time of diagnosis from first F combination was a median of 42 months (range 5-99). The estimated MDS-free rate at 10 years was 55.7% (95% CI 27.6-80.6%). Median overall survival post-t-MDS/AML diagnosis was 11 months. Patients developing t-MDS/AML included 11/57 with FL (crude rate 19.3%), 5/85 with CLL (5.9%), and 3/25 with WM/MZL (12%). Most patients had other cytotoxic treatments (median 4, range 0-7) but 3 with FL had F combination as their only line of treatment. The type and number of other cytotoxic treatments were not significant risk factors for t-MDS/AML but of thirteen patients (7.0%) who received M with their first F combination, four (30.8%) developed t-MDS/AML. Median MDSFS was shorter for F+M (p=0.004), being 6.3 years compared to > 10.1 years without M. The MDS-free rate at 5 years was 60.0% for F+M compared to 92.2% without M. There was a trend towards females having a higher risk for t-MDS/AML (p=0.068). Karyotypic analysis of t-MDS/AML was typically complex with 13/16 accessible cases having cytogenetic aberrations. Abnormalities of chromosomes 7, 5 and 13 were prominent. *Conclusion*. Fludarabine combination chemotherapy is associated with a moderate risk of t-MDS/AML particularly when combined with mitoxantrone. This complication should be considered when evaluating the potential benefit of this treatment in lymphoproliferative disorders.

7.14

PHARMACOKINETICS (PK) OF ALEMTUZUMAB AFTER SUBCUTANEOUS (SC) ADMINISTRATION FOR CONSOLIDATION IN PATIENTS WITH CLL

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The pharmacokinetic approach is an important tool for the clinician to select patients who could benefit from more individualized administration schedules and to design new therapeutic regimens. We evaluated the PK profiles of Alemtuzumab (CAM) in 29 patients with CLL receiving the MoAb consolidation treatment, 10 mg three times/week for six weeks by subcutaneous administration, and investigated possible correlations between serum levels and clinical response. Serum samples were collected on days 1, 3, 5, 15, 17, 22, and 31, immediately before sc administration. After the last dose on day 40, samples were collected about weekly up to day 101. On day 15, total systemic exposure to CAM (AUC 0-12huors) was evaluated. Serum concentrations were assayed by an ELISA developed and validated in our laboratory. The 29 patients were divided into Responders and Non-responders, considering as responders the patients who exhibited an improvement after CAM consolidation therapy. All values were significantly higher in Responders than in Non-responders and the difference was statistically significant. The median Cmax was 1.69 mcg/mL (IQR: 1.2-4.45 mcg/mL) for Responders versus 0.44 mcg/mL (IQR: 0.19-0.57 mcg/mL) for Non-responders (p=0.0002). On day 15, the median Cpre-dose before sc administration was 0.7 mcg/mL (IQR: 0.54-1.2 mcg/mL) and 0.21 mcg/mL (IQR: 0.1-0.41 mcg/mL) for Responders and Non-responders, respectively (p=0.0006). The median of total systemic exposure to CAM (AUC 0-12h) was 11.09 mcg*h/mL (IQR: 7.69-13.32 mcg*h/mL) and 2.26 mcg*h/mL (IQR: 1.185-6.495 mcg*h/mL), for Responders and Non-responders, respectively. The difference between the two groups was statistically significant (p=0.0073). CAM serum concentration quantified before each sc administration increased gradually during week 1, and then faster during weeks 2 and 3, and then approached the steady state. The accumulation ratio was 25-fold higher during week 3 (day 3 vs day 17) and over 75-fold higher during week 5 (day 3 vs day 31). This suggests that the steady-state is not reached in the first 2 weeks but rather during week 3, at about the seventh sc dose. No significant difference (p=0.6143) was seen on day 3 (week 1), when levels were low (around zero) but on day 15, when CAM levels began to increase, the intergroup difference became statistically significant (p=0.0026). The median Cpre-dose of all samples in the first 31 days was 0.63 mcg/mL and the median Ctrough exceeded 1.0 mcg/mL (1.14 mcg/mL) only after the last dose: we can hypothesize that CAM continues to be slowly absorbed through tissues for about 2-3 weeks after the last administration, providing steady serum levels, and thereafter starts to decrease. Complete response rates expressed as the percentage of patients with AUC0-12hours values correlating with effective treatment, increased with higher levels of systemic exposure to CAM. AUC0-12h was >5 mcg*h/mL in 90.5% of 21 Responders, while only 37.5% of 8 Non-responders had values >5 mcg*h/mL. Therefore, higher AUC0-12h values significantly correlated with better clinical response. The pharmacokinetic model of sc CAM disposition that we observed can be challenged with new doses and/or dosage schedules. A detailed pharmacokinetic profile may help design to more rapid and effective treatment regimens.

7.15

CLLU1, A NOVEL MOLECULAR MARKER FOR DISEASE MONITORING AND MINIMAL RESIDUAL DISEASE DETECTION IN CHRONIC LYMPHOCYTIC LEUKEMIA

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Eradication of minimal residual disease (MRD) following treatment for chronic lymphocytic leukemia (CLL) is associated with improved progression free and overall survival. However, CLL patients are often evaluated following therapy solely based on clinical features. Elevated expression of the CLLU1 gene (CLL Upregulated gene1) is restricted to CLL, and the CLLU1 expression level in the CLL population represent a continuum ranging from 0.0005- to 10.000-fold upregulation compared with that of normal B-lymphocytes. Furthermore, CLLU1 status has been shown to be an intrinsic, constant parameter for the CLL clone. We therefore hypothesized that the CLLU1 marker may be used to evaluate response to treatment and monitor for MRD in CLL patient samples. We performed a retrospective analysis on time-matched cryopreserved peripheral blood specimens from patients who underwent marrow biopsies for MRD determination by 4-color flow. MRD-negativity was based on leukocyte expression of CD5, CD19, CD20, and CD79b with a threshold of detection of 0.1%. First, we evaluated nine patients, who achieved MRD-negative remissions and were followed by serial marrow surveillance. CLLU1 levels were determined by QRT-PCR, and normalized to the individual's CLLU1 pretreatment value. Forty-six paired evaluations revealed a strong correlation between blood CLLU1 levels and the extent of leukemia in the marrow (analysis of variance for multiple regression, correlation coefficient=0.963, p<0.001). Detection of a rising CLLU1 level in the blood was observed in the absence of clinically detectable disease (7 patients) and preceded a clinical relapse (3 patients). Next, we assessed if the CLLU1 marker could be used to detect persistent marrow disease in patients in a clinical complete remission (CR). The level of CLLU1 in peripheral blood mononuclear cells (PBMC) of healthy individuals was determined as CLLU1 RQ=0.14 (95%CI:0.1-0.19, n=15). Twenty-eight patients in clinical CR, who required a marrow for confirmation of a suspected NCI-WG CR, underwent a total of 36 response assessments. In patients deemed to have MRD-negative remissions by 4-color flow, the mean CLLU1 RQ level was 0.06 (95%CI:0.03-0.09)(n=14). In contrast, patients with residual disease in the marrow had a mean CLLU1 RQ level of 31.66 (95%CI:4.68-58.65)(n=22). Using a cut-off of CLLU1 RQ=0.5, which represents a conservative threshold well above the level found in PBMCs from healthy donors, the sensitivity for CLLU1 for detecting residual disease in the marrow was 68%, and all cases with CLLU1 RQ>0.5 had persistent MRD by 4-color flow. The degree of disease reduction at CR, calculated as the relative change in CLLU1 levels between the pre- and post-treatment samples, was determined for patients with a CLLU1 pretreatment level above 10, for whom at least a 2.5-fold log-reduction in CLLU1 levels is attainable. Patients that had achieved a MRD-negative CR had a mean log-reduction in CLLU1 levels of 3.47-fold (95%CI:2.45-4.48)(n=7), while patients with a MRD-positive CR had a mean log-reduction in CLLU1 levels of 1.41-fold (95%CI:1.02-1.81)(n=15)(p=0.002). Assessment of blood CLLU1 expression holds promise for serial surveillance of patients following therapy, MRD detection, and monitoring for early relapse, and therefore might be of great benefit for the CLL community.

7.16

PENTOSTATIN, CYCLOPHOSPHAMIDE, RITUXIMAB, AND MITOXANTRONE: A NEW HIGHLY ACTIVE REGIMEN FOR PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKEMIA PREVIOUSLY TREATED WITH CHEMOIMMUNOTHERAPY

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Combination therapy with purine analogs, alkylators, and/or monoclonal antibodies has markedly improved the quality of responses in patients with chronic lymphocytic leukemia (CLL). Most regimens have utilized fludarabine as the purine analog but the severe myelosuppression and immunosuppression of these combinations require careful attention to dosing and schedule to minimize these toxic complications. Of the purine analogs active in CLL, pentostatin is the least myelosuppressive. Previously, we reported that combination pentostatin, cyclophosphamide, and rituximab was very active and acceptably safe to administer to patients with CLL and in the salvage setting this regimen appeared to have less myelosuppression and less frequent infectious complications than comparable fludarabine-based combinations. The current study combines pentostatin 4 mg/m^2, cyclophosphamide 600 mg/m^2, rituximab 375 mg/m^2 (omitted from cycle 1) and mitoxantrone (dose escalated in a phase 1 portion starting at 6 mg/m^2, 8 mg/m^2, and 10 mg/m^2) all administered on day 1 of 28-day cycles for a total of 6 treatments. Supportive measures included prophylactic administration of pegfilgrastim, sulfamethoxazole/trimethoprim, acyclovir, and antiemetics. Renal function was closely monitored and all patients received at least 1.5 liters of intravenous hydration with the administration of chemotherapy. Thirty-two patients (median age 58, range 44-75) with CLL (27 patients) or other low grade B cell neoplasms (5 patients) who have received prior chemotherapy (median number of prior treatment regimens 1, range 1-5) have been enrolled. There were 25 men and 7 women. Of the CLL patients all had either high-risk disease (67%) or "active" intermediate-risk disease (33%) and their median pretreatment WBC count was 30,600/µL, HGB 10.9 g/dl, and PLT 132,000/µL. The median -2 microglobulin was 3.85 mg/L. Most of the CLL patients (74%) had previously been treated with chemoimmunotherapy utilizing PCR, FCR, or FR. Response data is currently available for 25/27 of the CLL patients. In this group there were 21 responses (84%), including 6 CRs (24%), 1 NRs (4%) and 14 PRs (56%). Prior therapy with PCR, FCR, or FR did not adversely affect the frequency of response with 88% of these patients responding (CR in 24%, NR in 5%, and PRs in 59%). These preliminary results indicate that PCRM therapy is very active and well tolerated even in patients who have received prior chemoimmunotherapy.

7.17

REALISATION OF A SCREENING PROCESS FOR DATA QUALITY IMPROVEMENT WITHIN THE CLL10 TRIAL OF THE GERMAN CLL STUDY GROUP EVALUATING COMBINED CHEMOIMMUNOTHERAPY IN FIRSTLINE TREATMENT OF CHRONIC LYMPHOCYTIC LEUKEMIA

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Introduction. The clinical course of CLL is extremely variable, depending on different prognostic factors especially high risk cytogenetics. Moreover, the patient (pt) population in CLL is quite heterogenic, due to different physical conditions and the burden of comorbidity, which varies with the broad age spectrum observed in this disease. Protocol violations are a frequent problem in clinical trials, for example in the previous phase III trial of the GCLLSG (CLL8) 23% of the patients had a creatinine-clearance <70 mL/min. Primarily in order to enhance patient safety, but also to describe the patient population more precisely and to increase the data quality, the GCLLSG implemented a central screening process prior to randomisation. *Patients and methods.* The CLL10-study is an international multicenter phase III trial comparing FCR and BR in patients (pts) with low comorbidity score and normal renal function in pts with advanced CLL requiring treatment. Pts with del(17p) are excluded and are treated in a separate protocol for very high risk pts. Central laboratory testing for immunophenotyping, cyctogenetics and other prognostic parameters, as well as a medical review of all data, especially those regarding response assessments and safety data, are well established means of quality control in GCLLSG-trials. The CLL10-study is the first investigator initiated study with a screening process prior to the randomisation. Blood samples from all patients are sent to the central laboratories in Ulm and Cologne for testing of cytogenetics and immunophenotyping to confirm the diagnosis of CLL and to exclude del(17p). In addition, the pt´s comorbid conditions and renal function are evaluated by checking the CIRS-Score and concomitant medication and by recalculating the Creatinine-Clearance according to the formula of Cockroft and Gault. Moreover, the need of treatment according to the recently published guidelines by Hallek et al. is reassessed by one of the GCLLSG study physicians. *Results.* Between the study initiation in September 2008 and July, 13th 2009, 144 pts were screened for participation in the CLL10-trial. The median number of included pts by center is 1.9 pts. 20.8% (30) of the 144 pts were not eligible for randomisation, either due to violation of the inclusion-/ exclusion-criteria (27; 18.8%) or due to physician or pts wish (3; 2%). Twelve pts were not eligible because of comorbid conditions, including eight pts with an impaired renal function and four pts with an active secondary neoplasia. Four pts had to be excluded because of a del(17p-), four pts were pretreated, in two cases the diagnosis of CLL was not confirmed by the central immunophenotyping (one B-prolymphocytic leukemia and one mantle cell lymphoma) and in another two cases the absolute leukocyte counts were <5000/µL, e.g. were small lymphocytic lymphomas. Three pts were not in need of treatment according to the recently published guidelines. Moreover, two pts were not randomised due to the treating physicians decision, one pt withdrew the consent. At the beginning of the trial, the percentage of screening failures was around 25% (11 of 44 in January and 26 of 105 in April) and decreased to 21% in July. *Conclusions.* The high rate of screening failures underlines the importance of quality assurance in clinical trials not only to achieve comparability and transferability of results, but also for better patient-security. The decline in the rate of screening failures could be the result of a learning process, which could contribute to a higher quality of the data in clinical trials, as well as to a higher quality of care outside clinical trials. Moreover, these data show that central laboratory diagnostic for immunophenotyping and prognostic factors is essential in CLL.

7.18

SALVAGE THERAPY FOLLOWING RELAPSE AFTER FCR AS INITIAL THERAPY FOR CLL

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300 patients (pts) received fludarabine, cyclophosphamide, and rituximab (FCR) as initial therapy for chronic lymphocytic leukemia (CLL) (Tam CS; Blood 112(4):975-980, 2008). 15 pts failed to respond and 94% had complete (CR) or partial response (PR). Follow-up for the failure/relapse pts included 18 that developed leukemia/lymphoma transformation, 16 died in remission of infection (7), cancer (7), or cardiac events (2). 128 pts were considered for retreatment of refractory/relapsed CLL. 19 pts are on "watch and wait" and 109 have received salvage therapy. 77 received treatment at M. D. Anderson Cancer Center (MDACC) and the other 32 in their local community. Median time for the initial FCR response to salvage therapy was 43 months. Of the 109 pts, 19 achieved a CR, 12 a nodular PR, and 27% a PR. β2M and Rai stage IV were significant predictors for achieving a complete or partial response. Age was not a predictive factor. Quality of the initial response to FCR was predictive of response to first salvage. An initial time to FCR failure of > 36 months was favorable. Survival following first salvage was strongly determined by salvage regimen, Rai stage IV, and β2M level greater than twice the upper limit of normal. 17p deletion on FISH was predictive for response rate (p<.01). A variety of treatment regimens was administered during the 10 years of study. Rituximab regimens had an overall response rate of 37%, alemtuzumab plus rituximab 82%, CFAR (FCR + alemtuzumab) 77%, FCR +/- lumiliximab 72%. Other miscellaneous regimens had a response rate of 29%. Median survival after S1 therapy was 36 months, 36% predicted to be alive at five years (Figure 1).

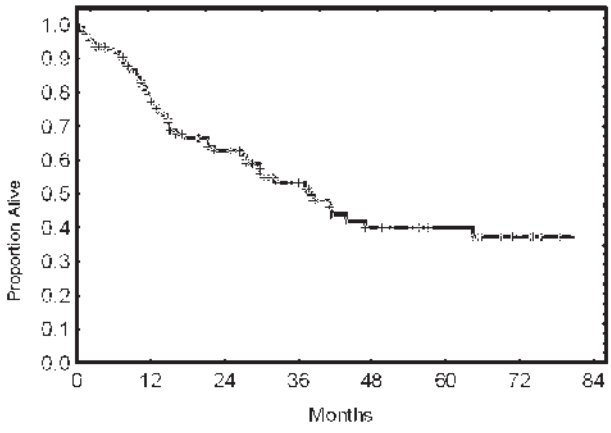

Figure 1.

Patients who failed to get a CR or NPR on first salvage therapy had a median survival of only 13 months. Monitoring of the second and subsequent salvage therapies is ongoing. 32 pts have subsequently received an allogeneic stem cell transplantation, 20 at MDACC and 12 elsewhere. 40% of these pts are predicted to be alive at three to five years. Characteristics associated with outcome of allogeneic stem cell transplantation are being analyzed. Biologic characteristics, response to initial FCR therapy, and treatment regimens offered all have significant impact on response to and survival after initial salvage therapy in FCR-treated pts with CLL. Continuous collection of such data is imperative now that FCR is emerging as a standard therapy for younger patients with CLL as initial therapy.

Stem cell transplantation

8.1

A HUMAN MONOCLONAL ANTIBODY DRUG AND TARGET DISCOVERY PLATFORM FOR B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA BASED ON ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION AND PHAGE DISPLAY

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Allogeneic hematopoietic stem cell transplantation (alloHSCT) is the only potentially curative treatment available for patients with B-cell chronic lymphocytic leukemia (B-CLL). Here we show that post-alloHSCT antibody repertoires can be mined for the discovery of fully human monoclonal antibodies (mAbs) to B-CLL cell surface antigens. Sera collected from B-CLL patients at defined time points after alloHSCT revealed selective binding to primary B-CLL cells. Pre-alloHSCT sera, donor sera, and control sera were negative. In order to identify post-alloHSCT serum antibodies and subsequently the B-CLL cell surface antigens they recognize, we generated a human Fab library from post-alloHSCT peripheral blood mononuclear cells (PBMC) and selected it on primary B-CLL cells by phage display. A panel of Fab with B-CLL cell surface reactivity was strongly enriched. The selection was dominated by highly homologous Fab predicted to bind the same antigen. One Fab was converted to IgG1 and analyzed for reactivity with PBMC from B-CLL patients and healthy volunteers. Cell surface antigen expression was found to be restricted to primary B cells and up-regulated in primary B-CLL cells. Mining post-alloHSCT antibody repertoires offers a novel route to discover fully human mAbs and identify antigens of potential therapeutic relevance to B-CLL and possibly other cancers.

8.2

ALLOGENEIC STEM CELL TRANSPLANTATION MAY OVERCOME THE POOR PROGNOSTIC FACTORS IN CHRONIC LYMPHOCYTIC LEUKEMIA: A SINGLE CENTER EXPERIENCE

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Background: We report a retrospective analysis of allogeneic stem cell transplantation(allo-SCT)in 29 patients (pts) with chronic lymphocytic leukemia (CLL) treated in years 2000-2009. Median age was 55 years (range, 42-63),overall 26 were men. Donors were 8 (28%) HLA -matched siblings and 21 (72%) unrelated volunteers, 10 out of them mismatched with recipient. Seventeen (59%) were treated with reduced intensity conditioning (RIC), twelve (41%) received myeloablative conditioning (MC). Vast majority of pts had poor prognostic features, that is 23 (79%) had unmutated IgVH status, 16 (55%) failed after fludarabine treatment, and 18 (62%) had poor cytogenetic abnormality (17p-, 11q- or ATM mutation or p53 mutation). Median interval from diagnosis to allo-SCT was 44 months (range, 8-112). *Results.* All pts engrafted. Relapse incidence was 14%. With a median folow-up of 36 months, estimated overall survival (OS) at 3 years is 78%, progression free survival (PFS) 70%, relapse risk 10% and NRM 16% resp. According to molecular/cytogenetic characteristics, OS and PFS for high risk group (n=18) were 70% and 57% resp, not significantly different to those with normal karyotype or 13q- and +12 (n=7). Acute graft-versus-host disease (aGVHD) occurred in 19 (66%) evaluable pts (3x grade I, 15x grade II and 1x grade III). Of the 26 evaluable pts, 10 (34%) developed chronic GVHD (4x extensive, 6x limited). From 9 pts with chemoresistant CLL at allo-SCT, seven (78%) achieved remission (3xCR, 4xPR, one patient in CR died 12 months after SCT of sepsis with GVHD and one patient in PR died at 52m of relapse), 2 pts died early with active disease. Overall 8 (28%) pts died, 5 for NRM and 3 for relapse. At time of last follow-up, 21 pts (72%) are alive, 16 in CR, 4 in PR and one at relapse. Overall 21 pts were evaluable for MRD (by allele specific IgVH - RT-PCR), overall 10 achieved molecular negativity (with median of 13 months). The only significant negative prognostic factor for OS (p=0.02) and TRM (p=0.01) was Binet stage C vs A or B at diagnosis. We didn´t found any significant difference

according to donor, conditioning, pretreatment, age or chemosensitivity. *Conclusion.* Allogeneic SCT represents an effective therapeutic option for patients with high risk CLL. In our conditions TRM was low as well as relapse risk, but median follow-up is too short to final conclusion. Our results in group of chemoresistant disease (78% responses) suppose that chemoresistance may be overcome by GVL effect. Transplants from unrelated donors may be considered comparable to related.

8.3

AUTOLOGOUS STEM CELL TRANSPLANTATION IN CLL. RESULTS FROM A PROSPECTIVE RANDOMIZED TRIAL INCLUDING 241 PATIENTS

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Background. We investigated the value of ASCT which has never been prospectively evaluated in a randomized trial in CLL. *Methods.* eligibility criteria were previously untreated stage B and C CLL patients under 66 years, characterized by Matutes score 4-5, absence of cyclin D1 expression, baseline flow assessment of ZAP 70 and CD38 expression, karyotype and FISH analysis, IGHV mutational status (centralized). Preceding randomization, initial chemotherapy consisted of three monthly courses of mini CHOP regimen as previously described, followed by three fludarabine monthly courses, IV or oral. Then, patients achieving CR (NCI 1996 criterias plus normal CT scan) were randomized between observation and ASCT. Non CR patients were offered cisplatin/cytosine-arabinoside/dexamethasone (DHAP) rescue and randomized whatever the response between ASCT or three subsequent monthly IV courses of Fludarabine-Cyclophosphamide (FC). Conditioning regimen for ASCT consisted of cyclophosphamide IV (60 mg/sqm d-5-4) and fractionated total body irradiation (10 Gy). The primary end-point of the study was event-free survival at 3 years. Responses after initial treatment (i.e.before randomization) and after completion of therapy, overall survival, side effects, prognostic significance of clinical and biological characteristics at baseline were other endpoints. *Results.* from March 2001 to December 2007, 241 patients were included in 37 centers. Baseline characteristics were: gender (M/F: 3), age (median : 56.4 years, range: 33.3-66), stage B (185 patients) or C (56 patients). All enrolled cases but five (236 patients) started the treatment. Among them, 206 completed the six planned courses of initial chemotherapy. For the 236 patients, CR rate was 43.6%, and OR was 89.8%. Forty two patients were not randomized because of uncontrolled progression (8), death or other SAE (13), major protocol violation (4), consent withdrawal (3), physician decision (12), cancer (2). At the reference date (1/1/2009), the median follow-up from randomization was 35.15 months. From the CR patients, 53 were allocated to ASCT, and 52 to observation. The EFS at 3 years in these groups are respectively 84.2% (95% CI: 74.0-95.7) and 30.1% (95% CI: 18.9-48.1), $p<0.0001$. The 36 months OS is 97% in the observation group (95% CI: 91.3-100), and 97.9% for the transplanted group (95% CI: 94-100), p=ns. From the 94 patients not achieving CR and rescued with DHAP, 46 were allocated to ASCT and 48 to FC. The EFS at 3 years in these groups are respectively 45.7% (95% CI: 31.7-66.0) and 43.7% (95% CI: 22.9-63.8), p=ns. The 36 months OS is 82.3% (95% CI: 71.0-95.5) in the transplanted group and 86.2% (95% CI: 75.5-98.5) in the FC group, p=ns. The overall survival for the whole group is 87.3% at 3 years (95% CI: 82.6-92.3) and 75% at 5 years (95% CI: 65.1-86.5). Twenty-eight patients allocated to ASCT did not receive actually this treatment mainly because of mobilisation failures (14 pts). Four MDS were recorded in this trial within the follow-up frame. Clinical and biological baseline characteristics predictive for response, EFS, OS, adverse events, will be presented. *Conclusions.* ASCT is a safe procedure which significantly improves response duration in patients attaining CR after a first line treatment. For patients not in CR, ASCT or consolidation with three FC courses provide similar results on response duration. Supported by grants from the French Department of Health (PHRC 2001), Chugai France, and Georgelin funds.

8.4

UNEXPECTED DETECTION OF MONOCLONAL B-CELL LYMPHOCYTOSIS IN A MATCHED RELATED SIBLING DONOR ON THE FIRST DAY OF ALLOGENEIC STEM CELL TRANSPLANTATION FOR A CLL PATIENT: CLINICAL OUTCOME

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Background. monoclonal B-lymphocytosis (MBL) is more common among first relatives of CLL patients and evolution from MBL to CLL is estimated to occur at 1% per year. Until now, allogenic stem cell transplantation (allo-SCT) remains the only potentially curative modality for CLL patients. Here, we describe a unique case of a first relative donor of the patient who was found to harbor MBL at the day of transplantation. The outcome of this case is also presented after a relatively long post-transplantation follow-up. In 2001, a 58 years old male was diagnosed with CLL (Binet stage C) and achieved a partial remission after treatment with fludarabine. Two years later, in 2003, there was rapid clinical progression and retreatment with chlorambucil, mitoxantrone and prednisone achieved a partial response. In March 2005, after completing therapy with CHOP for a second progression, the patient underwent a matched related (sister) reduced intensity allo-SCT. Unexpectedly, on the transplantation day we detected by flow cytometry, the presence of a monoclonal B-lymphocytosis within the collected cells, which were $CD5^+$,$CD19^+$,$CD20^+$,$CD23^+$, surface +, $CD38^-$ and $ZAP70^+$ (whereas the patient's CLL cells were +, $CD38^+$ and $ZAP\text{-}70^+$). We confirmed that the donor was asymptomatic, with no physical findings of note and apparently normal blood cell counts. The transplant was carried out uneventfully, achieving a mixed chimerism of 84% donor's cells. As planned, periodic donor lymphocyte infusion (DLI) was given, maintaining a mixed chimerism> 90% of donor's cells. Over time, the patient 's white blood cell (WBC) count normalized, with no signs of CLL. In November 2008, three years after transplantation, the patient's WBC counts increased reaching 16.700/uL, with 75.6% lymphocytes. An extensive study was carried out to determine whether the relapsed CLL was patient or donor in origin. This time, the peripheral blood lymphocytes had a typical CLL phenotype but $CD38^+$ cells. IgVH sequencing revealed that the relapsed cells were VH4-30.1/4-31 with a 100% germline homology whereas the donor cells harbored a different clone consisting of VH1-18 with 97.3% of germline homology. FISH analysis revealed mixed cell populations one with XY(62.5%) another of XX(38.5%) chromosomes, but only cells of male phenotype carried the aberration - 13q.14.3 deletion. Short tandem repeat (STR) DNA analysis of all the peripheral blood cells showed mixed chimerism (55% of donor origin). The CLL cells were further purified by CD19 magnetic microbeads and after STR analysis these cells were clearly of patient origin. Since the relapse was seen to be host-derived the patient was treated with interferon- , cyclophosphamide together with stem cells and DLI from the original donor. Today, 6 months after this therapy the patient has normal blood counts and no clinical evidence of CLL and is complete donor by STR. During the follow-up period, the donor did not progress and still has normal blood counts (absolute lymphocytes count-1,900/uL) *Conclusions.* Attempts should be made to detect MBL among first relative donors of CLL patients. MBL donors should generally be excluded but may perhaps be considered as donors only in cases where no other alternative therapeutic options exist.

8.5

AUTOLOGOUS HEMATOPOIETIC STEM CELL TRANSPLANTATION (AUTOHSCT) IN CLL: FIRST RESULTS OF AN EBMT RANDOMIZED TRIAL COMPARING AUTOTRANSPLANT VERSUS WAIT AND WATCH

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This phase-III randomized EBMT-intergroup trial studied the impact of a consolidating autoHSCT vs no consolidation for patients with CLL in Binet stage A progressive, B or C , in CR, nodular PR or VGPR after first or second line therapy. The primary objective was to show that autoHSCT increased the 5-year progression-free survival (PFS) by 30%. Although it had been calculated that 270 patients were to be randomized, the study was terminated by the steering committee in July 2007 due to poor accrual. Here we present a first analysis based on 69% of expected follow-up forms. *Results.* Between November 2001 and July 2007, 223 patients were enrolled (SFGM-TC/FCLLG n=98, MRC n=62, GCLLSG n=32, SAKK n=10, other EBMT centers n=17). There were 74% males and 26% females. Binet stages were progressive A 13%, B 67%, C 20%; 59% were in CR, and 41% in very good or nodular PR. Of note, SFGM-TC/FCLLG included only patients in CR. 82% of the patients were enrolled in 1st, and 18% in 2nd remission. Patients were randomized between group 1 (autoHSCT n=112) and group 2 (observation n=111) after an induction treatment which was left at the discretion of the investigators. Median PFS was 43 months in the observation group but not reached in the autoHSCT group; 5-year PFS was 48% and 65%, respectively (p=0.005). Accordingly, autoHSCT halved the relapse risk (5-year relapse incidence 25% vs. 51%; HR 0.4 [0.23-0.71], p=0.002). Cox modeling for randomization arm, Binet stage, disease status, line of treatment, contributing group (country), and the interaction between randomization arm and contributing group confirmed that autoHSCT significantly improved PFS (HR 0.41 [0.23-0.75] p=0.004). The beneficial effect of autoHSCT was stable over all contributing groups although patients accrued by SFGM-TC/FCLLG overall had a significantly better PFS than patients from other countries (HR 0.2 [0.08-0.55], p=0.001). At 5 years, the probability of OS was 92% and 91% for autoHSCT and observation, respectively. Significant differences in terms of non-relapse death were not observed. At the last follow up, among 205 evaluable patients, 186 are alive (147CR, 39 relapse), 19 died (14 from relapse and 5 from non-relapse causes) . In conclusion, in patients with CLL in first or second remission, consolidating autoHSCT reduces the risk of progression (PFS) by more than 50%, but has no effect on overall survival.

8.6

A LONG-TERM FOLLOW-UP AFTER ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATIONS FROM RELATED AND UNRELATED DONORS FOR CHRONIC LYMPHOCYTIC LEUKEMIA REPORTED ON THE EBMT REGISTRY (CLL SUB-COMMITTEE ON BEHALF OF EBMT CLWP

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Allogeneic hematopoietic stem cell transplantation (HSCT) remained till now a controversial strategy within treatment of CLL. There is now a well established "European Consensus" concerning the indications of allogeneic HSCT for CLL. In addition, some recent data showed the importance of HCT co-morbidity index of the quantitative MRD monitoring on transplant outcome. Our retrospective analysis concerned 374 patients (pts) (282 M, 92 F, median age 53 years) who underwent an allogeneic HSCT for CLL reported to the EBMT registry. The interval between diagnosis and transplantation was 53 months (3-308). At transplant, 302 among 323 evaluated patients had a good performance status (PS) (93%), 51 pts were in CR (14%), 163 in PR (45.5%), 39 in SD (11.5%) and 105 in PD (29%) among 353 evaluated patients. Two hundred and ninety-two pts received a reduced intensity conditioning regimen (RIC) and 82 a standard (Std); 314 pts received PBSC, 55 BM and 5 cord blood cells from 202 HLA siblings (Sib), 2 mismatched related donors and 170 unrelated donors (UD). There were 136 (36%) sex-mismatched (90 F/M and 46 M/F), 150 pairs (40%) had an ABO incompatibility. After transplantation, 359 pts engrafted. At day 100, the cumulative incidence (CI) of AGVHD for the total population was 31% (26-36) for gr ≥ II. At 1 year the CI of limited and extensive cGVHD were 15 % (6-24) and 29.5% (18-41) for Std; 18.6 (13-24) and 18% (13-23) for RIC respectively. With a median follow up of 38 months, the probability of 3-year and 5-year overall survival (OS) and disease-free survival (DFS) for the total group were 56% (51-62) and 47.4 % (42-53); 49 % (43-56) and 42% (36-48.5) respectively. We observed a significant difference concerning 5-year OS according to the pretransplant disease status [CR: 73% (60-89), PR: 57% (48-68) and PD: 35% (26-46)] (p<0.00001).There was no significant difference between standard and RIC HSCT in term of OS with 52.4% (42-66) and 47% (40-55.5) respectively (p=0.44) (Figure 1). The multivariate analysis using Cox model showed a significant impact of 3 factors on OS: age: HR=1.061 (1.02-1.10) p<0.0001, gender: HR=2.29 (1.02-5.11) p=0.04 and PS: HR=3.15 (1.40-7.10) p=0.005.The CI of non-relapse mortality and of relapse at 3 months and 1 year were: 10 % (7-13), 5% (3-7) and 24% (19-28), 15% (12-19) respectively [Std:23 % (11-35.6), 6% (0-14) 1 year; RIC: 22.5% (17.5-27), 18% (13-22) at 1 year]. This large retrospective analysis showed a high percentage of long-term OS after HSCT for CLL either after Std or RIC conditioning without any difference between the 2 groups and demonstrated a significant impact of disease status pretransplant, age, PS and sex-matching.

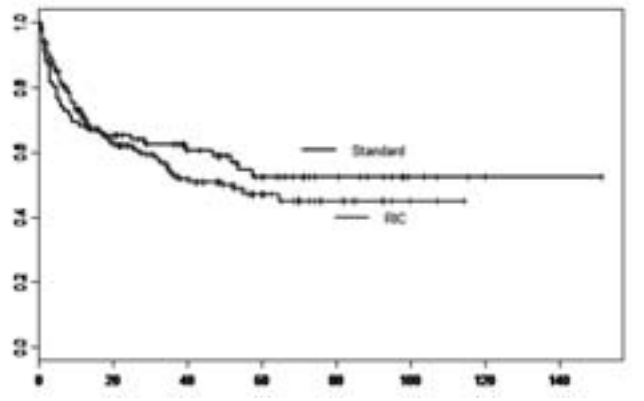

Figure 1. Probability of OS for Standard conditioning group and RIC group.

8.7

T-PROLYMPHOCYTIC LEUKEMIA (T-PLL) IS SENSITIVE TO GRAFT-VERSUS-LEUKEMIA EFFECTS: EVIDENCE FROM MINIMAL RESIDUAL DISEASE (MRD) KINETICS

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Introduction. T-cell prolymphocytic leukemia (T-PLL) is a postthymic T-cell malignancy with an aggressive clinical course. Only a small proportion of patients respond to conventional chemotherapy, and early relapse is common. With less than 3 years, median survival is short. The optimal therapy is still matter of debate. According several encouraging reports allogeneic stem cell transplantation (alloSCT) seems to be an option with the possibility of cure in T-PLL. However, evidence that GVL activity is indeed effective in T-PLL is sparse. Here we present post-transplant MRD kinetics from 3 cases of T-PLL who underwent an allogeneic SCT, suggesting that the disease can be sensitive to GVL effects. Patients: Three consecutive patients (2 male, 1 female; age 42-60 years at diagnosis) presented with progressive HTLV-negative leukocytosis (48-177/nL) of mature T cell immunophenotype fulfilling the diagnostic criteria of T-PLL. All 3 patients had increasing thrombocytopenia (49-102/nL), anemia (10.0-11.2 g/dL) and progressive splenomegaly. After induction of first complete remission by sc alemtuzumab, all 3 patients underwent alloSCT from an HLA-identical related (n=1) or unrelated

(n=2) donor after reduced-intensity conditioning with fludarabine (30 mg/m day 1-5) and cyclophosphamide (500 mg/m day1-5) +/- ATG. All patients experienced successful engraftment and there was no severe transplant-related toxicity.

Table.

Patient	*1*	*2*	*3*
Marker 1 (sensitivity)	J β 1.3 (0.001%)	J β 1.4 (0.001%)	J β 2.7 (0.005%)
Marker 2 (sensitivity)	J β 2.2 (0.05%)	J β 2.5 (0.005%)	V gamma 8 (0.05%)
MRD level (chimerism)			
At SCT	0.06%	0.03%	neg
Day +28	0.4% (80%)	n.a. (60%)	0.7% (25%)
Before CSA taper	2% (70%)	0.1% (70%)	4% (50%)
After CSA taper	<0.01% (100%)	0.08% (80%)	0.1% (>95%)
After DLI	-	<0.01% (>95%)	-
GVHD acute	none	none	none
GVHD chronic	limited	none	limited

All 3 patients were available for longitudinal post-transplant MRD monitoring of blood and bone marrow samples by Taqman-based clone-specific quantitative TCR β/gamma PCR using individual markers (Table) as well as by donor chimerism quantification using STR-PCR. *Results.* In all 3 patients, MRD levels were strongly suppressed by alemtuzumab pre-treatment at SCT but tended to increase over time post-transplant. In patients 1 and 3, CSA withdrawal resulted in a >1 log reduction of MRD levels. Patient 2 did not respond to cessation of immunosuppression but showed >1 log reduction of MRD levels upon DLI (Table). Despite these impressive molecular responses, however, MRD persists in all 3 patients at levels below the quantification threshold with a follow-up of 16 (11-19) months post-transplant. *Conclusions.* This data suggests that T-PLL is indeed sensitive to GVL activity conferred with alloSCT. It remains to be shown, however, whether GVL effects are sufficient to induce complete MRD eradication in this disease, thereby representing a curative treatment approach.

8.8

ADULT TO CHILD TRANSMISSION OF CHRONIC LYMPHOCYTIC LEUKEMIA THROUGH UNRELATED BONE MARROW TRANSPLANTATION

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The risk of CLL transmission is not currently considered in the SCT setting, but two cases were recently reported with sibling matched donors. We report here the case of a B-CLL disease occurring in a 12y old boy after unrelated SCT. When aged 8, he received an 10/10 antigen matched, sex-mismatched bone marrow (BM) graft after a myeloablative TAM (12 Gy TBI, Cytarabine, Melphalan) conditioning for a Philadelphia positive ALL in second CR. Post BMT evolution was uneventfull including a grade II acute GVHD and a limited cGVHD treated by CSA until month 34 post BMT. Imatinib was administered from D60 to D260, then stopped due to side-effects. Minimal residual disease showed undetectable Bcr/Abl transcripts post BMT. Chimerism studies (assessed by Short Tandem Repeat analysis) revealed a stable full donor chimerism from 1 month post-BMT in mononuclear and polynuclear cells in the peripheral blood (PB) and the BM. Four years after BMT, the blood count revealed a slight lymphocytosis at 3,5 Giga/l, composed of atypical small lymphocytes characteristic of B-CLL. Flow cytometry analysis confirmed a B-CLL profile in 16% of the total lymphocytes with a light chain Kappa restriction. The same circulating B-CLL clone was detected 3 months later. The recipient was asymptomatic. Molecular analysis of post-BMT immunoglobulin β heavy (IgH) and Kappa light (IgK) chain rearrangements in PB & BM samples allowed us to detect a clonal IgH and IgK rearrangement on a polyclonal background. Retrospective analysis identified the same IgH and IgK BCR rearrangements in BM or PB all over the available time points post BMT. A donor blood sample drawed at the time of the BM was thawed and retrospectively analysed: it contained the B-CLL population with the previously described immunophenotype, accounting for 34% of blood lymphocytes. The cells carryed the same IgH and IgK rearrangements than the recipient's cells post BMT. Moreover, the IgVH gene mutational status (92%) is similar between donor PB-circulating-B-CLL and the recipient clone. At time of the graft donation, the female donor was 44y and her blood lymphocyte count was 3.5 Giga/l, thus below the 4 Giga/l threshold considered abnormal in french blood or marrow donors. However, one year after BM graft donation, a stage A B-CCL was detected while doing a routine check-up before hysterectomy. In situ fluorescence hybridation (FISH) study revealed a del13q14.3 abnormality. At 4,5 years post marrow donation, the del13q14.3 abnormality is detected in 62% of analysed nuclei in the donors PB, whereas it is detected in 73% of the recipient's PB cells. The slow lymphocyte doubling time in the donor, the presence of a del13q14.3 abnormality associated with a mutated IgVH status are all indicative of a low rate evolution. However, given the young age of the recipient, this first report to our knowledge, of a B-CLL transmission through unrelated BMT raises several ethical and practical questions, such as the proper information to disclose about the risk of donor to recipient disease transmission. The introduction of a systematic PB immunophenotypic analysis in donors older than 40 should be evaluated.

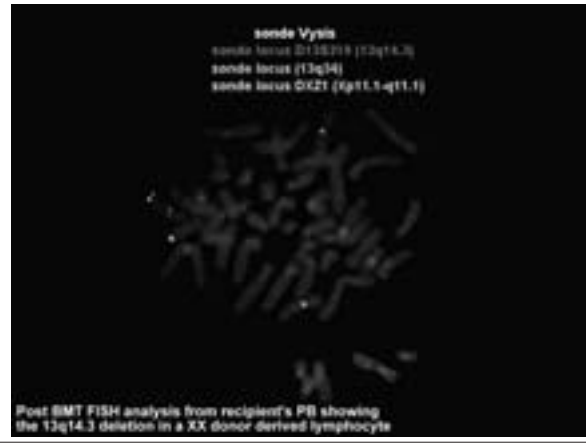

Figure 1.

8.9

SECONDARY MALIGNANCIES AFTER EARLY AUTOLOGOUS STEM CELL TRANSPLANTATION (SCT) IN GENETICALLY POOR-RISK CHRONIC LYMPHOCYTIC LEUKEMIA: RESULTS FROM THE GCLLSG CLL3 TRIAL

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German CLL Study Group

Introduction. As previously reported by the German CLL Study Group (GCLLSG), early SCT as conducted in the CLL3 protocol is a feasible and effective therapy option for younger patients with genetically poor-risk chronic lymphocytic leukemia (CLL). Purpose of the present analysis was to study incidence and type of secondary malignancies occurring in this trial. Trial design and patients: The protocol comprised optional cytoreduction with CHOP, fludarabine, or FC, PBSC mobilization using the Dexa-BEAM regimen, and myeloablative therapy with TBI/CY followed by reinfusion of purged stem cells. Inclusion criteria were age <61 years, stage Binet B/C or poor-risk stage A as defined by short lymphocyte doubling time plus elevated STK, and one line of pretreatment or less. From December 1996 through September 2002, 216 patients were registered with the protocol. As 47 cases had to be excluded due to screening failure (n=21), withdrawn consent (n=19) or other reasons (n=7), 169 patients were eligible for the current analysis. Male to female ratio was 5:1 and the median age at diagnosis was 51 years (range 27-60). An unmutated VH rearrangement was present in 70%. *Results.* SCT was performed in 131 patients (78%) at a median time of 17 months (range 4-159) after initial diagnosis, whereas 38 patients did not proceed to SCT due to mobilization failure (n=14), disease progression

(n=4), early death (n=3), patients preference (n=6), or unknown reasons (n=11). At a median follow-up of 99 months (range 4-137) after initiation of first cytoreductive therapy within the protocol, median overall survival (OS) of all 169 patients was 10.5 years, with 10.5 years for those treated with and 6.1 years for those treated without SCT, yielding a hazard ratio of 0.26 (95% CI 0.13-0.54; $p<.0001$). Median progression free survival (PFS) was 6.3 years, with 6.8 years for those treated with and 4.8 years for those treated without SCT (HR 0.39; 95% CI 0.23-0.67; $p=0.0007$). Altogether, 20 secondary malignancies were observed, with the most frequent ones being t-MDS/ t-AML (n=6), genito-urinary (n=3) and gastrointestinal cancers (n=3), translating into a 10-year incidence of 20% (95% CI 11-30%). There was no significant difference in the 10-year incidence of any secondary malignancy among individuals treated with and without SCT ($p=0.68$). However, all cases of t-MDS/ t-AML occurred after SCT, yielding a 10-year incidence rate of t-MDS/ t-AML of 9% (1-18%). While 8 secondary malignancies were documented in the absence of CLL relapse, 12 occurred after CLL recurrence, of which 5 (including 3 t-MDS/AML cases) were observed only after CLL-specific re-treatment. Overall survival after onset of secondary neoplasm was 22 months (2-50), with no difference between t-MDS/AML and other malignancies. *Conclusions.* Secondary neoplasms are a serious problem after early SCT for poor-risk CLL, but do not appear to occur more frequently than reported after SCT for other diseases. The overall survival provided by the early SCT approach is, nevertheless, promising for this high-risk patient selection.

8.10

LONG TERM IMMUNE RECONSTITUTION FOLLOWING STEM CELL TRANSPLANTATION IN CHRONIC LYMPHOCYTIC LEUKEMIA

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Chronic lymphocytic leukemia (CLL) is characterized by the accumulation of monoclonal CD5+ B lymphocytes. In addition, immune disturbances are commonly present. Although CLL is still considered incurable, long term remissions can be observed after allogeneic stem cell transplantation (SCT). Whether immune function is restored in patients responding to treatment is largely unknown. We report on the immune status in 16 patients with CLL in long-lasting complete remission (CR) after SCT (11 allogeneic, 5 autologous). Median age was 51 (range, 33- 58) and median follow-up since transplantation was 5 years (range, 2-17). Three patients had chronic graft-versus host disease at sample collection, two of whom were receiving immunosuppression. Lymphocyte subsets were studied using multiparameter flow cytometry and compared to healthy controls. We quantified immunoglobulin subtypes, complement proteins, and beta2-microglobulin (β2M) by standard techniques and IL-10 and VEGF by using flow-based cytometric bead array technology. $CD8^+$ T cell response to CMV was assessed using a pentameric HLA-A2 binding CMV pp65-derived peptide. Two patients (both following allogeneic SCT) had detectable residual CLL cells (>10-4) in peripheral blood at the time of the analysis. In the remaining 14 patients with no detectable minimal residual disease (MRD) normal $CD19^+$ $CD5^-$ and $CD19^+CD5^+$ B cell populations were lower than in healthy individuals (10.2% vs. 16.3%; $p=0.02$ and 1.5% vs. 3.9 %; $p=0.002$ respectively). A significant increase in the proportion of $CD8^+$ T cells (median 27.9% vs. 19.5%, $p<0.05$), particularly those with a chronically activated phenotype $CD3^+CD8^+DR^+$ (10.13% of $CD3^+$ cells vs. 5.16% in controls), was observed ($p=0.003$). Eight CMV^+ patients showed specific cytotoxic $CD8^+$ T cells which exhibit predominantly a $CD45RA^+$ $CD27^-$ phenotype, being better preserved in autologous SCT than in allogeneic SCT patients. Also, higher numbers of $CD8^+CD45RA^+CD27^-$ T cells were observed in patients with a longer follow-up. $CD4^+$ T cell count was normal except in 4 patients ($<400/mm^3$). An abnormal CD4:CD8 ratio was seen in 5 out of 16 patients. Interestingly, a significant increase of double positive CD4 and CD8 T cells was detected in most patients comprising 2.76% vs.1.63% of lymphocytes in normal subjects ($p=0.02$). There were no quantitative abnormalities in $CD3^-CD56^+$ cells. Not surprisingly, hypogammaglobulinemia was present in all but two patients immediately prior to transplant. Whereas IgM levels normalized in all patients, 4 and 6 patients respectively still had abnormal IgG and IgA levels more than two years after transplantation. Of note, abnormalities in serum immunoglobulin levels were seen in 6 out of 14 MRD negative-CR patients and both MRD positive-CR patients. Complement proteins C3 and C4 were within the normal range in all cases. The direct Coombs test (DCT) was also negative in all patients although one patient had indirect signs of hemolysis. Regarding serum markers, β2M was increased (>2.5 mg/dL) in 5 out of 16 patients. No significant differences were found in IL-10 and VEGF levels between patients and normal controls (median levels, 2.14 vs. 1.06 pg/mL and 156.88 vs. 104.14 pg/mL), but there were 2 patients with markedly elevated IL-10. In summary, these data demonstrate that immune defects persist over time in CLL patients with a long lasting CR (including MRD-negative CRs) after SCT.

8.11

BETA-2 MICROGLOBULIN IS A STRONG PROGNOSTIC MARKER IN PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKEMIA SUBMITTED TO ALLOGENEIC STEM CELL TRANSPLANTATION

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Allogeneic stem cell transplantation (SCT) is the only curative treatment for chronic lymphocytic leukemia (CLL). Advanced age, extensive prior therapy, lack of response to treatment, and T-cell depletion of the graft are poor prognostic factors which have been identified in many studies. Beta-2 microglobulin (β2M) has important prognostic value in patients treated with chemoimmunotherapy, but has been scarcely investigated in the context of allogeneic SCT. In two studies (Khouri et al. Cytotherapy 2002; Sorror et al. J Clin Oncol 2008) no correlation was found between β2M and transplant outcome. Against this background, we analyzed the influence of β2M and other prognostic parameters in 32 patients (median age 50 yrs [range, 29-63], 20 males) who received an allogeneic SCT in our institution between 1991 and 2006. Interval between diagnosis and transplantation was 44 months (range, 6-116). Median number of prior therapies was 2 (range, 1-6). Six patients had previously received an autologous SCT. Most patients had adverse biologic features (high ZAP-70 expression, unmutated IGHV, poor cytogenetics). Serum β2M was increased (≥2.5 mg/L) in 13 out of 29 patients prior to transplant. Creatinine levels and glomerular filtration rate were normal. Median follow-up after transplantation was 7 years (range, 1.8-16.9). The relapse risk (RR) at 5 and 10 years was 5 % (95% CI, 0-14%) and 23% (95% CI, 2-44), respectively. At one and 10 years the cumulative non-relapse mortality (NRM) was 34% (95% CI, 17-51) and 38% (95% CI, 20-55), respectively. Five and 10-year progression free survival (PFS), event free survival (EFS) and overall survival (OS) were 85% (CI, 66-100) and 65% (CI, 35-94), 58% (CI, 40-76) and 40% (CI, 19-62), and 62% (CI, 45-79) and 57% (CI, 38-75). In the univariate analysis, factors associated with a higher NRM were prior autologous SCT ($p=0.006$), chemorefractory disease ($p=0.04$), and high serum β2M levels at the time of SCT ($p=0.03$). Parameters associated with EFS and OS were high β2M levels ($p=0.001$ and $p=0.002$), prior autologous SCT ($p<0.001$ and $p=0.001$), and number of prior lines of chemotherapy (≤1 vs. ≥2) ($p=0.018$ and $p=0.042$). In the multivariate analysis, prior autologous SCT (RR=4.4) and chemorefractory disease (RR=3.82) were associated with a higher NRM whereas β2M at the time of SCT was a strong independent factor associated with EFS (RR=5.34) and OS (RR=6.20). In contrast, IGHV mutational status, high ZAP-70 expression, > 30% bone marrow infiltration, and disease status (CR vs. no CR) at the time of SCT were not associated with outcome. In summary, this study indicates that, as in patients treated with chemoimmunotherapy, β2M is a strong predictor of clinical outcome in patients with CLL submitted to allogeneic SCT.

8.12

TREATMENT WITH ALEMTUZUMAB PRIOR TO ALLOGENEIC STEM CELL TRANSPLANTATION INTERFERES WITH EARLY T-CELL ENGRAFTMENT

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Objectives. The majority of patients with chronic lymphocytic leukemia (CLL) who receive allogeneic hematopoietic cell transplantation (HCT) have fludarabine-refractory disease. The most active single agent in this disease stage is alemtuzumab. Alemtuzumab has a long half-life and induces profound T-cell depletion (TCD). Since TCD may mitigate graft-versus leukemia effects we evaluated „pre-conditioning" with alemtuzumab followed by a washout period in order to minimize *in vivo* T-cell depletion of the graft in a phase II study (NCT 00337519). *Methods.* Patients received cytoreductive treatment with 3 x 30 mg alemtuzumab weekly prior to HCT. The scheduled interval between last dose of alemtuzumab and HCT was increased from two weeks to one month during the study. The conditioning regimen contained fludarabine (150 mg/m^2) and busulfan (8 mg/kg). Cyclosporine (CSA) and methotrexate (MTX) were applied as GVHD-prophylaxis. Medically fit patients with relapsed CLL were elible. *Results.* 62 patients with a median age of 57 years were included between April, 2004 and October, 2008. A median of 3 prior regimens had been given. 55% of the patients had fludarabine-resistant disease. Two patients failed to reach HCT due to progressive disease during alemtuzumab therapy. Donors were matched siblings for 26 and matched unrelated donors for 34 patients. The median level of alemtuzumab in peripheral blood at HCT was 62 ng/mL (range, 0 to 490 ng/mL) after a washout period of two weeks and 0 ng/mL (range, 0 to 256 ng/mL) after a delay of four weeks (p=0.005). Despite one month time between the last dose of alemtuzumab and HCT 4 out of 30 patients (13%) had alemtuzumab levels greater than 200 ng/mL. No primary or secondary graft failure occurred. A linear relationship between the alemtuzumab level at HCT and the time to complete CD4-T-cell chimerism (TCC) was observed (p=0.003). At day +100 a CD4$^+$ T-cell-chimerism (TCC) >95% had been achieved by 84% of patients with alemtuzumab levels <100 ng/mL, 83% of patients with antibody levels between 100 and 200 ng/mL and 25% of patients with antibody levels >200 ng/mL (p=0.006). All patients had a complete neutrophil-chimerism at day +100. After early taper of immunosuppression (N=2) or the application of donor lymphocyte infusions in incremental doses (N=5) mixed TCC has been converted to complete TCC in all patients. The median follow-up is 17 months (1 to 61 months). Day +100 non-relapse mortality was 2%. At two years non-relapse mortality and relapse incidence were 21% and 29%, respectively. Two-year overall survival and progression-free survival were 67% (95% CI, 51% to 83%) and 50% (95% CI, 31% to 69%). *Conclusions.* In patients who received alemtuzumab prior to HCT, residual drug levels may interfere with T-cell engraftment. Lineage specific T-cell chimerism should therefore be assessed prospectively in this group of patients. Persistent mixed T-cell chimerism can be converted by an early taper of immunosuppression and incremental doses of donor lymphocyte infusions.

Immunotherapy

9.1

MONOCLONAL ANTIBODY AGAINST THE RECEPTOR TYROSINE KINASE ROR1 AS A POTENTIAL THERAPEUTIC DRUG FOR HUMAN B CELL CHRONIC LYMPHOCYTIC LEUKEMIA

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Targeted therapy for cancer has been gaining support as a feasible modality, and antibodies that recognize specific antigens on the cancer cell surface have been utilized to induce direct or indirect cytotoxicity. Rituximab, a mouse-human chimeric monoclonal antibody (mAb) and alumtuzumab, a humanized mAb are being widely used as immunotherapeutic agents for the treatment of human B cell malignancies including chronic lymphocytic leukemia (B-CLL). The target antigens CD20 and CD52, respectively, are expressed not only by malignant B cells but also by normal cells. Targeting leukemia-specific antigens may help increase treatment efficacy and reduce non-specific side effects. Gene expression profiling on a genomic scale led to the discovery of a distinctive B-CLL gene signature composed of genes selectively expressed by B-CLL cells. However, the expression and function of the corresponding proteins and their suitability as targets for therapeutic intervention remains largely unknown. Receptor tyrosine kinases have been shown to play a crucial role in cellular signaling and several members of this family have been implicated in the pathogenesis of a variety of cancers including hematologic malignancies. Previously, we and others have reported the selective cell surface expression of the receptor tyrosine kinase ROR1 protein in human B-CLL. Although the functional role of ROR1 in B-CLL remains to be elucidated, its uniform and restricted expression on B-CLL cells makes it an attractive therapeutic target for monoclonal antibodies (mAbs). We report here the development and characterization of mouse mAbs against human ROR1. Mice were immunized with a fusion protein consisting of the human Fc domain and the extracellular domain of human ROR1, and four hybridomas secreting mAb against human ROR1 were derived. All four mAb are of IgG1-kappa isotype and showed specific binding and recognition of purified human ROR1. Both the hybridoma supernatants and affinity purified IgG of these mAbs demonstrated specific recognition of cell surface ROR1 on ROR1-expressing transfectants as well as cell lines, and more importantly, on primary B-CLL cells from all patients tested. Two of these mAbs were further characterized by ELISA, flow cytometry and surface plasmon resonance measurements. Antigen domain mapping studies suggest that the epitope(s) recognized by these mAbs are located in the Ig and Frizzled domains of human ROR1. These mouse mAbs also cross-reacted with mouse ROR1 albeit weaker when compared to the human ROR1. The antigen binding avidity of one of these mAbs (2A2-IgG) is in the range of 100 pM with a very low dissociation rate. The Ig variable domains (VH and VL) of 2A2 mAb were used to construct mouse/human chimeric antibody and the antigen binding activity of the chimeric mAb (Chi-2A2-IgG) was similar to that of the parent 2A2-IgG. The activity of these anti-ROR1 mAbs and their engineered derivatives are under investigation.

9.2

VACCINATION OF CLL PATIENTS WITH AUTOLOGOUS DENDRITIC CELLS LOADED WITH APOPTOTIC BODIES (APO-DC): A PHASE I-II CLINICAL TRIAL

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Background. In preclinical studies we showed that dendritic cells (DC) that have endocytosed apoptotic CLL cells (Apo-DC) are a suitable antigen presentation platform to stimulate multiple-epitope autologous antileukemic immune responses. We validated a method for large-scale production of Apo-DC from a single leukapheresis product. DC were

generated from immunomagnetically enriched monocyte precursors, cultured ex vivo with GM-CSF and IL-4 and loaded with autologous apoptotic leukemic cells as antigen source. Materials and Methods. CLL patients with slowly increasing leukemic cell count but no expected (> 6 mo) need of antitumor therapy, received 10^7 Apo-DC at five immunizations timepoints (wk 0, 2, 4, 6, 14). Three cohorts consisting of 5 patients/group were accrued stepwise. Cohort I received Apo-DC alone; Cohort II: Apo-DC + GM-CSF 75 mg/day dd 1-4; Cohort III: same schedule as Cohort II + cyclophosphamide (CTX) 300 mg/m^2 i.v. at day –2 at week 0, 6 and 14. The total period of clinical and immunological follow-up is 52 weeks. A positive immune response is defined as a fold increase greater than 2 compared to pre-immunization values in either proliferation or ELISpot assay, at least at 1 time point during follow-up. Levels of regulatory T cells (Tregs) were also evaluated. Results. To date, cohort I and II have been completed; cohort III is still ongoing. The Apo-DC vaccine was well tolerated. Local, transient grade I skin reactions were observed following GM-CSF administration. The clinical and immune responses are reported in the Table 1. Tregs levels were lower in immune responders vs non-immune responders. Conclusions. Vaccine production was successfully achieved in all the 15 patients accrued. No significant toxicity was associated with this therapeutic approach. Immune responses were noted in 8/15 pts and of the 10 pts who reached 1-year follow-up, 7 pts were clinically stable. These data indicate that the Apo-DC vaccine can induce/boost anti-leukemia T-cell immune responses that seem to correlate with Tregs levels. This therapeutic approach shall be explored further in CLL patients without emerging need of chemotherapy.

Table 1.

	Pt	Follow-up (wk)	Clinical outcome	Immune response			
				proliferation		ELISpot	
				Preexisting	Vaccine-induced	Preexisting	Vaccine-induced
Apo-DC	I - 01	52	SD	+	-	+	↑ (wk 8-52)
Apo-DC	I - 02	40	PD (wk 16)	+	-	+	-
Apo-DC	I - 03	40	PD (wk 16)	+	-	+	-
Apo-DC	I - 04	52	SD	+	↓ (wk 8, 16, 40)	+	
Apo-DC	I - 05	52	SD	+	↑ (wk 40)	+	↑ (wk 40)
Apo-DC + GM-CSF	II - 01	52	SD	-	↑ (wk 28)	+	↑ (wk 8-40)
Apo-DC + GM-CSF	II - 02	40	SD	+	-	+	↑ (wk 8 =28)
Apo-DC + GM-CSF	II - 03	52	SD	+	-	+	-
Apo-DC + GM-CSF	II - 04	52	SD	+	-	+	↓ (wk 40)
Apo-DC + GM-CSF	II - 05	52	PD (wk 40)	+	↑ (wk 8)	+	↑ (wk 8)
Apo-DC + GM-CSF + CTX	III- 01	28	SD	+	↑ (wk 8)	-	-
Apo-DC + GM-CSF + CTX	III- 02	8	PD (wk 8)	+	-	+	-
Apo-DC + GM-CSF + CTX	III- 03	28	SD	+	↑ (wk 16)	+	↑ (wk 28)
Apo-DC + GM-CSF + CTX	III- 04	28	SD	+	↑(wk 8)	-	↑ (wk 28)
Apo-DC + GM-CSF + CTX	III- 05	8	SD	+	-	+	-

9.3

A NEW CHIMERIC ANTIGEN RECEPTOR TARGETING THE CD23 ANTIGEN EXPRESSED BY CHRONIC LYMPHOCYTIC LEUKEMIA CELLS

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B-Chronic lymphocytic leukemia (B-CLL) is characterized by a progressive accumulation of mature CD19/CD5 double positive, CD20dim B-lymphocytes. Moreover, B-CLL cells over-express the B-cell activation marker CD23. Chimeric Antigen Receptors (CAR) are engineered molecules able to redirect T-cell killing/effector activity towards a selected target in a non MHC-restricted manner. Selective CD23 overexpression on B-CLL cells renders it an optimal target for a CAR-mediated anti-B-CLL strategy. A new CAR targeting the CD23 antigen (CAR-CD23) to redirect T-cells against CD23$^+$ B-CLL has been generated. CAR-CD23 modified T cells showed specific cytotoxic activity against CD23$^+$ tumor cell lines (average lysis 72%, range 24%-100%, at an Effector-Target (E:T) ratio 40:1) and autologous CD23$^+$ B-CLL leukemic cells (average lysis 58%, range 26%-84%, at an E:T ratio 20:1). On the contrary, CAR-CD23 transduced T cells displayed no relevant cytotxic activity against normal B cells (average lysis 13%, range 7%-17%, at an E:T ratio 40:1), differently from anti-CD19 CAR-redirected T cells (average lysis 77%, range 57-100%, at an E:T ratio 40:1, p=0.05). Moreover CAR-CD23$^+$ T cells showed a significant increase in cytokine release (INF-gamma, TNF α, TNF β and IL2) in response to a CD23$^+$ target. Altogether these results suggest that CD23-targeting by CAR-expressing T cells is associated with a selective and potent killing of tumor cells, while sparing normal B cells, and triggers a selective release of immunostimulatory cytokines, that might amplify and sustain an effective anti tumoral immune response against B-CLL.

9.4

T CELLS FROM CHRONIC LYMPHOCYTIC LEUKAEMIA PATIENTS HAVE ABNORMAL GENE EXPRESSION PROFILE AND SUPPORT IN VITRO SURVIVAL OF AUTOLOGOUS LEUKEMIC CELLS

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Background; There is substantial evidence that T cell functions is dysregulated in B-cell chronic lymphocytic leukemia (CLL) patients and the T cells may contribute to the survival and growth of the leukemic clone. *Material and Methods.*; T and B cells were purified from PBMC of 7 healthy donors and 20 untreated CLL patients with indolent disease. Purified B cells were cocultured with different ratios of autologous T cells for 72 h in transwell plates. B cell apoptosis was measured by flow cytometry following staining with Annexin-V and propidium iodide. Supernatants of cocultures were collected for cytokine analysis by Luminex assay. The panel of cytokines assayed included RANTES, IL-10, IL-8, TNF- , IFN- , IL-4, MCP-1, CD40L and GM-CSF. Using Affymetrix arrays, the gene expression profiles of highly purified T cells from the peripheral blood of untreated, indolent CLL patients were compared with healthy donors and multiple myeloma (MM) stage I patients in an attempt to delineate T cell factors that may have an impact on supporting the malignant CLL cells. The results of gene expression profiling was verified using quantitative real time PCR (qRT-PCR) and immunoblotting on highly purified CD4 and CD8 T cells of patients with CLL, MM and healthy donors. Results; The apoptosis rate in purified tumor B cells cocultured with different ratios of autologous T cells was significantly lower (p<0.01) in higher T cells / B cells ratios compared to lower ratios. The apoptosis rate in purified B cells cultured alone was also higher than that of the B cells cocultured with T cells (p<0.01). Statistical comparison of apoptosis rate in B cells cocultured with different ratios of T cells in transwell plates did not show any significant difference. Luminex assay revealed that in CLL pts the levels of IL-4, RANTES, MCP-1, TNF- and IFN- are significantly higher in B cell / T cell cocultures compared to B cells alone. The gene expression profile demonstrated that diverse (356) genes were upregulated or downregulated in T cells from CLL patients compared to healthy donors and MM patients. These genes are potentially involved in different cellular pathways and activities including signaling, proliferation control, apoptosis, metabolism, immune response, and cytoskeleton formation. Three genes that demonstrated the greatest upregulation were the chemokines XCL1, XCL2, and the cytokine IFN- . Additionally CCL4 and CCL5 were two other chemokines that also was found to be specifically upregulated in T cells of CLL patients. Conclusion; Our data confirm that T cells support CLL cells survival *in vitro*. Also, the genes of chemokines that showed the greatest upregulation as well as other molecules identified by gene expression profiling, may have a significant effect on the survival of neoplastic cells. The results of the present study may be of significance for the better understanding of CLL pathobiology and development of therapeutic strategies.

9.5

CONTROL OF MRD POST-FCR FRONTLINE: CORRELATION TO IMMUNOLOGIC RECOVERY RATHER THAN TO DISEASE-RELATED ADVERSE PROGNOSTIC FACTORS GIVES RATIONALE FOR IMMUNOMODULATION THERAPY

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Aims. FCR is the gold standard for frontline therapy in CLL. Response rates (95-100% ORR) and Minimal Residual Disease (MRD) eradication (66%) are impressive. However, both immunological reconstitution and reexpansion of the leukemic clone have received little attention. The aims of the study were to: 1) measure the immunological reconsitution in FCR-treated patients by determining the absolute number of immune cells, 2) to evaluate Rituximab-mediated ADCC capacity of PBMC against autologous leukemic cells in FCR-treated patients; 3) to correlate immunological reconstitution with MRD control over time after FCR. METHODS Thirty patients without del17p and given FCR frontline were studied. Hematologic recovery ($CD4^+$ and $CD8^+$ T cells, NK, Tgammadelta, monocytes, and neutrophils absolute counts) were monitored at the End Of Treatment (3 months after the 1st day of last cycle of FCR), 6, 12, 18 and 24 months (EOT, M6-24). Four-color flow cytometry was used to evaluate MRD follow-up (% of CD5/19/22/81+ among leucocytes) in peripheral blood at the same timepoints. For Rituximab (RTX)-induced Antibody Dependent Cell Cytotoxicity (ADCC), Peripheral Blood Lymphocytes (PBL) were collected, incubated with IL-2 or not, with MEC-2 cell line or autologous CLL cells (frozen before FCR treatment) as target cells. Effector:Target ratio (E:T) were calculated acoording to the number of NK cells among the collected PBL. *Results*. In our cohort, FCR yielded a 72% CR+PRnod/22% PRi (PR due to incomplete bone marrow recovery)/6% PRd (PR disease-related). Considering a 10e-4 threshold, MRD eradication was obtained in 16/30 patients (53.3%, as compared to 66.4% in the CLL8 trial). CR was statistically correlated to Binet stage only (90.5% in A/B vs 55.5% in C patients, p=0.038), but MRD detection was not linked to disease-related prognostic factors. We then performed MRD follow-up, and classified results into 2 cohorts at M12 in 26 informative patients. One cohort showed stable or even decreasing levels over time (MRD group A), indicating an immunomodulation effect to occur post-FCR (irrespective of MRD level). There were 14/26 patients (9/14 had been classified as MRD EOT $<10^{-4}$). All patients remained between 10^{-4} and $<10^{-2}$ for up to 2 years, none relapsed. The second cohort encompassed 12/26 patients (5/12 had been classified as MRD EOT $<10^{-4}$) with constant increase of MRD levels over time (MRD group B). 10 patients within this group displayed $>10^{-2}$ MRD levels during follow-up: 3 patients died (1 from Richter syndrome (RPd), 2 from pneumonia with severe sepsis (1 CR MRD+ and 1 PRd)), 1 experienced Acute Myeloid Leukemia (CR MRD+, 2 years from completion of FCR), 4 had clinical relapse (only 1 requiring re-treatment), and 2 are still in clinical CR. Event Free Survival (EFS) was 62% at 2 years for these 26 patients. Starting from M12 (to assess the impact of MRD kinetics during the 1st year post-FCR), EFS was significantly different between MRD groups A and B (median EFS not reached group A vs 15 months groupB, n=24, log-rank p=0.0014), suggesting immune mediated control of MRD the first year after FCR was critical for prolonged EFS. Interestingly, median $CD4^+$ T cell reconstitution was correlated with MRD group B (median $CD4^+$ at M12 496/mm^3 group B vs 233/mm3 group A, n=23, p=0.03). We also observed a trend in an inverse correlation of the kinetics of MRD and the kinetics of Tgammadelta cells recovery (median count at M12 14/mm^3 group A vs 3.5/mm^3 group B, p=0.06). We next assessed NK cell functions post-FCR. Natural or IL-2 triggered cytotoxicity and RTX-induced ADCC against autologous CLL cells were significantly stronger after than before FCR. At M12, significantly higher NK activity against MEC-2 (±IL-2 or RTX) was found in MRD group A vs B patients even at low E:T ratio of 0.5:1 (p<0.05, n=10), similar to results obtained with healthy purified NK cells. *Conclusion.* FCR is an efficient frontline regimen but furthermore sets the stage for efficacious anti-leukemic response and good MRD control to occur. It still remains uncertain which cell population harnesses this process.

9.6

ENHANCED *IN VIVO* ACTIVATION OF ADOPTIVELY TRANSFERRED GENETICALLY TARGETED T CELLS FOLLOWING CYCLOPHOSPHAMIDE CHEMOTHERAPY: INITIAL RESULTS FROM A PHASE I CLINICAL TRIAL TREATING CLL PATIENTS WITH AUTOLOGOUS CD19-TARGETED T CELLS

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Patient T cells may be genetically modified to express chimeric antigen receptors (CARs) targeted to antigens expressed on tumor cells. We have initiated a clinical trial treating chemotherapy-refractory chronic lymphocytic leukemia (CLL) patients with autologous T cells modified to express the 19-28z CAR targeted to the CD19 antigen expressed on most B cell malignancies. In the first cohort of this trial, patients were infused with the lowest planned dose of modified T cells alone. All patients treated in this cohort experienced low-grade fevers following modified T cell infusion, and 2 of 3 treated patients exhibited subjective and laboratory evidence of transient reductions in tumor burden. The first patient treated on the second cohort of this study received prior cyclophophamide chemotherapy followed by the same dose of modified T cells administered to the first cohort of patients. This patient experienced persistent fevers, dyspnea, hypotension, renal failure, and died 44 hours following modified T cell infusion, likely secondary to sepsis. Although modified T cells were not detectable in the peripheral blood of the patient 1 hour following completion of T cell infusion, post mortem analyses revealed a rapid infiltration of targeted T cells into anatomical sites of tumor involvement. Serum levels of the inflammatory cytokines IL-5, IL-6, IL-8, and GM-CSF, but not TNF , markedly and rapidly increased following infusion of genetically targeted T cells in this patient, mirroring the *in vitro* cytokine secretion profile of this patient's T cells, and consistent with marked *in vivo* activation of the modified T cells in the infused patient. Similar cytokine signatures were not found in patients from the first cohort. Significantly, serum cytokine analyses from the second cohort patient revealed a marked increase in the pro-proliferative cytokines IL-2, IL-7, IL-12, and IL-15 following cyclophosphamide therapy, in contrast to the baseline levels found in the first cohort. This report demonstrates the high efficiency trafficking of CD19-targeted T cells and *in vivo* activation of T cells encoding a second generation CD28/zeta chain-based chimeric antigen receptor. Furthermore, these data highlight mechanisms whereby cyclophosphamide may generate an *in vivo* milieu that enhances the anti-tumor efficacy of autologous tumor targeted T cells.

9.7

HIGH RESPONSE RATES AND CLINICAL IMPROVEMENTS WITH SINGLE-AGENT OFATUMUMAB IN FLUDARABINE-REFRACTORY CHRONIC LYMPHOCYTIC LEUKEMIA (CLL) ALSO REFRACTORY TO ALEMTUZUMAB OR WITH BULKY LYMPHADENOPATHY

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Background. Patients with fludarabine-refractory CLL who are also refractory to alemtuzumab (FA-ref) or less suitable for alemtuzumab due to bulky (>5 cm) lymphadenopathy (BF-ref) have poor outcomes with available salvage regimens (overall response rate [ORR] 23%; median overall survival [OS] 9 months; Tam *et al.* Leuk Lymphoma 2007;48:1931-9). Ofatumumab is a human mAb that binds a unique small-loop epitope on the CD20 molecule close to the cell membrane and elicits more efficient *in vitro* lysis of B-cell lines and primary CLL cells via complement-dependent cytotoxicity compared with rituximab. Here we report updated results from the planned interim analysis of an international pivotal trial of ofatumumab in patients with FA-ref and BF-ref CLL, including outcomes by prior rituximab exposure. *Methods.* Patients received 8 weekly infusions of ofatumumab followed by 4 monthly infusions (Dose 1, 300 mg; Doses 2-12, 2000 mg). The primary endpoint was ORR (1996 NCI-WG criteria) over the 24-week treatment period assessed by an Independent Endpoint Review Committee (IRC). Secondary endpoints included progression-free survival (PFS), OS and safety. *Results.* The interim analysis included 59 patients with FA-ref and 79 with BF-ref CLL (median age, 64 and 62 years); 63% had Rai stage III/IV disease; 91% received ≥8 infusions; 54% received all 12 infusions. ORR (99% CI) by IRC evaluation was 58% (40, 74%) in the FA-ref and 47% (32, 62%) in the BF-ref groups. Median PFS (95% CI) was 5.7 (4.5, 8.0) and 5.9 (4.9, 6.4) months, and median OS (95% CI) was 13.7 (9.4, [upper limit not yet reached]) and 15.4 (10.2, 20.2) months in the FA-ref and BF-ref groups, respectively. The ORR was similar across subgroups based on pre-treatment factors. Of 35 (59%) FA-ref and 43 (54%) BF-ref patients who previously received rituximab-containing treatments, the ORR (95% CI) was 54% (37, 71%) and 44% (29, 60%), respectively, and median PFS (95% CI) was 5.5 (3.7, 8.0) and 5.5 (3.8, 6.4) months, respectively. In FA-ref and BF-ref patients refractory to fludarabine in combination with rituximab and cyclophosphamide (FCR; n=16 in each group), ORR was 50 and 44%, respectively. In addition to NCI-WG responses, improvements (maintained for ≥2 months) in disease symptoms, physical findings and blood parameters were observed in a large proportion of patients (Table). Infusions were well tolerated; transient grade 1 or 2 infusion-related reactions occurred in approximately 60% of patients, which largely subsided during the course of treatment. The most common grade 3 or 4 adverse events (occurring between first infusion and up to 30 days after last infusion) judged by investigators to be related to ofatumumab were infections (FA-ref, 12% [n=7]; BF-ref, 8% [n=6]) and neutropenia (FA-ref, 14% [n=8]; BF-ref, 6% [n=5]). *Conclusions.* Single-agent ofatumumab results in high ORR, provides clinical improvements in disease symptoms and physical findings, and has a favorable safety profile in heavily pretreated patients with FA-ref and BF-ref CLL. Furthermore, ofatumumab activity was independent of prior rituximab treatment; high response rates were seen irrespective of prior rituximab exposure, including in patients refractory to fludarabine-based regimens containing rituximab (e.g. FCR).

Table. Improvements in clinical and laboratory findings with a minimum duration of 2 months.

	FA-ref		BF-ref	
Categories of improvement	*N**	*n (%)†*	*N**	*n (%)†*
Complete resolution of B-symptoms‡	31	15 (48)	46	29 (63)
Complete resolution of lymphadenopathy (<1 cm nodes)	55	9 (16)	74	8 (11)
≥50% reduction in lymphadenopathy	55	34 (62)	74	36 (49)
Complete resolution of splenomegaly	30	14 (47)	46	16 (35)
Complete resolution of hepatomegaly	18	9 (50)	21	11 (52)
Neutrophil count from $<1.5x10^9$/L to $\geq1.5x10^9$/L	19	1 (5)	17	5 (29)
Hemoglobin from ≤11 g/dL to >11 g/dL	26	8 (31)	42	11 (26)
Platelet count from $\leq100x10^9$/L to 50% increase or $>100x10^9$/L	29	12 (41)	44	17 (39)

*Total number of patients with abnormal baseline parameters; †Number of patients with improvement (lasting for at least 2 months) from baseline to Week 24; ‡Including weight loss, fever, night sweats and extreme fatigue. FA-ref=fludarabine- and alemtuzumab-refractory; BF-ref=bulky fludarabine-refractory

9.8

ALEMTUZUMAB AND RITUXIMAB COMBINATION THERAPY IN PATIENTS WITH UNTREATED CLL – A PHASE II TRIAL

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Despite the availability of multiple therapeutic regimens, without a stem cell transplant, chronic lymphocytic leukemia (CLL) remains an incurable disease. A dramatic response rate with intensive chemoimmunotherapy in patients with CLL is frequently associated with irreversible long term consequences to the bone marrow, limiting further therapeutic options. For this reason we have initiated a clinical trial combining Rituximab (RIT) and Alemtuzumab (ALEM), two monoclonal antibodies with established activity and side effects profiles, as an initial therapy for patients with CLL requiring intervention. Methods Currently, data is available on 24 out of 29 enrolled patients. Therapy duration is 18 weeks. Subcutaneous (SC) ALEM dose escalation: 3 mg - 10 mg - 30 mg on days 1, 3, 5, followed by the 30 mg Monday, Wednesday and Friday for 17 weeks. RIT at a dose of 375 mg/m^2/dose IV is administered every other week staring on the 3d week for 8 doses. All patients received PCP, herpes virus, and fungal prophylaxis as well as CMV viral DNA monitoring. Responses were based on NCI-WG 1996 criteria; however, lymphadenopathy and organomegaly were also assessed by serial CT scans. Minimal residual disease (MRD) was measured in peripheral blood and bone marrow aspirate using flow cytometry for $CD19^+/CD5^+$/CD23 lymphocytes. Patients' characteristics: Since September 2005, 29 patients have been enrolled and 24 completed the therapy. All patients met ECOG criteria for requiring treatment. Median age was 53 years (28-87) with 15 males and 9 females; 22 Caucasian and 2 African American. The median time from the diagnosis to treatment was 25 months (2-144 months). Clinical stage (Rai) was I in 2 patients, II in 10 patients, III in 6, and IV in 6 patients. Median 2 microglobulin was 3.25 (0.34-15.3). Median WBC was 60 $x10^9$/L (5.2 – 158), Hgb 12.6 g/dL (7.8-14.7), and platelet count $157x10^9$/L (66 – 307). Cytogenetic

analysis, by FISH panel, was 13q- in 9, trisomy 12 in 8, and 13q-/11q- in 3, 11q- in 1, 11q-/p53/13q- in 1, 11q-/6q- in 1, and 13q-/11q-/6q- in 1 patient. Eleven patients were Zap70$^+$ and 4 patients were CD38$^+$. Mutational analysis is pending. *Results.* Based on the NCI-WG 1996 criteria, 19 patients (79%) achieved CR, 3 patients (13%) achieved PR, and 2 patients (8%) had stable disease. With utilization of CT scans responses were: 10 CR (42%), 11 PR (46%), and 3 SD (12%). At the completion of the study 16 patients (67%) had no evidence of MRD by flow cytometry. All 6 patients with 11q- achieved CR based on the NCI-WG 96 criteria. Median duration of the response has not been reached with a median follow-up of 26 months (1-42+). Four patients (18%) required alternative therapy for the disease progression at 6, 16, 25, and 34 months after the completion of study. Seven patients (29%), all of whom were baseline CMV IgG$^+$, had CMV reactivation by PCR. Two of them developed symptom of malaise and required hospitalization, none suffered organ involvement, and all of them cleared the infection with valgancyclovir administration. One patient suffered neutropenic fever requiring empiric antibiotic therapy followed by Clostridium difficile and adenovirus infections. No other serious infectious complications were documented. All patients developed grade 1-2 skin rash at the site of ALEM injection after the 1st dose of 3mg only; none required intervention. All patients developed grade 3-4 lymphopenia; neutropenia: grade 2 in 5, grade 3 in 5, and grade 4 in 3 patients; anemia: grade 1 in 9, grade 2 in 2 patients; thrombocytopenia: grade 1 in 10, grade 2 in 4, and grade 3 in 3 patients. Ten patients have not achieved full T cell recovery (CD4 >200) by 1 (n=7), 2 (n=1), 24 (n=1), and 34 (n=1) months. The other 14 patients achieved T cell recovery by 1 (n=2), 4 (n=2), 6 (n=1), 7 (n=2), 9 (n=2), 10 (n=1), 12 (n=1), 13 (n=1), 15 (n=1), and 16 (n=1) months. All of the patients who suffered CMV reactivation achieved faster T cell recovery than those who did not (median time 5 vs. 22 months). Two patients developed clinically significant autoimmune hemolytic anemia and one patient who had large cells present at the diagnostic BM biopsy suffered Richter's transformation. No death occurred. *Discussion.* Combination of ALEM and RIT is well tolerated and active regimen for patients with CLL and may represent a viable alternative to the combination chemotherapy. Complete data on all 29 patients will be presented at the meeting.

Novel treatment approaches

10.1

ANTILEUKEMIC ACTIVITY OF VALPROIC ACID IN CHRONIC LYMPHOCYTIC LEUKEMIA B-CELLS DEFINED BY MICROARRAY ANALYSIS

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Epigenetic code modifications by histone deacetylase inhibitors (HDACi) have recently been proposed as potential new therapies for hematological malignancies. Chronic Lymphocytic Leukemia (CLL) remains incurable despite the introduction of new treatments. CLL B-cells are characterized by an apoptosis defect rather than excessive proliferation, but proliferation centers have been found in organs such as bone marrow and lymph nodes. Here, we investigated gene expression modifications in CLL B-cells following treatment with valproic acid (VPA), a well-tolerated anti-epileptic drug with HDAC inhibitory activity. CLL B-cells obtained from 14 patients were treated in vitro with a concentration of 1mM VPA for 4 hours. VPA effects on gene expression were thereafter studied using Affymetrix technology, and some identified genes were validated by real-time PCR and western-blot. We observed that VPA induced apoptosis by downregulating several anti-apoptotic genes and by upregulating pro-apoptotic genes. Furthermore, VPA significantly increased chemosensitivity to fludarabine, flavopiridol, bortezomib, thalidomide and lenalidomide. VPA could inhibit the proliferation of CpG/IL2 stimulated CLL B-cells and modulated many cell cycle mRNAs. In conclusion, exposure of CLL B-cells to VPA induced apoptosis, potentiated chemotherapeutic agent effects and inhibited proliferation. These data strongly suggest the use of VPA in CLL treatment, particularly in combination with antileukemia agents.

10.2

A NOVEL HSP-90 INHIBITOR SNX7081 SHOWS IN VITRO ACTIVITY AND SYNERGY WITH FLUDARABINE IN CHRONIC LYMPHOCYTIC LEUKEMIA

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Introduction. The molecular chaperone heat-shock protein 90 (Hsp-90) has been proposed as a therapeutic target in CLL. This represents a promising strategy since Hsp-90 inhibition induces apoptosis in CLL cells irrespective of poor risk features, including aberrations of the p53 pathway. Many of the Hsp90 inhibitors which have reached clinical trial are derivatives of geldanamycin (GA). A recent clinical trial of the GA derivative, 17-AAG in CLL was terminated due to toxicity. Serenex (Pfizer) have developed a family of novel synthetic inhibitors of Hsp-90, which lack the benzoquinone structure believed to confer the toxicity of the GA derivatives. In preclinical and early phase I trials for CML the Serenex compounds displayed superior pharmacodynamic properties to the GA derivatives. The current study is a comparison of the activity of one of the Serenex compounds, SNX7081, with 17-AAG *in vitro* and investigates whether there are synergistic combinations of this Hsp90 inhibitor with fludarabine (FdA) that may represent a novel therapeutic strategy in CLL. Methods: Mononuclear cells were isolated by density centrifugation from CLL patients following informed consent. MTT (3-4, 5-dimethylthiazol-2,5-diphenyl tetrazolium bromide) and flow cytometric assays were used to evaluate sensitivity to 17-AAG and SNX7081 alone and in combination with FdA at doses determined by the Ic50 of the single agents. ZAP-70 expression and p53 functional status were evaluated by flow cytometry using previously published methodologies [Orchard *et al.*, Lancet, 2004; Best *et al.*, Leukemia, 2008]. Importantly, the p53 functional assay employed represents a sensitive method for identifying and discriminating between cases which harbour ATM or p53 mutations [Best, 2008]. All samples were defined as functionally normal (CatN), p53 (Cat1) or ATM (Cat2) dysfunctional. *Results.* 15 patient samples were selected on the basis of their ATM/p53 and ZAP-70 status; 3 and 4 were Cat 1 or Cat 2 dysfunctional respectively and 6 were ZAP-70 positive. A good correlation between functional status and loss of the ATM/p53 regions by FISH was observed; all 7 ATM/p53 dys-

functional cases had loss of the corresponding chromosomal locus. 1 of the 8 functionally normal cases had loss of 11q23, none had loss of 17p13. As single agents 17-AAG and SNX7081 significantly reduced viable cell number irrespective of the level of ZAP-70 expression or ATM/p53 functional status. The Ic50 of SNX7081 (0.94±0.53 μM) was significantly lower than that of 17-AAG, (7.25±3.26 μM) (p<0.0001). No significant difference in the Ic50 was observed between the ZAP-70 or functional categories for either agent. PBMCs obtained from healthy donors were resistant to both inhibitors. Marked synergy between SNX7081 and FdA was observed at clinically achievable FdA concentrations in the 7 cases with ATM/p53 dysfunction. In 2 of the 8 Cat N and all 3 samples of normal PBMC antagonism was observed. The combination index for each of the functional categories was 1.38±0.93 (CatN), 0.12±0.06 (Cat 1) and 0.43±0.31 (Cat 2), where values <1 or >1 represent a synergistic or antagonistic response respectively. As ZAP-70 is a `client' protein of Hsp90, we investigated the effect of SNX7081 treatment on the proportion of both B and T-cells expressing ZAP-70. In 8/8 ZAP-70 positive patient samples, exposure to 1 μM SNX7081 for 24 hours significantly decreased the percentage of B, but not T-cells, expressing ZAP-70; all 8 cases were deemed ZAP-70 negative following treatment according to the assay used [Orchard, 2004]. *Conclusions.* The Hsp-90 inhibitor SNX7081 is active in CLL. The combination of SNX7081 and FdA shows a high level of *in vitro* synergy, particularly against cells from CLL patients with aberrations of ATM and p53. Our data suggest that the inhibitors developed by Serenex may represent a novel and potent method of targeting Hsp-90 in CLL cells. Work is ongoing to investigate the mechanism of the synergy between SNX7081 and FdA.

10.3

THE HISTONE DEACETYLASE INHIBITOR SUBEROYLANILIDE HYDROXAMIC ACID (SAHA) DOWNREGULATES THE CXCR4 CHEMOKINE RECEPTOR AND IMPAIRS MIGRATION OF CHRONIC LYMPHOCYTIC LEUKEMIA CELLS

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Chronic lymphocytic leukemia (CLL) is a neoplastic disorder that arises primarily as a result of defective apoptosis. The SDF-1/CXCR4 axis has been shown to play an important role in CLL cell trafficking and survival. Since histone acetylation is involved in the modulation of gene expression, we evaluated the effects of suberoylanilide hydroxamic acid (SAHA), a histone deacetylase inhibitor, on CLL cells and in particular on cell survival, CXCR4 expression, and migration. Here, we showed that a 48 hour treatment of SAHA induced a dose-dependent decrease in CLL cell viability via apoptosis (n=20, p=0.0032). This effect was also seen in previously untreated and chemo-resistant CLL patients. Using specific caspase inhibitors, we demonstrated the participation of caspases-3, -6 and -8, suggesting an activation of the extrinsic pathway. Additionally, SAHA significantly decreased CXCR4 mRNA (n=10, p=0.0010) and protein expression (n=25, p<0.0001). As a result, CLL cell migration in response to SDF-1 (n=17, p=0.0003) or through bone marrow stromal cells was dramatically impaired. In conclusion, SAHA induces apoptosis in CLL cells via the extrinsic pathway and downregulates CXCR4 expression leading to decreased cell migration. SAHA (alone or in combination with other drugs) represents a promising therapeutic approach to inhibiting migration, CLL cell survival and potentially overcoming drug resistance.

10.4

RESISTANCE TO THE NOVEL TRANSLATION INHIBITOR SILVESTROL IS MEDIATED BY UPREGULATION OF MCL-1 IN A B-LEUKEMIA CELL LINE

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We recently reported the potent anti-tumor effects of silvestrol in CLL and demonstrated that this agent *in vitro* shows nanomolar potency, B-cell selectivity relative to T-cells, and activity that is equivalent in cells from patients with or without del(17p13). In vivo, silvestrol maintains B-cell selectivity in Tcl-1 mice and significantly prolongs survival in an ALL xenograft model. We further showed that silvestrol's target in B-cells is selective translational inhibition, with subsequent loss of Mcl-1 and concomitant increase of Bmf and Bak. Silvestrol was confirmed by Bordeleau et al. to directly block translation at the initiation step. However, no information is available regarding mechanism of resistance to silvestrol. We first investigated this question using HL-60 cells with overexpression of MDR-1 or MRP-1, members of the ATP-binding cassette transporter (ABC) family. MRP-1 over-expressing cells show moderate resistance to silvestrol, and MDR-1 over-expressing cells are completely resistant to this agent at concentrations up to 80 nM (IC_{50} of parental HL-60 < 5 nM). These data indicate that silvestrol is a substrate of ABC proteins, and is therefore unlikely to be effective in tumors with strong expression of these factors. We then incubated the ALL cell line 697 with gradually increasing concentrations of silvestrol to generate a cell line (697-R) with resistance to 30 nM silvestrol (IC_{50} of parental 697 < 5 nM). Expression of MDR-1 or MRP-1 remains undetectable in both 697 and 697-R, suggesting that increased ABC protein expression is not involved in the observed silvestrol resistance. However, Mcl-1 protein expression is strikingly increased in untreated 697-R cells relative to the parental line, although these cells still show similar percent-wise reduction in Mcl-1 upon re-exposure to 80 nM silvestrol. To investigate whether the observed silvestrol resistance is reversible, 697-R cells were maintained without silvestrol for 6 weeks (~18 passages). During this time, viability remained near 99%. Cells were then treated with 30 nM silvestrol. Viability was 94% at 48 hr post-treatment and returned to 99% within a week, while parental 697 cells with the same treatment were completely dead. Baseline Mcl-1 levels remained elevated in 697-R even with prolonged silvestrol-free incubation. This result indicates that the resistance phenotype is not rapidly reversible, as is seen with transient upregulation of multi-drug resistance or stress-response proteins. No differences between 697-R and the parental line were detected upon detailed immunophenotyping. However, cytogenetic analysis revealed a balanced 7q;9p translocation in 697-R not present in the parental 697 cell line, which may be related to the development of resistance. These results suggest that increased Mcl-1 protein mediates resistance to silvestrol, underscoring the importance of Mcl-1 in the mechanism of silvestrol-mediated cytotoxicity. We are now investigating the mechanism of Mcl-1 upregulation in 697-R cells to identify a factor or pathway that can be targeted therapeutically to circumvent resistance. Furthermore, silvestrol is now undergoing preclinical pharmacology and toxicology investigation by the U.S. National Cancer Institute Drug Development Group at the Stage IIA level, which will facilitate its progression to Phase I clinical testing.

10.5

OXALIPLATIN, FLUDARABINE, CYTARABINE, AND RITUXIMAB COMBINATION THERAPY INDUCES HIGH RESPONSE RATES IN AGGRESSIVE CHRONIC LYMPHOCYTIC LEUKEMIA AND RICHTER'S SYNDROME

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Introduction. The first Phase I-II clinical trial of oxaliplatin, fluradabine, Ara-C, and rituximab (OFAR1) demonstrated significant activity in refractory CLL and RS (Tsimberidou *et al*., J Clin Oncol, 2008;26:196). To enhance the response rate with a decrease in myelosuppression, the dose of oxaliplatin was increased to 30mg daily, the dose of Ara-C was decreased to 0.5 g/m^2 daily and the optimal number of days of fluradabine and Ara-C administration was explored (OFAR2). METHODS: In a Phase I-II study of OFAR2, patients were treated with oxaliplatin 30 mg/m^2, D1-4; fludarabine 30 mg/m^2, Ara-C 0.5 g/m^2; rituximab 375 mg/m^2, D3; and pelfigrastim 6 mg D6. Fludarabine and Ara-C were given on D2-3 (dose level 1) D2-4 (dose level 2) or D2-5 (dose level 3); courses were repeated every 4 weeks. Prophylaxis for tumor lysis, DNA viruses, and PCP was given. *Results.* A total of 82 pts have been treated to date: 12 pts enrolled in Phase I (dose level 1, n=3; dose level 2, n=6; and dose level 3, n=3). DLTs were noted in 2/3 pts on dose level 3 (G4 diarrhea, 1; G4 neutropenic sepsis, 1), therefore, level 2 was the MTD. Seventy patients were treated in the Phase II portion of the study (relapsed CLL, 54; RS, 16). Patient characteristics were as follows: age > 60 years, 63%; 17p deletion, 38%; 11q deletion, 22%; 13q deletion, 16%; trisomy 12, 19%; no findings, 5%; unmutated IgVH, 83%; ZAP70-positive, 67%;

and CD38 ≥30%, 60%. Response was evaluable in 56 patients (Table). The overall response rates in patients with 17p and 11q deletions were 50% and 63%, respectively. The median survival duration was 20 months. Twelve patients underwent stem cell transplantation after OFAR2 (as post-remission therapy, n=10; as salvage, n=2). Overall, 149 cycles were administered. Grade 3-4 neutropenia, thrombocytopenia, and anemia were noted in 73%, 83%, and 40% of patients and in 61%, 70%, and 26% of cycles and Grade 3-4 infections in 17% of cycles. *Conclusion.* Preliminary results demonstrate that OFAR2 induced response in 38% of patients with Richter's syndrome and 60% of patients with CLL. OFAR2 had antileukemic activity in patients with 17p deletion and clinical outcomes appeared to be superior to those of OFAR1. Accrual is ongoing.

Table 1.

	RS (N=13)		*CLL (N=43)*	
	No. of pts	*% RS pts*	*No. of pts*	*% CLL pts*
CR	1	8	2	5
nPR	-	-	6	14
PR	4	31	18	42
Fail / NE	7	54	15	35
Early death	1	8	2	5
OR	5	38	26	60

10.6

ENZASTAURIN ACTIVATES PROTEIN PHOSPHATASE 2A-DEPENDENT PRO-APOPTOTIC PATHWAYS IN CHRONIC LYMPHOCYTIC LEUKEMIC CELLS : RATIONALE FOR COMBINATION THERAPY

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Aims. Small molecule inhibitors of BCR-dependent signaling pathways in CLL open new opportunities for treatment. Protein Kinase C β inhibition with Enzastaurin (Enza) could be important, as this enzymes plays a role in the onset of disease in transgenic Tcl-1 mice. METHODS 30 CLL patients were included in this study. IC50 were measured over 5 days of culture with 05-10 µM Enza (pharmacologic doses in humans), and then correlated with disease parameters (Binet stage, CD38, IgVH mutational status, FISH+caryotype) to isolate biomarkers of efficacy. Protein Phosphatase 2A (PP2A) activity was measured with Upstate non radioactive kit. As a control, known activators of PP2A (forskolin and FTY720) were used in some experiments alone or in combination with Enzastaurin. Okadaic acid (OA) is a specific PP2A inhibitor at low nanomolar range (0.5-5 nM). Cell viability was assessed by trypan blue exclusion test or Annexin V-FITC staining, Fludarabine was used at 1µg/ml, Rituximab at a saturating dose of 10 µg/mL. RESULTS Median IC50 at 5d was 5µM (range 1 to >10 µM), delineating resistant (>50% cell survival after 5d with Enza 5 µM, n=11) and responding patients (<50% cell survival after 5d with Enza 5µM, n=19, $p<0.05$). The subgroup of patients with deletion 11q or 6q (del 11q/6q) was found to harbour significantly more resistance (45.5% vs 11% resistant patients without del11q/6p, $p=0.046$). Interestingly, neither IgVH UNMUT nor del17p seemed to confer specific refractoriness to Enza. Apoptotic death is triggered (evidenced by PARP cleavage), which is blocked by pan-inhibition of caspases with Z-VAD-fmk ($p<0.05$). Two major pro-survival proteins in CLL (Mcl-1 and Bcl-2 phosphorylated on Serine 70 residue) were decreased upon treatment in responding samples only. Moreover, dephosphorylation of Glycogen Synthase Kinase 3beta (GSK3β), a widely used biomarker of Enza activity, was complete in responding patients, only partial in resistant samples. Interestingly, serine kinases upstream of GSK3β (Akt, mTOR, ERK, and even PKC as assessed by Ser660 phosphorylation) that control its inhibition by phosphorylation were not modulated by Enza. The hypothesis of a phosphatase activation rather than a kinase inhibition with Enza seemed plausible. To this end, PP2A activity was shown to be significantly increased with Enza 5 µM (+40% as compared to DMSO, $p<0.01$), but prevented by co-incubation with OA 5nM ($p<0.05$). OA restored Mcl-1 expression, Bcl-2 and GSK3β phosphorylation levels, and partially cell death upon Enza 5 µM treatment for 48h (cell survival DMSO=100%, Enza=56.7%, OA+Enza=87%, n=6, $p=0.019$), thus indicating the importance of PP2A activation in Enza activity. Moreover, Forskolin 20µM pre-treatment sentized cells to low dose Enza (1µM) induced cell death. We next focused on the mechanism by which PP2A activation occurs, since death induced by FTY720 does not involve Bcl-2 or GSK3β dephosphorylation. Enza did not induce down-regulation of PP2A inhibitors (SET (its main transcriptional repressor) or PP2A-A), nor up-regulation of its subunits (PP2A-B' regulatory or PP2A-C catalytic). On the other hand, dephosphorylation of Tyrosine 307 (an inhibitory residue in the catalytic site) was noticed, a surrogate for PP2A-C activation. Since Bcl2-Ser70 phosphorylation and/or Mcl-1 have been implicated in many drug resistance phenomenon (to immunochemotherapy, Rituximab, but also BH3 mimetics), CLL cells from 5 Enza resistant patients were incubated with Fludarabine (F) ±Enza (E) for 4d. Chemosensitization was obvious, as well as enhanced Mcl-1 degradation and Bcl-2 dephosphorylation with F+E (cell survival : DMSO=100%, E=87.4%, F=74.4%, F+E=48.9%, n=5, $p=0.046$). Same results were obtained with Enza+Rituximab in 3 CLL samples with del17p (% Annexin V + cells : DMSO IgG control=1.2% vs RTX 2.9%, $p=0.3$, ENZA IgG control=19.3% vs RTX 38.7%, $p=0.012$) CONCLUSION We describe a new mode of action of Enzastaurin in CLL cells through phosphatase activation, and propose PP2A activation could be a relevant pro-apoptotic switch able to induce multi-drug chemosensitization. To this end, FTY720 and Enza could be used in combination since they do not activate PP2A in the same way.

10.7

TREATMENT WITH THE PKCBETA-SELECTIVE INHIBITOR ENZASTAURIN DELAYS LEUKAEMIA DEVELOPMENT IN A TCL1ATG MURINE MODEL OF B CELL CHRONIC LYMPHOCYTIC LEUKAEMIA

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B cell chronic lymphocytic leukaemia (B-CLL) follows a disease course that varies widely among patients depending on several factors that in part relate to the signalling capacity of the B cell receptor (BCR) complex. These include the mutational status of the IgVH genes that make up the BCR, as well as the expression of several BCR-associated molecules such as CD38 and ZAP-70 that can modulate the BCR signal. In addition, evidence for the role of antigenic input in the establishment or maintenance of the malignant clone highlights the BCR as a key molecule in the pathogenesis of CLL, and the components of the BCR signalling machinery as important therapeutic targets. We have recently validated PKCb as a potential therapeutic target in B-CLL. PKCb, a modulator of BCR signal strength, proved to be essential for the development of B-CLL. Targeted deletion of PKCb in TCL1a transgenic mice completely abolished CLL development in these mice. Importantly, loss of even one allele of PKCb significantly delayed CLL onset, implying that inhibition of PKCb to 50%, as is clinically achievable using chemotherapeutic compounds, could translate into a survival benefit in patients. We have used the murine model as well as primary patient-derived CLL cells to investigate the efficacy of the PKCb-selective inhibitor LY317615. HCl (Enzastaurin) in the treatment of CLL. Treatment of patient PBMCs with Enzastaurin led to an induction of Bmf and a decrease in Mcl-1, resulting in a significantly reduced viability of the $CD5^+$ $CD19^+$ B-CLL cells. Moreover, the sensitivity of the B-CLL cells to Enzastaurin was similar in all patient samples tested, regardless of their B-CLL risk group, or whether they were cultured in the presence of stromal cells which are known to confer a protective effect and survival advantage to CLL cells in culture. Finally, Enzastaurin treatment of C57BL/6 mice engrafted with tumor cells derived from a TCL1a tg mouse led to an increased overall survival compared to untreated controls. Taken together, we provide a rationale for the further development of PKCb inhibitors for the treatment of B-CLL.

10.8

QUALITY-ADJUSTED SURVIVAL (Q-TWIST) ANALYSIS OF RITUXIMAB PLUS FLUDARBINE AND CYCLOPHOSPHAMIDE (R-FC) VERSUS FC ALONE AS FIRST-LINE TREATMENT FOR CHRONIC LYMPHOCYTIC LEUKAEMIA

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Aim. The phase III randomized controlled trial, CLL-8, demonstrated that the addition of rituximab to fludarabine and cyclophosphamide (R-FC) versus FC alone significantly delayed disease progression in previously untreated chronic lymphocytic leukaemia (CLL) patients (Hallek et al., ASH 2008). The objective was to to assess the impact of treatment-related adverse events (AE) on overall survival from the CLL-8 trial by conducting a Quality-Adjusted Time Without Disease Symptoms or Treatment Toxicity analysis (Q-TWiST Gelber et al., 1995). *Methods*. Patients receiving at least one study treatment were included in the analysis (n=793; 26.4 months median follow-up). The area under survival curves was partitioned into health states: (1) Relapse (REL) - area between overall and progression free survival curves; (2) Toxicity (TOX) - time period with all treatment-related adverse events from start of study treatment to up to 28 days following last dose and/or progression; and (3) Time without disease symptoms or treatment toxicity (TWiST), defined as the area between the PFS and TOX curves. Utility weights were applied to each health state to reflect the QoL value relative to time in TWiST. Each utility weight ranged from 0 to 1, where 0 represents a state as bad as death, 1 represents a state as good as TWiST. For example, a utility weight of 0.5 for REL would indicate that 2 month of time after relapse is valued the same as 1 month in the TWiST state. Mean differences and 95% confidence intervals (CI) were calculated for each health state. *Results.* The unadjusted mean difference in survival between R-FC and FC was 1.35 months (95% CI, 0.01-2.65, p=0.1856). The combination of R with FC versus FC alone did not significantly increase the duration of toxicity (mean difference 0.43 months, 95% CI, 0.03-0.79, p=0.2777). The mean time R-FC patients spent in REL was 4.33 months (95% CI, 2.17-6.33, p<0.001) shorter than observed with FC (FC 7.42 vs R-FC 11.75 months). Patients in both treatment arms experienced TWiST, but R-FC patients achieved a 5.25 month additional benefit compared to FC alone (95% CI: 3.37-7.16, p<0.0001). Q-TWiST for R-FC versus FC was calculated as (uTOX x TOX)+(uREL x REL)+(uTWiST x TWiST). With uTOX = 0.618, uREL= 0.618 (Hancock et al., 2002) and uTWiST =1, R-FC patients experienced a mean of 2.91 months longer Q-TWiST compared with FC (95% CI, 1.75-4.08 , p<0.01). Utility combinations (between 0.1-0.9) for TOX and REL with a TWiST utility of 1 resulted in a statistically significant (p<0.05) gain in Q-TWiST for R-FC patients. *Conclusions.* This Q-TWiST analysis based on 26.4 months follow-up from the CLL-8 trial demonstrates that patients treated with R-FC experience longer time without disease progression with less time spent with disease symptoms and no significant increase in treatment toxicity compared to FC alone.

10.9

THE TREATMENT OF RELAPSED/REFRACTORY CHRONIC LYMPHOCYTIC LEUKEMIA/SMALL LYMPHOCYTIC LYMPHOMA (CLL) WITH EVEROLIMUS RESULTS IN CLINICAL RESPONSES AND MOBILIZATION OF CLL CELLS INTO THE CIRCULATION

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Patients with relapsed/refractory chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL) often have chemotherapy resistant disease resulting in a poor prognosis. Rapamycin (sirolimus) is a highly specific inhibitor of the mammalian target of rapamycin (mTOR) which is a multifunctional signal transduction kinase with a critical role in the signal transduction pathway linking growth stimuli with cell cycle progression. Rapamycin has been demonstrated to induce apoptosis in CLL cells *in vitro* although the required concentration was higher than is achievable *in vivo* and the clinical significance of this finding remains uncertain. In addition, the rapamycin analog temsirolimus is effective in the treatment of relapsed/refractory mantle cell lymphoma. The aim of this study was to learn if inhibition of mTOR by an oral drug (everolimus) would produce tumor responses in indolent lymphoid malignancies. *Methods.* This was a phase II study of oral single-agent everolimus (10 mg/day) for relapsed/refractory indolent lymphoid malignancies including CLL. *Results.* Four of 22 patients with CLL (18%, 95% CI: 5-40%) achieved a partial remission to therapy. An unanticipated finding in this study was an increase in the absolute lymphocyte count (ALC) associated with a decrease in lymphadenopathy in 8 (36%) patients. The ALC increased a median of 4.8 fold (range, 1.9-25.1), and the clinically measurable lymphadenopathy decreased a median of 75.5% (range, 38-93) compared to baseline measurements (Figure). Fourteen patients experienced grade 3-4 hematologic toxicity and 7 patients experienced grade 3-5 non-hematologic toxicity which was at least possibly attributable to treatment. Grade 3-4 anemia, neutropenia, and thrombocytopenia occurred in 23%, 32%, and 50% of patients, respectively. The most common non-hematologic toxicities were pneumonia (n = 2) and hypertriglyceridemia (n = 2). Two patients died from infections. *Conclusion*. Everolimus has modest anti-tumor activity against CLL but can mobilize malignant cells from nodal masses into the peripheral circulation in a subset of CLL patients. Because CLL cells in lymphatic tissue and bone marrow are more resistant to therapy than circulating CLL cells, the ability of everolimus to mobilize CLL cells into the circulation could be utilized in combination therapeutic regimens. This is being tested in a recently opened phase I/II trial of everolimus and alemtuzumab for the treatment of relapsed/refractory CLL. This study was supported by University of Iowa/Mayo Clinic NIH SPORE Grant CA97274 and Novartis Oncology.

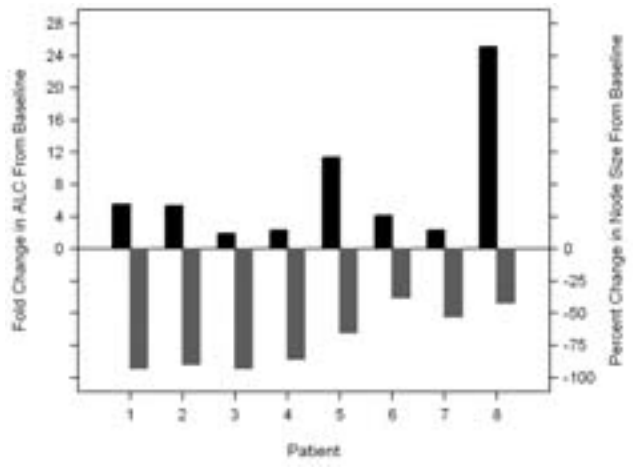

Figure 1. Maximum decrease in the clinically measurable lymphadenopathy (node size grey bars) and associated increase in the absolute lymphocyte count (ALC, black bars) in 8 patients with CLL treated with everolimus.

10.10

TARGETING THE LEUKAEMIC MICROENVIRONMENT: THE ROLE OF SRC/ABL TYROSINE KINASE INHIBITORS IN COMBINATION THERAPIES FOR CHRONIC LYMPHOCYTIC LEUKAEMIA

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Introduction. Chronic lymphocytic leukaemia (CLL) is the most common leukaemia in the western world, and remains incurable with current chemotherapy. CLL cells are dependent on micro-environmental signals for survival, evidenced by significant rates of spontaneous apoptosis observed on *in vitro* culture. Recent advances in CLL cell biology implicate signalling through the B cell antigen receptor (BCR) in the pathogenesis and progression of the disease. The absence of significant somatic hyper-mutation of the immunoglobulin heavy chain variable region (IgVH), which largely correlates with the expression of ZAP-70, is a significant adverse prognostic marker in the disease. CLL cases expressing an unmutated IgVH gene (U-CLL) retain the ability to signal through the BCR. The non-receptor tyrosine kinases Lyn (a Src kinase) and c-Abl are both required for effective BCR signalling, and both are over-expressed, and constitutively active in CLL. Dasatinib is a dual Src/Abl tyrosine kinase inhibitor in clinical use for chronic myeloid leukaemia. We have studied the anti-leukaemic effects of dasatinib on CLL cells *in vitro*. *Results.* Clinically relevant concentrations of dasatinib (100 nM) both inhibited ERK-MAPK and Akt phosphorylation, and prevented calcium mobilisation following BCR crosslinking. CLL cell apop-

tosis (confirmed by annexin V staining) was also observed following 48 hr continuous exposure to dasatinib, with an average reduction in viability of 34.8±19.8% (n=24). Neither ZAP-70 expression nor cytogenetic abnormalities were predictive of response. In addition, dasatinib exhibited synergy with both fludarabine and chlorambucil, with mean ED50 combination indices of 0.29 and 0.62 respectively. Additional micro-environmental elements responsible for the maintenance and progression of CLL include stromal or 'nurse-like' cells and activated T lymphocytes, the latter expressing CD40 ligand and secreting IL-4. CLL cell co-culture with the murine bone marrow stromal cell line NT-L significantly inhibited both spontaneous and dasatinib induced apoptosis. As we observed that stromal co-culture significantly increased phosphorylation of both ERK-MAPK and the Akt/mTOR target p70 S6 kinase in CLL cells, we investigated whether pharmacological inhibition these signalling pathways may re-sensitise CLL cells to dasatinib in co-culture. Both the MEK inhibitor PD98059 and PI3K inhibitor LY294002 significantly increased CLL cell sensitivity to dasatinib in co-culture with NT-L cells. Dasatinib retained the ability to potentiate the effects of fludarabine and chlorambucil in stromal co-culture, however CLL cells co-cultured with NT-L cells stably transfected with CD40 ligand (CD154-L cells) were resistant to these combinations. Moreover, dasatinib treatment of CLL cells co-cultured with CD154-L and IL-4 failed to prevent up-regulation of Bcl-xL and Mcl-1, or inhibit CLL cell proliferation. *Conclusions.* Dual src/abl tyrosine kinase inhibitors are promising novel agents for CLL, able to inhibit BCR signal transduction and induce apoptosis of CLL cells *in vitro*. However, our co-culture data suggest that combination of these agents with drugs that target additional micro-environmental signalling pathways may be required to effectively target CLL cells within lymph node and bone marrow niches, and achieve optimum clinical responses.

10.11

ACTIVITY OF 4-HPR ON IN VITRO CULTURED LEUKEMIC B CELLS DERIVED FROM CLL PATIENTS

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N-(4-hydroxyphenyl)retinamide (4-HPR) is a synthetic retinoid with chemopreventive and cytotoxic activity against various cancers. Although its mechanism of action has not been fully elucidated, it is recognized that 4-HPR triggers p53-independent apoptosis that is elicited up-stream by Reactive Oxigen Species (ROS) generation in several solid and hematologic tumor cells and inhibited by Bcl-2 overexpression. It involves mitochondrial apoptotic pathways and was observed to decrease anti-apoptotic Bcl-2 family members and activate pro-apoptotic BH3-only and effector Bcl-2 family proteins. Importantly, 4-HPR exerts no or very mild cytotoxicity on normal peripheral blood lymphocytes or normal bone marrow cells *in vitro*. Also, evaluated in a number of large therapeutic and chemopreventive clinical trials, HPR has demonstrated minimal toxicity. To date, no studies have examined the *in vitro* sensitivity of ex-vivo derived B-CLL cells to 4-HPR. One multi-Institutional Phase I Trial of Intravenous Fenretinide (4-HPR) (NCI-06-C-0227) has started for patients with lymphomas or chronic haematologic malignancies with poor prognosis, including B-CLL, but only for those patients with relapsed disease and refractory to any standard treatments. No data are yet available. By multiparameter flow cytometry and confocal microscopy we aimed to elucidate the effects of 4-HPR on ex-vivo isolated B-cells from B-CLL patients, with particular regard to the cytotoxic pathway elicited by the drug, and to the biological factors that regulate the cellular sensitivity to the drug. We found that 4-HPR induces dose-dependent apoptosis B-CLL cells and that its activity is mediated by induction of ROS generation and dissipation of the mitochondrial transmembrane potential. Activation of the pro-apoptotic effector Bcl-2 family members Bax and Bak is preceded by up-regulation of the "sensitizer" BH3-only Bcl-2 family molecules Noxa and Puma, and down-modulation of the anti-apoptotic protein Mcl-1. Decreased, though still significant, apoptosis is observed when B-CLL cells *in vitro* are subjected to microenvironment stimuli such as ligation of CD40 by CD40L (CD154). Possibly due to the limited sample size of this still ongoing study the susceptibility to apoptosis upon 4-HPR treatment does not seem to be related to the molecular prognostic markers VH mutational status or CD38 expression, nor to the basal level of Bcl-2 family members. The results provide a first glance on the cytotoxic activity of 4-HPR on B-CLL cells and may be exploited for designing further and more detailed studies to assess whether 4-HPR could represent an attractive candidate for B-CLL treatment.

10.12

A PHASE I STUDY OF LENALIDOMIDE IN COMBINATION WITH FLUDARABINE RITUXIMAB IN PREVIOUSLY UNTREATED CLL/SLL

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Lenalidomide is an immunomodulatory drug recently reported to have single agent activity in relapsed/refractory CLL, with ORR 35-50% in patients with poor risk features. Recent efforts to improve initial therapy of CLL have led to chemoimmunotherapy combinations like FCR, which have high ORR and improved PFS, but at the cost of myelosuppression and infection. Given the high ORR reported with lenalidomide in the relapsed setting and its potential to spare immune function, we undertook this Phase I study to explore the safety and tolerability of lenalidomide in combination with fludarabine and rituximab. Eligibility criteria included a confirmed diagnosis of CLL/SLL, previously untreated with systemic therapy and in need of therapy by NCI-WG 1996 criteria; ANC > 1000, platelets > 50K, and adequate organ function. Six dose levels were planned, beginning with fludarabine 25 mg/m^2 days 1-3, rituximab 375 mg/m^2 day 1, and lenalidomide 2.5 mg administered on days 1-21 of a 28-day cycle. The study used a standard 3+3 dose escalation design, with dose limiting toxicity (DLT) assessed in the first 28 days. Nine patients were enrolled on this study, with a median age of 59 yrs (range 37-65). Six patients had Rai 3-4 disease, and three patients had bulky lymphadenopathy >5 cm on physical exam. The time from diagnosis to therapy ranged from 12-87 months. The first cohort enrolled four patients, of whom two developed DLTs and came off study: patient 2 had grade 4 neutropenia that persisted through day 50 and patient 3 developed a syndrome of fever, grade 3 rash, myalgias and grade 4 CK elevation. The two remaining patients in this cohort came off study after 3 cycles: one for inadequate response and the second for a second malignancy requiring other therapy. The study proceeded to enroll to dose level -1, with FR as above and lenalidomide 2.5 mg every other day for days 1 to 21 of a 28-day cycle. Five patients enrolled on this cohort, of whom only two completed the planned six cycles of therapy. The other three patients discontinued after 3 cycles due to toxicity: one had grade 2 thrombocytopenia persistent for >4 weeks, preventing cycle 4; the second had recurrent grade 3-4 neutropenia and thrombocytopenia, despite growth factor support, dose reduction and holding lenalidomide; and the third had intermittent recurrent grade 3 tumor flare, rash and hand-foot syndrome, along with recurrent grade 3 neutropenia despite similar measures as above. At that point the study was terminated due to significant myelotoxicity and idiosyncratic tumor flare, resulting in only two of nine patients completing the planned therapy. Correlative science studies are in progress but a preliminary evaluation of T cell numbers suggests a decline during study therapy despite the addition of lenalidomide. We conclude that administering lenalidomide concurrently with fludarabine and rituximab is poorly tolerated and limits the ability to deliver adequate therapy with either drug. Other trials currently in progress are exploring alternative schedules of lenalidomide administration with standard CLL chemotherapy regimens; our data favor a sequential schedule.

10.13

GENE EXPRESSION PROFILE OF B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA CELLS EXPOSED TO THE NON-GENOTOXIC P53 ACTIVATOR NUTLIN-3

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Background. p53 plays a key role in determining the clinical features of B cell chronic lymphocytic leukemia (CLL). Disruption of p53 by point mutations, deletion at 17p13, or both, occurs in a fraction of cases at diagnosis and predicts poor survival and chemorefractoriness. In cells with functional p53, p53 activity is inhibited through interaction with MDM2. In fact, p53 can be activated upon exposure of cells to inhibitors of p53/MDM2 interaction, like Nutlins. Exposure of CLL cells to Nutlin-3 is effective in raising the levels of p53 protein with subsequent induction of cell cycle arrest and/or apoptosis, independently of the most relevant prognostic markers. *Aim*. To analyze the gene expression profile (GEP) induced by Nutlin-3 exposure in primary CLL cells from p53(wt) and p53(del/mut) cases. *Methods.* Purified cells from 24 PB CLL samples, all characterized for IGHV mutational status, CD38 and ZAP-70 and p53 mutations (16 p53(wt) CLL, 8 p53(del/mut) CLL of which 6 with del17p13 and p53 mutations, 1 with del17p13 alone, and 1 with p53 mutations alone), were exposed to 10 microM Nutlin-3 for 24 hours. GEP was performed using a dual labelling strategy; the differential expression of the below reported genes were validated by quantitative real-time PCR. *Results.* i) signature of Nutlin-3 exposure in p53(wt) CLL: 144 differentially expressed genes (143 up-regulated, 1 down-regulated) were correlated with response to Nutlin-3. Among the over-expressed genes, several genes were related to apoptosis (e.g. BAX, BBC3, E124, IKIP, FAS, LRDD, FLJ11259, TRIAP1, GADD45, TP53INP1, ISG20L1, ZMAT3, TNFRS10C, TNFRSF10B/TRAIL-R2), while other genes (e.g. MDM2, CDKN1A, PCNA) were up-regulated by Nutlin-3 as a part of a negative feed-back mechanism. Of note, this signature was not shared by 3/16 p53(wt) cases (identified as "non-responder" p53(wt) CLL) and 7/8 p53(del/mut) cases (identified as "non-responder" p53(del/mut) CLL); consistently, cells from these cases were also significantly resistant to the in-vitro cytotoxic effects of Nutlin-3; ii) signature of Nutlin-3 "non-responder" p53(wt) CLL: by comparing the constitutive GEP of 13 "responder" versus 3 "non-responder" p53(wt) CLL, we obtained 278 differentially expressed genes, 149 up-regulated and 129 down-regulated in "non-responder" p53(wt) CLL. Among up-regulated genes, we focused on MDM4/MDMX, a gene whose product was known to have an inhibitor activity of p53-dependent transcription and to form Nutlin-3 resistant complexes with p53. Among down-regulated genes, validations were made for BIRC4BP, whose product is known to act as an antagonist of the anti-apoptotic protein XIAP; iii) signature of Nutlin-3 "non-responder" p53(del/mut) CLL: by comparing the constitutive GEP of 13 "responder" versus 7 "non-responder" p53(del/mut) cases, we obtained 72 differentially expressed genes, 26 up-regulated and 46 down-regulated (31/46 located at the 17p segment) in "non-responder" p53(del/mut) CLL. Among down-regulated genes, validations were made for several genes whose products display pro-apoptotic activities (e.g. PSMB6, RPL26 and ZBTB4, located at 17p segment, and GNAZ located at chromosome 22). Among up-regulated genes, we focused on ARHGDIA, whose gene product displays anti-apoptotic activities and mediates cellular resistance to chemotherapeutic agents. *Conclusions.* specific genesets and GEP were documented to be associated with response or resistance to Nutlin-3 exposure in p53(wt) or p53(del/mut) CLL. These findings may help to identify novel molecular targets for CLL therapy.

10.14

NOT PUBLISHED

10.15

BRYOSTATIN ENHANCES THE CYTOTOXIC EFFECTS OF ANTI-CD22 IMMUNOTOXINS IN CLL BY TWO DISTINCT MECHANISMS: IMPLICATIONS FOR A SEQUENTIAL THERAPY

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Recently, treatment of CLL has been improved by the addition of Rituximab, a monoclonal antibody directed against CD20. Although Rituximab displays modest activity when used as monotherapy, the combination of Rituximab and chemotherapy has demonstrated impressive efficacy in CLL. However, even though remissions can be achieved in the majority of patients, CLL still remains an incurable disease for most patients. Therefore, alternative treatment options are needed. BL22 is a recombinant protein composed of the variable region of a monoclonal antibody, which binds to CD22 on the surface of normal and malignant B-cells and of PE38, a truncated Pseudomonas exotoxin. Previously, we demonstrated that BL22 induced cell death in CLL, involving the intrinsic apoptotic pathway. However, apoptosis induction correlates with expression of CD22 on the surface of CLL cells and was only moderate in CD22low expressing cells. The aim of this study was to increase BL22 cytotoxicity by modulating the surface expression of CD22 on CLL cells. Bryostatin is a macrocyclic lactone which structurally mimics the PKC-activating second messenger diacylglycerol. This PKC modulator has demonstrated anti-leukemic effects in CLL by itself *in vivo*. Here we show that Bryostatin up-regulates the expression of CD22 on CLL cells in a PKC dependent pathway, thereby increasing the cytotoxic effects of BL22. However, PKC-mediated up-regulation of Mcl-1 attenuated the anti-leukemic effects of BL22. We unravel that CD22 and Mcl-1 up-regulation by Bryostatin occurs in a dose dependent manner. Importantly, higher doses of Bryostatin prevented the undesirable up-regulation of Mcl-1 by inhibition of PKC in spite of CD22 up-regulation. Therefore, combined treatment of BL22 and Bryostatin seems to be a promising approach to enhance the cytotoxic effects of the immunotoxin by modulating PKC activity and CD22 up-regulation. In addition, our data provide evidence for a sequential therapy with both drugs, since CLL cells can be primed with Bryostatin. After a single dose of Bryostatin, PKC-modulation lasts for several days, allowing to administer BL22 at later time points, thereby decreasing the risk of potential drug interactions. Finally we demonstrate that stromal cell mediated drug resistance in CLL can be overcome by Bryostatin/ BL22 therapies. Conclusively, Bryostatin enhances the anti-leukemic effects of BL22 in CLL. Clinical trails are needed to prove if such an approach has clinical impact on the treatment and prognosis of B-CLL.

10.16

SORAFENIB BUT NOT BEVACIZUMAB INDUCES APOPTOSIS IN CLL

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Although disease remission in CLL can usually be achieved by conventional chemotherapy, nearly all patients relapse due to persistent leukemic cells in the bone marrow. Here, bi-directional interactions between stromal cells and leukemic cells provide sufficient protection from cytotoxic drugs. A role of VEGF in stromal cells support of CLL cells was observed. Bone marrow infiltrated by CLL cells displays increased vascular density, indicating active pro-angiogenic processes in the course of the disease. However, bone marrow stroma also induces a pro-angiogenic phenotype in CLL cells, suggesting mutual paracrine cross-talk involving angiogenic factors. Of known pro-angiogenic molecules, VEGF and its receptors (VEGF-R) have been extensively studied in CLL: VEGF signalling has been described to promote the survival of leukemic cells due to an autocrine feed-back loop, based on the secretion of VEGF by CLL cells. Furthermore, therapeutic approaches to inhibit VEGF signalling have previously been

shown to be effective against CLL. However, no currently approved inhibitor of VEGF has previously shown efficacy against CLL cells. To this end, we assessed the effects of the VEGF inhibitors Bevacizumab and Sorafenib on the survival of CLL cells. Strikingly, Sorafenib, but not Bevacizumab readily induced cell death of CLL cells, nearly killing all leukemic cells after 72 hours. Based on subgroup analysis (ZAP70 expression, CD38 expression, Binet stages A-C), Sorafenib was equally effective in all CLL subgroups; particularly heavily pre-treated patients as well as patients expressing ZAP-70 responded to the VEGF inhibitor. Importantly, Sorafenib overcomes stromal cell mediated, acquired drug-resistance. Sorafenib induced cell death was accompanied by substantial changes in the expression of anti-apoptotic proteins, namely Bcl-2, Mcl-1 and XIAP. Because disease progression in CLL is based on proliferative cells rather than on a "passive" accumulation of G1-arrested leukemic B-cells, we investigated whether Sorafenib was an active drug also in cycling CLL cells. Here we report that Sorafenib induces cell cycle arrest, accompanied by an increase in apoptotic cells. These data demonstrate that different cellular compartments in CLL patients can be targeted by Sorafenib. Because Sorafenib, but not Bevacizumab demonstrates efficacy in CLL cells, other targets than VEGF-R must mediate Sorafenib induced cell death. Results will be presented at the meeting. Conclusively, based on our pre-clinical data, Sorafenib constitutes a novel, very powerful therapeutic option for any patient requiring treatment for CLL. Our results warrant further clinical trials.

10.17

THE DUAL EFFECTS OF TARGETING DNA-DEPENDENT PROTEIN KINASE AND ATAXIA TELANGIECTASIA MUTATED KINASE IN CLL: DOUBLE HIT ON DNA REPAIR AND NF-KB

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Dysfunction of p53 (including del(17p) and p53 mutation) and defects in ataxia telangiectasia-mutated kinase (ATM) confer poor prognosis in CLL. This is due to the resulting impaired DNA damage response following drug treatment, which leads to therapeutic resistance. DNA-dependent protein kinase (DNA-PK) and ATM also mediate the repair of DNA double strand breaks (DSBs). We previously examined the function of DNA-PK and ATM in CLL using selective small molecule inhibitors developed in collaboration with KuDOS Pharmaceuticals, Cambridge, UK. We showed that high levels of the catalytic subunit of DNA-PK (DNA-PKcs) predict for shorter time to treatment, and that inhibition of DNA-PK sensitizes CLL cells ex vivo to a number of chemotherapeutic agents. Our recent studies demonstrate that inhibitors of DNA-PK (NU7441) and ATM (KU-55933) not only inhibit DNA repair, but also prevent activation of the stress-inducible transcription factor, NF-kappaB. Recent reports have shown that consititutive activation of NF-kappaB is frequent in CLL, and correlates with chemoresistance and poor survival. Using a cohort of well-characterized CLL cases (n = 113) in ex vivo experiments, we examined mitoxantrone sensitivity, DNA-PK and ATM activation, and NF-kappaB expression and kappaB DNA binding activity. NU7441 prevented drug-induced autophosphorylation of DNA-PKcs at ser2056. Similarly, KU-55933 inhibited ionising radiation-induced autophosphorylation of ATM at ser1981. Both inhibitors increased mitoxantrone-induced cytotoxicity, even in drug-resistant cases with del(17p), p53 mutation, del(11q) or unmutated IgVH genes. RT-PCR analyses revealed a strong correlation between DNA-PKcs mRNA levels and DNA-PKcs protein levels (p=0.008). High DNA-PKcs mRNA and protein levels predicted for shorter time to treatment (p=0.05), substantiating our previous data showing a similar effect for DNA-PKcs protein levels. Measurement of gammaH2AX foci following mitoxantrone treatment showed dose-dependent formation of DSBs that was increased (at 24 hr) by co-incubation with NU7441, indicating that DNA-PK mediates repair of mitoxantrone-induced DSBs in CLL. In contrast, KU-55933 reduced the level of mitoxantrone-induced gammaH2AX foci, consistent with the known role of ATM in gammaH2AX formation. We examined constitutive and drug-induced NF-kappaB DNA binding activity in a subset of cases. Preliminary data show that high nuclear levels of the p50 and p65 subunits of NF-kappaB correlate with poor survival. Furthermore, NF-kappaB activity, which was induced merely by culturing CLL cells, increases with time, (maximal at 6hr) and was highly stimulated by treatment with mitoxantrone. Co-incubation of cultured cells with either NU7441 or KU-55933 significantly reduced the kappaB DNA binding activity of both p65 and p50 and increased drug-induced cytotoxicity. These data suggest a novel role for these inhibitors, in driving apoptosis in drug-resistant CLL by inhibition of aberrantly activated NF-kappaB. Future studies will investigate the mechanism by which DNA-PK and ATM impact on NF-kappaB function. These results support the novel approach of targeting DNA-PK and ATM in CLL and provide a new insight into the multiple functions of these proteins. Since overexpression of DNA-PKcs and aberrant activation of NF-kappaB are a common feature of poor prognosis CLL, these data underline the potential of this targeted therapy to overcome drug resistance in CLL.

10.18

PRELIMINARY EVIDENCE OF CLINICAL ACTIVITY IN A PHASE 1 STUDY OF CAL-101, A POTENT SELECTIVE INHIBITOR OF THE P110DELTA ISOFORM OF PHOSPHATIDYLINOSITOL 3-KINASE, IN PATIENTS WITH RELAPSED OR REFRACTORY CHRONIC LYMPHOCYTIC LEUKEMIA

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Background. The class I phosphatidylinositol 3-kinases (PI3Ks) regulate diverse cellular functions relevant to oncogenesis, including metabolism, proliferation and survival. The four members of this PI3K family are designated p110α, p110β, p110delta and p110gamma. Expression of the p110delta isoform is largely restricted to cells of hematopoietic origin where it plays a key role in B cell maturation and function. Selective targeting of p110 delta may provide a therapeutic approach to inhibiting the PI3K pathway without adversely affecting insulin signaling mediated by p110α. CAL-101 is an oral, potent inhibitor of p110delta (IC_{50} of 2.5 nM against purified enzyme and EC50 of 65 nM in a whole blood assay) with 40 to 300-fold selectivity compared to other PI3K isoforms. Primary leukemic cells from patients with chronic lymphocytic leukemia (CLL) showed consistently high levels of p110delta expression and incubation of these cells *in vitro* with 1 to 10 uM CAL-101 resulted in induction of apoptosis. *Methods.* An ongoing Phase 1 study of CAL-101 is enrolling patients with relapsed or refractory CLL, select B-cell non-Hodgkin's lymphoma and acute myeloid leukemia; data from patients with CLL are presented. CLL patients were required to have had at least 2 prior therapies, including fludarabine. CAL-101 was administered orally twice daily (BID) for 28 days per cycle, with continuous dosing. Clinical response was evaluated according to the 2008 IWCLL criteria at the end of Cycles 1 and 2 and every 2 cycles thereafter. The study had an initial dose escalation component with a 3+3 cohort design, followed by cohort expansion. *Results.* To date, 16 patients with CLL have been enrolled across 4 dose levels: 50 mg BID (n=2), 100 mg BID (n=1), 200 mg BID (n=6) and 350 mg BID (n=7). Patient characteristics were median age 69 years, 88% male, 50% refractory disease and median number of prior regimens 4 (range 2 to 15), with 100% having prior fludarabine therapy, 81% prior alkylating agent, 100% prior rituximab and 50% prior alemtuzumab. At the time of data submission, mean duration of CAL-101 treatment was 12 weeks (range 2 to 36). At the cohort expansion dose levels of 200 mg and 350 mg, 12 patients were evaluable and 11 had >50% reduction in lymphadenopathy. Four patients had partial response (1 with del(17p), 1 with del(11q) and 2 with bulky lymphadenopathy) with the longest duration of response at 5 cycles and continuing; the other 7 patients had concurrent >50% increase in peripheral lymphocyte counts and were considered to have stable disease. No patient had Grade 3 or 4 hematological toxicity; one had Grade 3 transaminase elevation. At the end of Cycle 1, plasma concentrations were similar between the 200 mg and 350 mg dose groups, with mean peak and trough concentrations of 5 uM and 1 uM, respectively. Enrollment is continuing and updated data will be presented.

Conclusions. Preliminary results from a Phase 1 study of the oral PI3K p110delta inhibitor CAL-101 show that it has clinical activity in patients with relapsed or refractory CLL with acceptable toxicity.

10.19

ANTIBODY THERAPY WITH ALEMTUZUMAB, RITUXIMAB, AND GM-CSF FOR EARLY TREATMENT OF HIGH RISK CHRONIC LYMPHOCYTIC LEUKEMIA

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The standard of care in CLL is to treat patients with progressive and advanced stage disease. In patients with aggressive disease, this could increase the tumor burden prior to treatment and the risk of clonal evolution. More accurate prognostic markers and therapeutic monoclonal antibodies (MoAb) could be used to develop effective earlier treatments for patients with high risk CLL. This could be improved by GM-CSF which can enhance effector cell activity. We report the planned interim analysis of a clinical trial designed to test this hypothesis. *Methods.* This two stage phase II study measured the complete and overall response and safety of early treatment of patients with high-risk CLL with alemtuzumab, rituximab, and GM-CSF. CLL was diagnosed with NCI-WG1996 criteria. Eligibility required early-intermediate stage disease (Rai 0-II) without indications for treatment. High risk CLL was defined as at least 1 of the following: 1. 17p13-; 2. 11q22- and; 3. VH3-21/umutated IGVH and CD38/ZAP-70 expression. Patients were treated with GM-CSF (250 mcg sc 3 x week) for 6 weeks starting on day 0, alemtuzumab 3-10-30 mg sc over 3 days starting on day 3 then 30 mg sc 3x 4 weeks, and rituximab 375 mg/m^2 IV weeks x 4 weeks starting on day 10. Patients received PCP and herpes virus prophylaxis for 7 months. CMV was monitoring by PCR weekly during treatment and then monthly x 6 and patients with detectable CMV were treated. Response was evaluated by NCI-WG1996 criteria at 2 months after therapy and the BM was examined with immunohistochemical staining for residual CLL. *Results.* We report the results of the planned interim analysis on the first 16 patients with a median age of 58.5 years (range 42-77) with 11 male and 5 females, who were treated a median of 10 months (range 1.3-52) after CLL diagnosis. Risk factors for study eligibility were 17p13- (n = 1), 11q22- (n = 5), and unmutated IgVH/VH3-21 and ZAP70$^+$/CD38$^+$ (n = 10). Fifteen (94%) patients responded to treatment with 4 (25%) CR, 2 (13%) nPR, and 9 (56%) PR. Only 1 patient with CR had no evidence of residual disease on BM re-examination. The median follow up was 10 months (range 2-3) with median time to progression of 7+ months (range 3-13+). All patients are alive and one patient has required additional treatment for CLL. Four patients had CMV reactivation responding to oral valganciclovir. Grade 3-4 toxicities at least possibly attributable to treatment were neutropenia (n = 1), febrile neutropenia (n = 1), and rash (n = 1). *Conclusions.* Interim analysis of this trial suggests that alemtuzumab, rituximab, and GM-CSF is effective and tolerable early treatment for high risk CLL. The trial is planned to accrue a total of 30 patients. This study was funded by University of Iowa/Mayo Clinic NIH SPORE Grant CA97274, Bayer Pharmaceuticals, Genzyme, and Genentech.

10.20

THE ANTI-INFLAMMATORY INVESTIGATIONAL AGENT LMP-420 DEMONSTRATES IN VITRO CYTOTOXIC ACTIVITY AGAINST CHRONIC LYMPHOCYTIC LEUKEMIA CELLS.

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Chronic Lymphocytic Leukemia (CLL) is a common incurable hematologic malignancy. When therapy is required, maximizing durable responses is often at the risk of increasing toxicity. Thus, developing novel therapeutic agents that have minimal overlapping toxicity with currently used chemotherapy would be advantageous. To this end, we investigated LMP-420, a boronic acid containing purine nucleoside analogue, that potently inhibits tumor necrosis factor α (TNF) transcription in stimulated peripheral blood mononuclear cells (PBMCs) without affecting cell viability. Since TNF has been implicated in promoting CLL cell viability and can be produced by CLL cells themselves, we hypothesized that LMP-420 would be cytotoxic for CLL lymphocytes, either alone or in combination with fludarabine. To test the activity of LMP-420, we negatively selected circulating CLL cells from blood collected from patients using the RosetteSep B-cell enrichment cocktail (StemCell Technologies) and a Ficoll-Hypaque gradient, which yields greater than 95% purity of malignant lymphocytes. We assessed the fractional toxicity and 50% effective dose (ED50) of LMP-420 with the MTS colorimetric cytotoxicity assay, in which CLL cells were incubated for three days in Hybridoma media + 10% fetal bovine serum and serial dilutions of LMP-420. The median ED50 of LMP-420 for CLL cells was 423 nM (range 0.01 to 2224 nM, n = 21). Two patients had high-risk cytogenetics (17p or 11q deletions), and their ED50 values for LMP-420 were 691 and 90 nM, respectively. The cytotoxic effect of fludarabine was potentiated on average 80 or 261 fold with the addition of LMP-420 at concentrations of 62 or 250 nM, respectively (ranges 1.14-947 and 1.19-2754). This agent killed malignant lymphocytes by apoptotic mechanisms in a dose-responsive fashion, as demonstrated by both Annexin V staining and caspase 3/7 activity assays. While LMP-420 has potent anti-CLL activity, it has minimal effects on normal hematologic cells. For example, fludarabine suppresses erythroid and myeloid colony formation by greater than 50% at a concentration of 1 uM, while this level of inhibition is seen for LMP-420 at a concentration of 90 uM. The average cytotoxic ED50 of LMP-420 on normal PBMCs using the MTS assay was greater than 90 uM, whereas for fludarabine, it was 5.3 uM. This finding was confirmed with apoptosis assays. The results of these experiments demonstrate that LMP-420, a novel inhibitor of TNF expression, has cytotoxic activity against CLL cells, including those with high-risk features. LMP-420 appears to increase the cytotoxic effect of the chemotherapy agent fludarabine, while imparting minimal increase in hematologic toxicity. Thus, LMP-420 is promising new therapeutic agent in CLL.

10.21

A SYSTEMATIC REVIEW OF TREATMENTS FOR PATIENTS WITH RELAPSED OR REFRACTORY CHRONIC LYMPHOCYTIC LEUKAEMIA

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Over the last decade CLL research has focused on different treatments and combinations with the goal of improving response, progression free survival (PFS), and ultimately overall survival(OS). The objective of this systematic review was to evaluate the evidence base for the efficacy and safety of alkylating or monoclonal antibody (MoAb) treatments as monotherapy or in combination with other chemotherapeutic agents for relapsed or refractory CLL. A systematic literature search was undertaken to identify randomised controlled trials (RCTs) and observational studies (non-RCTs) reporting efficacy and safety data in relapsed or refractory CLL. Medline, Embase, The Cochrane Library, and relevant conference proceedings were searched using both free text and MeSH search terms relevant to CLL treatments and study methodology. Outcomes of interest included rates of complete response (CR), overall response (OR), PFS, OS, disease-free survival, and duration of remission. Safety data were also collected. Studies were critically reviewed by at least two reviewers and data extracted into data extraction tables. Study quality was assessed according to Cochrane criteria for adequacy of randomisation and allocation concealment procedures, adequacy of blinding procedures and completeness of follow-up. Methods for data extraction and meta-analyses were pre-specified. The systematic review identified 2,318 articles, the majority of which failed to meet inclusion criteria. The most frequent reasons for exclusion were inappropriate disease (708 studies), no relevant outcomes (385 studies), and no relevant interventions (314 studies). Thirty-eight publications were retrieved in full but subsequently excluded. Thirty-one potentially relevant articles/abstracts were identified from reference lists and conference proceedings. Nine articles reported data from eight RCTs and 90 reported data from 86 individual non-RCTs. All the RCTs included an appropriate control treatment arm. The size and duration of RCTs ranged from 16-552 patients and six to 60 months. OS and PFS ranged from 24-33.8 (five RCTs) and 6-30.6 months (two RCTs), respectively, depending on the treatment regimens compared. Any meta-analysis of RCTs was deemed inappropriate due to heterogeneity in treatment comparisons, methodologies, and reported outcomes. Adverse event data were infrequently reported

and were often incomplete, selective or combined across treatment groups and/or study populations. Non-RCTs typically followed a (small) cohort group of patients undergoing treatment without a comparator treatment or patient group. Compared to RCTs, the non-RCTs were of similar size (5-724 patients), and duration (3-60 months) but reported on a wider range of treatment comparisons. OS and PFS were reported in 26 and 10 non-RCTs and ranged from 5.9-48 months and 6.5-24.5 months, respectively. The incidence of adverse events and serious adverse events was reported by only 6 and 5 publications and ranged from 19-82% and 5-90%. Mortality rates varied 0-91%. Multiple different treatment and treatment combinations were described. For MoAbs, the largest body of evidence was identified for rituximab (two RCTs, 22 non-RCTs) with the REACH RCT (Robak *et al.,* 2008) being the largest available study, to date. No RCTs were identified for alemtuzumab or ofatumumab. Unfortunately there is a lack of large, fully published, high quality RCT data in relapsed or refractory CLL, however a number of typically small non-RCTs have reported on various treatment regimens. Overall, disease response outcomes and OS were most frequently reported outcomes and survival outcome; however, PFS, which is potentially less affected by confounding and cross-over effects arising from retreatment in the the relapsed/refractory setting was infrequently reported. Although no quantitative meta-analyses were possible, the value of a systematic qualitative overview of available evidence should not be ignored.This systematic review provides a summary of available evidence and can form the basis for future quantitative analyses should more data become available.

10.23

THE PARP INHIBITOR, AZD2881, SUPPRESSES GROWTH OF ATM MUTANT LYMPHOID TUMOUR CELLS IN VITRO AND *IN VIVO*

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Inactivation of the tumour suppresser gene, Ataxia Telangiectasia Mutated (ATM), is a frequent event in lymphoid malignancies such as chronic lymphocytic leukaemia (CLL), T-prolymphocytic leukaemia (T-PLL) and Mantle Cell Lymphoma (MCL). The ATM gene plays a crucial role in the activation of DNA damage-induced apoptosis, and consequently its inactivation leads to resistance to standard chemotherapeutic agents. ATM also participates in the repair of DNA double strand breaks (DSBs) through both homologous recombination (HR) and non-homologous end joining (NHEJ) repair pathways and ATM mutant cells exhibit defects in these processes. It has recently been shown that tumour cells defective in the HR repair pathway, due to mutations in BRCA1/2 genes, are sensitive to PARP inhibition. In this study we addressed the ability of the PARP inhibitor, AZD2881, to selectively target ATM mutant lymphoid tumour cells both *in vitro* and *in vivo* and we explored the mechanism/s by which this process might take place. First, we addressed the ability of AZD2218 to target a range of ATM mutant and ATM wild type LCL, CLL, and MCL cell lines as well primary CLL tumours that were first induced to proliferate. We observed a significant dose-dependent reduction in ATM mutant cell number *in vitro* between the doses 1 and 3 M AZD2281. This observed sensitivity to PARP inhibition was even more prominent when we addressed only the reduction of actively proliferating, BrdU-positive ATM mutant cells by FACS analysis, suggesting that AZD2281 targets proliferating, ATM mutant cells. Western blotting and immunofluoresence revealed cellular killing of ATM mutant tumour cells by PARP inhibition that was independent of apoptosis but included the gradual accumulation of DNA damage. To determine whether sensitivity to AZD2281 was specifically a consequence of ATM inactivation, we downregulated ATM gene expression by shRNA in the CLL cell lines PG, CII, WacC3CD5$^+$ and MECI and subsequently compared AZD2281-induced killing between the isogenic cell line pairs. We observed a significant difference in AZD2281 sensitivity between cell lines with ATM knockdown (KD) and GFPshRNA. To confirm AZD2281-induced cytoxicity in ATM mutant tumours *in vivo*, we generated a xenograft model in irradiated NOD/SCID mice of the ATM mutant MCL cell line Granta 519 and compared the degree of engraftment between 9 mice treated with AZD2881 and 9 mice receiving placebo. Analysis at week 5 post-intravenous injection revealed a significant reduction in engraftment of Granta 519 cells in the bone marrow and reduced engraftment in spleen of mice treated with AZD2281 compared with those receiving placebo (p=0.02). Finally, we addressed the ability of AZD2881 to sensitise ATM mutant lymphoid tumour cells to a range of chemotherapeutic drugs currently used for treatment of lymphoid malignancies, including CLL. We observed an additive effect of AZD2281 with fludarabine as well as the HDAC inhibitor, Valproic acid. We conclude that the PARP inhibitor, AZD2881, is a promising new agent for the treatment of chemo-resistant ATM mutant lymphoid malignancies. Consequently, we are in the process of initiating a phase II clinical trial for refractory ATM mutant CLL, MCL and T-PLL tumours with this agent as monotherapy.

10.24

THE HSP90 INHIBITOR 17-DMAG TARGETS NF-KAPPAB TO INDUCE APOPTOSIS IN CLL AND PROLONGS SURVIVAL IN A CLL MOUSE MODEL

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One attractive therapeutic target currently being explored in CLL is Hsp90, a chaperone which stabilizes various client proteins (Akt, Raf, ZAP-70) which are important for survival of CLL cells. Interfering with Hsp90 protein binding to these client proteins leads to their rapid degradation. Our group and others have demonstrated that 17-allylamino-17-demethoxy-geldanamycin (17-AAG) depletes only select chaperone proteins and promotes modest cytotoxicity in CLL patient cells. 17-dimethylaminoethylamino-17-demethoxy-geldanamycin (17-DMAG) is a novel Hsp90 inhibitor with improved solubility, bioavailability and cytotoxicity in cancer cell lines as compared to 17-AAG. We demonstrate that 17-DMAG more potently induces caspase-dependent apoptosis of primary CLL cells compared to 17-AAG. This enhanced cytotoxicity is tumor cell selective as it spares normal T-cells and NK-cells, and only modestly affects normal B cells. In addition to the broad range of Hsp90 client proteins which are regulated by 17-AAG (Akt, Cdk9, ZAP-70), we found that 17-DMAG was also able to deplete both IKKα and IKKβ, the activating kinases of the NF-kappaB family of transcription factors. The impact of 17-DMAG on both IKKα and β is very relevant, as there have been several studies investigating the effect of IKK inhibitors on *in vitro* apoptosis in CLL but no clear therapeutic option for targeting these kinases in the clinic. Most IKK inhibitors including 17-AAG are specific for the IKKβ subunit that mediates classical NF-kappaB signaling, leaving IKKα and the alternative NF-kappaB pathway intact. This is particularly important given recent evidence by Lam *et al.* demonstrating that IKKα is able to compensate for the loss of IKKβ in DLBCL cells lines. Therefore the ability of 17-DMAG to target both subunits of the IKK complex potentially makes it a very potent and effective NF-kappaB inhibitor in CLL. To validate the downstream significance of this NF-kappaB regulation, we show that 17-DMAG effectively reduces NF-kappaB nuclear localization DNA binding in CLL patient cells resulting in decreased transcript and protein levels of NF-kappaB targets Mcl-1 and Bcl-2, known to be major factors in CLL cell survival and drug resistance. The decrease in Mcl-1 and Bcl-2 following 17-DMAG treatment was not prevented by treatment with a caspase inhibitor BocD even though viability was rescued, suggesting that the decrease in these survival proteins precedes cell death and is not simply a consequence of the apoptotic process. Finally, we determined the *in vivo* significance of 17-DMAG treatment using a Tcl-1-SCID transplant model. We found that NF-kB targets genes (A20, Bcl-2, cIAP, Mcl-1 and XIAP) were decreased *in vivo* following treatment with 17-DMAG. Furthermore, 17-DMAG treatment significantly prolonged the survival of these mice (75 days vs. 66 days, p=0.027, n=10/group). Together, our data demonstrate that the Hsp90 inhibitor 17-DMAG represents a novel multi-subunit IKK inhibitor leading to a decrease in anti-apoptotic genes relevant to CLL survival. Given its oral formulation, which allows administration of 17-DMAG by continuous dosing and uninterrupted inhibition of Hsp90, initiation of phase II clinical trials in CLL that include detailed pharmacodynamic studies monitoring NF-kappaB target genes are indicated.

10.25

THE SYK INHIBITOR R788 (FOSD) INHIBITS TUMOR GROWTH IN THE TCL1 TRANSGENIC MOUSE MODEL OF CLL BY BLOCKING ANTIGEN-DEPENDENT BCR SIGNALING

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CLL B-cells depend on various signals from the microenvironment for survival and proliferation. Among these, antigenic stimuli that are propagated through the B-cell receptor (BCR) are considered particularly important for the development and progression of CLL, suggesting that the BCR signaling pathway could be an important target for therapeutic intervention. We have previously characterized some of the critical components of the signaling pathway downstream of the BCR in CLL B cells and identified the protein tyrosine kinase Syk as a promising therapeutic target. In a recent study we showed that CLL B-cells frequently have increased basal/constitutive Syk activity and are moderately sensitive to the cytotoxic effect of the selective Syk inhibitor R406.[1] More importantly, the survival signal induced by sustained BCR engagement was completely abolished by R406, suggesting that this compound may exert an even greater effect *in vivo* by inhibiting antigen-dependent Syk activation. We have now tested this possibility in the E -TCL1 transgenic mouse model of CLL. Aged E -TCL1 mice develop $CD5^+$ B-cell leukemias that, similar to aggressive human CLL, show features of an antigen-driven process, including expression of stereotyped BCRs and reactivity with common autoantigens and microbial agents.[2] For our experiments we used a TCL1 leukemia (TCL1-002) that does not grow *in vitro*, but can be propagated in syngeneic recipients *in vivo*. TCL1-002 cells express an unmutated stereotyped BCR that reacts with phosphatidylcholine, an autoantigen exposed on the surface of senescent erythrocytes. *In vitro* experiments showed that R406 is not cytotoxic for TCL1-002 cells, although it completely inhibited both the basal and BCR-induced activation of signaling pathways downstream of Syk. The absence of a direct cytotoxic effect provided a unique opportunity to investigate whether inhibition of BCR signaling will affect leukemia growth *in vivo*. For this purpose, $1x10^7$ TCL1-002 cells were injected intraperitoneally in 18 syngeneic mouse recipients. Three days later treatment was started in 8 mice with R788, which is the water-soluble prodrug of R406, at a daily dose of 80mg/kg during 18 consecutive days. Because of the rapid clearance of the drug (serum half-life <2 hours) R788 was administered in 3 divided doses at 4 hour intervals. Two weeks after the end of treatment leukemia developed in all mice from the control group (median WBC counts $131x10^6$/mL, range 12-$300x10^6$/mL), whereas all R788-treated mice showed normal WBC numbers (median $6x10^6$/mL, range 3-$8x10^6$/mL, $p<0.001$). Three weeks later all mice in the control group had died (median survival 46 days), whereas all mice in the R788 group were still alive and only two of them had detectable leukemic cells. The primary mechanism of R788 activity was related to inhibition of leukemic cell proliferation, as evidenced by a substantial decrease in the percentage of Ki67-positive cells following 7 days treatment of mice with overt leukemias (30% before, 5% after therapy, $p<0.001$). In conclusion, this study provides the first *in vivo* experimental evidence that inhibition of antigen-dependent BCR signaling should be an effective therapeutic strategy in CLL. Studies are ongoing to determine whether R788 will also be effective against other TCL1 leukemias. *Supported by The Leukemia & Lymphoma Society (White Plains, NY, grant no. 6043-06) and the Italian Association for Cancer Research (Milan, Italy, grant no. 5917).*

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10.26

EVALUATION OF NOVEL S-ALLYL DERIVATIVES OF 6-MERCAPTO-PURINE AGAINST B-CLL: COMBINING THE EFFECTS OF DIFFERENT COMPOUNDS IN A SINGLE MOLECULE

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Previously, we have shown that Allicin, the highly active compound of freshly crushed garlic, produced by the reaction of the enzyme Alliinase with its substrate Alliin, induced the apoptotic killing of B-CLL cells *in vitro*. Furthermore, we reported that overcoming the short half-life of Allicin by its generation in situ on the surface of B-CLL cells by targeting the enzyme Alliinase to the cell surface of the $CD20^+$ cells by Rituximab, resulted in the eradication of primary B-CLL in a human-mouse chimeric model, denoting the marked anti B-CLL potential of combining these two different molecules, with different mechanism of action, into a single drug entity (Arditti *et al.*, Mol Cancer Ther 2005;4(2)325-331) . Indeed, monotherapeutic approaches, even if effective, are usually not sufficient to fully eradicate B-CLL and the most effective therapeutic protocols requires the utilization of more than one agent. With this in mind, we took advantage of the high reactivity of Allicin with SH-containing compounds and created novel chimeric compounds by the combination of Allicin with 6-Mercapto-Purine (6MP) and 6MP-riboside (6MPR), both SH-containing purine analogs used for decades for the treatment hematologic malignancies. The resulting novel compounds, S-Allyl-6MP (SA-6MP) and S-Allyl-6MPR (SA-6MPR), were examined against primary B-CLL cells obtained from the peripheral blood of patients at Binnet stage C. In our *in vitro* assays, Annexin-V staining indicated that SA-6MP acted in a dose dependent manner, inducing the apoptotic death of 37.9% and 95.2% of plated $CD19^+CD5^+$ B-CLL cells (10.9% in untreated cells) incubated for 16 h at 37 degrees in the presence of 50 uM or 100 uM, respectively. In contrast, the original 6MP compound had no impact on the viability of plated B-CLL cells (9.7% and 8.7% Annex+) at doses of up to 150 uM. In preliminary *in vivo* experiments, we compared the anti-BCLL activity of SA-6MP with that of SA-6MPR and the original 6MP compound on primary B-CLL cells from 3 different patients (Binnet stage C) in the human-SCID/Beige mouse model. Following the engraftment of the human B-CLL cells, mice were treated with i.p. injections of 2.5 mg/kg body weight of SA-6MP, SA-6MPR, or 6MP on a daily basis with a single dose throughout 7 consecutive days, after which, the engraftment of primary B-CLL cells was examined by the recovery of $CD45^+CD19^+CD5^+$ cells from engrafted mice. An additional group of mice injected with vehicle (1% DMSO) was also examined as a control. In close similarity to our *in vitro* results, engraftment of primary B-CLL cells was considerably reduced following treatment with SA-6MP (>90% reduction), as compared with treatment with the original 6MP drug, which has no effect on the primary human cells. In addition, the chimeric riboside 6MP derivative, SA-6MPR, induced a potent anti B-CLL effect comparable to that of SA-6MP. In summary, our results *in vitro* and *in vivo* suggests that combining the pro-apoptotic effects of Allicin with 6MP or 6MPR in a single molecule is highly effective against B-CLL. Additional combinations of Allicin with other SH containing chemotherapeutic agents will be examined.

10.27

SELECTIVE TARGETING OF CLL CELLS BY THE DUAL PI3K/MTOR INHIBITOR NVP-BEZ235

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The anti-apoptotic PI3-K/Akt pathway appears to be critically involved in pathogenesis and progression of CLL. Recently, a new oral-

ly available PI3-K inhibitor, NVP-BEZ235 has been developed. Here we show, for the first time, the effects of NVP-BEZ235 on the viability of CLL cells *in vitro*. Primary CLL cells from 37 patients with known clinical stages, IgVH gene mutational status and cytogenetics were used in this study Co-culture model using human bone marrow stromal fibroblasts was applied to overcome spontaneous apoptosis of CLL cells *in vitro*. CLL cells were exposed to NVP-BEZ235 at (1 nM-10 μM) and incubation times (1, 3, 7 days). Cell viability was assessed by annexin-V/propidium iodide staining, flow cytometry and MTT assays. Cell viability was significantly higher in co-cultures compared to suspension cultures (the percentage of apoptotic cells after 3 days in co-culture was 5±4 compared to 23±12 in suspension cultures, $p< 0,01$). NVP-BEZ235 induced apoptosis in the majority of CLL samples under both experimental conditions. However, this effect tends to be more remarkable in co-culture than in suspension: 4-10 fold versus 3-fold increase in apoptosis rate respectively. The pro-apoptotic effect was dose and time dependent and could be observed within 16 hours after incubation at 10 nM. The IC50 values varied between patients and were in a range of 250-750 nM. NVP-BEZ235 inhibited the adhesion of CLL cells to stromal cells suggesting that it may interfere with the survival signal provided by the lymphoid microenvironment in addition to its direct effect on the leukemic cells. FACS analysis demonstrated that NVB-BEZ235 specifically targets the leukemic $CD19^+$ cells while a minimal effect on the viability of T cells and monocytes could be observed. The pro-apoptotic effect of NVP-BEZ235 was independent from the mutational status and cytogenetics. In addition, it induced apoptosis *in vitro* in CLL cells from patients resistant to fludarabine treatment. In parallel to induction of apoptosis in CLL cells, western blotting demonstrated that NVP-BEZ235 significantly inhibited Akt phosphorylation at Ser-473. Microarray analysis revealed more than 200 genes which were at least 2 fold up- or down-modulated by NVP-BEZ235 *in vitro*. These genes include LY9, DUSP10, CCR6, RGS2, IRS2, PI4K2A, ISG20, TFRC, EGR1, HSP90, LCK, TNFRSF17, LYZ, TGFBI and TLR10. In conclusion, the results demonstrate a significant and selective pro-apoptotic effect of NVP-BEZ235 in CLL cells. The data also suggest that targetining the PI3K-pathway represent novel therapeutic concept for CLL.

10.28

CIRCULATING OFATUMUMAB CONCENTRATIONS CORRELATE WITH RESPONSE AND PROGRESSION-FREE SURVIVAL IN FLUDARABINE-REFRACTORY CHRONIC LYMPHOCYTIC LEUKEMIA

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Background and Objective. Ofatumumab, a unique human monoclonal antibody (mAb) targeting a membrane-proximal small-loop epitope of CD20, elicits efficient complement-dependent cytoxicity *in vitro* and has single-agent activity in treating patients with CLL. Ofatumumab is being evaluated in a pivotal trial for patients with fludarabine-refractory CLL also refractory to alemtuzumab (FA-ref; n=59) or unsuitable for alemtuzumab due to bulky (>5 cm) lymphadenopathy (BF-ref; n=79). Remarkable overall response rates (ORR) of 58% in FA-ref and 47% in BF-ref patients were reported at an interim analysis; median progression-free survival (PFS) for FA-ref and BF-ref patients was 5.7 and 5.9 months, respectively. The objective of the current work was to evaluate relationships between pharmacokinetic (PK) parameters and ORR and PFS in this pivotal trial. *Methods.* Patients with fludarabine-refractory CLL (n=154) received IV ofatumumab 300 mg (Dose 1) followed by 7 weekly doses of 2000 mg, then 4 monthly doses of 2000 mg. Response (1996 NCI-WG criteria) was assessed over 24 weeks of treatment by an Independent Review Committee. Blood samples to measure free ofatumumab levels were collected at Dose 1, Dose 8 (last weekly dose), and Dose 12 (last monthly dose), and a population PK approach including data from a previous study [Coiffier, 2008] was used. For Dose 1, Cmax was determined; for Doses 8 and 12, Cmax, Cmin, AUC, clearance (CL), volume of distribution (Vss), and t were determined. Associations between PK and ORR or PFS were identified using univariable logistic regression or Cox regression analyses. *Results.* 90% of patients received 8 weekly infusions; 55% received all 12 infusions. PK was similar between FA-ref and BF-ref patients. Higher Cmax at Dose 1 was associated with lower B-cell counts ($p<0.001$). Ofatumumab CL, Vss, and t were similar between Doses 8 and 12. At Dose 8, the majority of responders and non-responders were still receiving treatment, therefore representing an informative time point for the present analysis. Higher Cmax ($p=0.034$) and Cmin ($p=0.025$) at Dose 8 were associated with increased likelihood of response, and significantly higher mean Cmax, Cmin, and AUC were observed for responders versus non-responders (Table). Higher Cmax, AUC, and Cmin and lower CL at Dose 8 were significantly correlated with longer PFS. Because samples collected at Dose 12 were from patients on continued treatment, the majority were from responders. At Dose 12, higher AUC, Cmax, and Cmin and lower CL and Vss were correlated with significantly longer PFS (Table). *Conclusion.* Understanding the pharmacokinetics of anti-CD20 mAb monotherapy with regard to treatment response is important for optimizing dose and schedule and is previously unappreciated for patients with CLL. These results demonstrated that free ofatumumab concentrations at the 8th weekly dose correlated with response; longer PFS was associated with higher AUC, Cmax, Cmin, and lower CL at Doses 8 and 12. Ofatumumab pharmacokinetics and treatment response may be affected by disease status (e.g., tumor burden, CD20 expression level, circulating CD20, leukemia growth kinetics) and other factors such as complement levels. Further work is underway for an integrated understanding of ofatumumab pharmacokinetics and pharmacodynamics in patients with CLL.

Table.

PK parameter	*PK at Dose 8 All patients*				*PK at Dose 8 by response Responders*		*Non-responders*		
	n	Mean	PFS HR	*p*	n	Mean	n	Mean	*p*
C_{max} (mg/L) 0.036	130	1482	>1.000	0.007	76	1614	54	1315	
C_{min} (mg/L) 0.026	129	579	.999	0.004	75	759	54	397	
AUC (mg*h/L) 0.013	127	674,463	>1.000	0.014	73	772,051	54	561,851	
CL (mL/h) 0.101	127	9.5	1.047	0.029	73	9.1	54	10.1	
V_{ss} (mL) 0.963	127	5127	<1.000	0.473	73	5123	54	5132	
$t^1/_2$ (d) 0.121	127	15.8	0.999	0.057	73	16.5	54	14.9	

10.29

SAFETY AND EFFICACY OF SLOW DOSE-ESCALATION OF LENALIDOMIDE FOR FRONT-LINE TREATMENT IN CHRONIC LYMPHOCYTIC LEUKEMIA (CLL) – RESULTS OF A PHASE II STUDY

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Introduction. Lenalidomide is an immunomodulatory compound that has shown activity in both relapsed and untreated CLL. Lenalidomide-related toxicities such as tumor flare (TF) and tumor lysis are notable when standard myeloma doses (25mg daily) are initiated. The mechanisms for this enhanced toxicity are unclear. We present updated results from a phase II study of single-agent lenalidomide in previously untreated, symptomatic CLL, using a conservative dose-escalation approach. *Methods.* Previously untreated patients (pts) were eligible if symptomatic (cytopenias, symptomatic adenopathy/organomegaly, constitutional symptoms, LDT <12 mos). The starting dose for lenalidomide was 10 mg po daily with weekly 5mg dose escalations to the target dose of 25 mg daily x 21 days every 28 day cycle. Toxic events in the first 2 pts (tumor lysis requiring dialysis; neutropenic sepsis leading to death) led to a study halt with data safety review. Subsequently, protocol amendments reduced both starting and target doses (2.5 mg and 10 mg, respectively, days 1-21), slowed the dose escalation rate (2.5 mg cycle 1, 5 mg cycle 2, 10mg cycle 3 and thereon), extended allopurinol tumor lysis prophylaxis to minimum 3 cycles, and increased frequency of tumor lysis lab monitoring. Steroids were allowed for management of TF symptoms as needed. Lenalidomide was continued until progression or 2 cycles past CR. *Results.* Twenty-five pts were accrued to the amended protocol. Median age 60 (range 33-78), RAI stage III-IV 40%, baseline median Hb 119 g/L (range 80-173), lymphocytes 88.8x10^9/L (range 2.8-220), β2microglobulin 221 nmol/L (range 139-626; normal <170), bulky nodes 36%, organomegaly 92%, del17p/del11q on FISH 8 pts 32%, ZAP70 60%, unmutated IgVH 75%. A median of 13 cycles (range 2-28) administered; median follow-up 13.7 mos (range 2.3-26.2). Hematologic toxicity: 18 pts (72%) developed Gr 3-4 neutropenia during at least 1 cycle, the most common cause for dose reductions/delays. Five pts developed febrile neutropenia and 8 pts (32%) received intermittent GCSF support. Seven pts (28%) developed Gr 3-4 thrombocytopenia. Nonhematologic toxicity: Fatigue (88%), TF (88%), non-desquamating rash (64%) were common and generally mild (one grade 3 rash required drug discontinuation). Mild respiratory/sinus/skin infections were common (76%). Major infections requiring hospitalization included pneumonia (2), aspergillus infection (1), disseminated zoster (1). TF presented with painful, enlarged node(s) often associated with nasal congestion, coryza, and scalp pruritis. Most TF symptoms resolved spontaneously but 8 pts required steroids on at least one occasion. TF was most common during cycle 1 but repeat flare symptoms in subsequent cycles were also noted (20% of 394 cycles). No further tumor lysis occurred on the amended protocol. *Responses.* 20 pts (80%) achieved at least a 50% drop in peripheral lymphocytes, as early as 1 week on lenalidomide (2.5mg) although rebound lymphocytosis during cycle days 22-28 off-therapy was common. Fourteen pts have achieved a PR (56%), no CR, 10 pts stable disease (SD) (40%). One pt progressed on therapy (4%) and died with Richter's transformation after stopping drug. Median response duration is 13.7 mos (range 6.7-25.2); estimated 2 year PFS 87% and OS 89%. Dose modifications/study withdrawals: Eight pts (32%) required dose reductions (most due to cytopenias). Seven pts have stopped study: 2 for lack of response, 5 for toxicities (infections, cytopenias, rash). Eighteen pts remain on study, 2 pts have died off-study (Richter's, stroke). Correlatives: Analysis of gene expression profiles from baseline and day 8 samples (cycles 1 and 2) shows that predominant changes occur in genes involved in immunomodulation (LAG3, TNFa, IFIT4, AIF1, LAK) which may be implicated in the mechanism of tumor flare and/or anti-tumor activity. *Conclusion.* Lenalidomide appears safe and tolerable when initiated with a conservative dose escalation schedule in previously untreated CLL patients. Although rapid peripheral lymphocyte reductions are noted, complete responses may not be achievable with the low doses used in this study. The rebound lymphocytosis during off-therapy days of the treatment cycle suggests that continuous therapy may be required. A follow-up study using this slow dose escalation approach and a combination of lenalidomide and dexamethasone for response synergy and to attenuate toxicities is ongoing. In this study, continuous daily dosing and a higher target dose of lenalidomide are used.

10.30

EXPERIENCE WITH LENALIDOMIDE AS INITIAL TREATMENT OF ELDERLY PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKEMIA

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Introduction. Lenalidomide is an immunomodulatory agent with clinical activity in a variety of B-cell malignancies including CLL. Lenalidomide restores the impaired ability of T-cells to form immunological synapses *in vitro.* This mechanism may play a role in correcting the immune paresis characteristic of CLL. We designed a phase II trial to assess the efficacy and safety of lenalidomide as initial therapy of elderly patients with CLL. This population does not have an established standard of care in front line therapy due to the high risk for myelosuppression and associated infections with chemo-immunotherapy. These patients may benefit from an oral agent which improves defective immune function. *Methods.* Sixty patients over 65 years with untreated CLL fulfilling NCI-WG criteria for therapy initiation were enrolled between 11/07 and 04/09. Patients with any ANC or platelet count were eligible. Patients were evaluated prior to treatment by conventional cytogenetic and FISH analysis, IgVH mutation status and ZAP-70 expression. Patients started with 5 mg lenalidomide orally daily for the first 56 days and could be titrated up by 5 mg every 28 days to 25 mg daily. Lenalidomide therapy was continued indefinitely until disease progression or excessive toxicity. Allopurinol was administered as tumor lysis prophylaxis. Patients were assessed for response after 3 months and every 6 months thereafter. Primary endpoint for efficacy was time to treatment failure (TTF).

Table 1. Patient characteristics and responses with lenalidomide therapy.

Patient characteristic		*Number*	*% NPR*	*CR/ %*	*%*	*ORR*	*%*	*p*
All patients		60	100	10	17	32	53	
Age	65-70y	26	43	3	12	17	65	
	71-75y	19	32	6	32	11	58	
	>75y	15	25	1	7	4*	27	0.034
Sex	F	26	43	3	12	15	58	
	M	34	57	7	21	17	50	
Rai Stage	0-II	42	70	8	19	25	60	
	III	7	12	0	0	2	29	
	IV	11	18	2	18	5	45	
β2M	< 4 mg/dL	26	43	5	19	14	54	
	≥4 mg/dL	34	57	5	15	18	53	
CD38	< 30%	29	48	3	10	13	45	
	≥30%	30	50	7	23	19	63	
ZAP70 FCM¥	< 20%	14	23	1	7	6	43	
	≥20%	17	28	4	24	11	65	
	ND	29						
ZAP70 IHC¶	NEG	17	28	2	12	8	47	
	POS	31	52	6	19	17	55	
	ND	12						
IgVH	Mutated	22	37	1	5	7	32	
	Unmut	33	55	8*	24	23*	70	0.011, 0.006
FISH hierarchy								
	Del 13q	15	25	4	27	9	60	
	Negative	12	20	2	17	5	42	
	Tri 12	13	22	2	15	11*	85	0.013
	Del 11q	14	23	2	14	7	50	
	p53	6	10	0	0	0*	0	0.0075
Number of lymph node groups	0	8	13	0	0	3	38	
	1	10	17	2	20	6	60	
	2	18	30	5	28	11	61	
	3	24	40	3	13	12	50	
Bulky disease≠	No	53	88	10	19	28	53	
	Yes	7	12	0	0	4	57	

*Statistically significant results denoted by * with respective p-values. ¥ZAP70 measurement by flow cytometry. ¶ZAP70 determination by immunohistochemistry stain of bone marrow. ≠Bulk defined by lymph node >5cm, spleen >6cm below costal margin.*

Results. Sixty patients were evaluable with a median follow up of 15.5 months (3.0-20.7 months). Median age was 71y (66-85y), 18 patients had Rai stage III-IV disease and median β-2M was 4.5 mg/L. Twenty patients carried unfavorable genomic abnormalities (17p deletion in 6 pts, deletion 11q in 14 pts) and 19 patients (32%) had unmutated IgVH. 57 of 60 patients (95%) are alive and 38 patients (63%) remain on therapy. Three deaths have occurred from progressive disease. Median TTF has not been reached. At the time of analysis, 3 patients (5%) have achieved CR, 7 patients (12%) nPR and 22 patients PR for an OR of 53%. The presence of trisomy 12 predicted for a higher OR, whereas age > 75 years and presence of p53 abnormality were each associated with a lower OR. Unexpectedly, patients with unmutated IgVH had a better OR. The most common G3/4 toxicities were hematological including neutropenia (31% cycles), thrombocytopenia (4.7%) and anemia (<1%). There was no G4 non-haematological toxicity and G3 toxicity included infection or fever (12% pts) metabolic or electrolyte abnormalities (8%), respiratory symptoms (3%), pain (3%) or fatigue (3%). Mild (G1/2) tumor flare reactions and tumor lysis were observed in 50% patients. *Conclusion.* Lenalidomide induces complete and partial responses in previously untreated elderly patients with CLL. Response to therapy is slower than with traditional chemotherapy and the quality of responses improves with continuation of therapy. Most CR and nPR were achieved between 9 and 15 cycles of treatment. Responses are durable with continuation of therapy and median time TTF has not been reached in this study. Treatment is associated with myelosuppression, but few serious infections have been observed.

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LENALIDOMIDE THERAPY IS ASSOCIATED WITH NORMALIZATION OF LYMPHOCYTE POPULATIONS AND INCREASE IN IMMUNOGLOBULIN LEVELS IN ELDERLY PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKEMIA

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Introduction. Lenalidomide is a novel immunomodulatory drug with demonstrated efficacy in B-cell malignancies. Multiple mechanisms of action have been attributed to the efficacy of lenalidomide in these diseases including modification of tumor microenvironment, restitution of synapse formation between B-cell and T-cells, and activation of T-cells and NK-cells. In order to evaluate the therapeutic mechanisms and immunological changes occurring with lenalidomide, we analyzed changes in lymphocyte populations in the bone marrow and peripheral blood of patients with CLL undergoing frontline treatment with lenalidomide. *Methods.* Between 10/07 and 04/09, 60 patients were entered into a study to assess the activity of lenalidomide in patients over the age of 65 years with untreated chronic lymphocytic leukemia. Twenty-two patients who have completed 15 cycles of lenalidomide have been included in this longitudinal analysis of lymphocyte populations. Evaluations were performed at baseline and after 3, 9 and 15 cycles of therapy and included complete blood count (CBC), analysis for CD3, CD4 and CD8 lymphocyte populations in the peripheral blood by flow cytometry (FCM)and serum immunoglobulin (Ig) levels. Measurement of CD5, CD19 and CD20 positive lymphocytes and light chain restriction was performed by FCM on bone marrow aspirates. Three patients had an expanded lymphocyte panel (including CD3, CD4, CD8, CD56) on bone marrow aspirate to further characterize lymphocyte changes. Statistical analysis for differences was performed using Wilcoxon Matched Pairs Test. RESULTS Treatment with lenalidomide was accompanied by a decrease in median peripheral blood absolute lymphocyte count (ALC) from $51x10^9$/L (2.5-$179x10^9$/L) pre-therapy to $2.1x10^9$/L (0.95-$38.3x10^9$/L) after 15 cycles, most pronounced during the initial three cycles of therapy (median decrease $46x10^9$/L). The decrease in ALC was accompanied by a relative increase in median $CD3^+$ T-lymphocyte from 7.3% (2.7-67%) to 73% (14-91%) after 15 cycles (n=17), including a rise in $CD4^+$ T-lymphocytes from 3.9% (1.2-36%) to 37% (5.6-66%) and $CD8^+$ T-lymphocytes from 3.6% (1.0-33%) to 24% (7.6-52%). We observed a significant rise in median IgG levels from 724 mg/dL (180-1820 mg/dL) at baseline to 941 mg/dL (217-2270 mg/dL) after 15 cycles of therapy (n=22), mostly occurring between 3 and 9 cycles of therapy (median increase 140 mg/dL). The analysis of bone marrow lymphocytes demonstrated a decrease in total lymphocytes from 80% (46-92%) to 37% (18-82%) after 15 cycles (n=22). The median proportion of aberrant $CD19^+CD5^+$ cells decreased significantly from 72% (22-87%) to 8.6% (0.2-63%) with a simultaneous rise in $CD5^+$ T-cells from 5.0% (1.4-21%) to 19% (6.0-45%). All changes were statistically significant with $p<0.001$. Normal CD19+ B-cells population did not change significantly. Lymphocyte subset analysis was available on bone marrow for 3 patients at 15 cycles demonstrating a similar proportion of $CD4^+$ T-cells (27-45%) and $CD8^+$ T-cells (22-40%) with smaller numbers of $CD56^+$ NK-cells (3.4-17%). *Conclusion*. Therapy with lenalidomide induced a significant decrease in aberrant lymphocytes in the peripheral blood and bone marrow accompanied by a normalization of T-lymphocyte proportions in both. There was also an increase in immunoglobulin levels in the blood. This analysis suggests a functional immune reconstitution of the peripheral blood and bone marrow during therapy with lenalidomide. The early kinetic T-cell response observed after 3 cycles in these patients may indicate that this phenomenon is required for the anti-leukemic activity of lenalidomide therapy in CLL.

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LONG TERM RESULTS OF CHEMOIMMUNOTHERAPY WITH FLUDARABINE, CYCLOPHOSPHOMIDE AND RITUXIMAB (FCR) FOR PATIENTS WITH RELAPSED AND REFRACTORY CHRONIC LYMPHOCYTIC LEUKEMIA

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Introduction. The majority of patients (pts) requiring therapy for CLL will relapse and require salvage therapy. Salvage treatment options include single agent or combination chemo-immunotherapy with complete remissions (CR) below 40%. Outcome is dependent on patient characteristics, prior treatment and response. The combination of fludarabine, cyclophosphomide and rituximab (FCR) has demonstrated efficacy in untreated pts with CLL with long remission duration. We present an update of FCR in relapsed CLL and identify patients likely to respond to this salvage therapy.

Table 1. Response assessment for salvage FCR chemotherapy by prognostic factor.

Risk Factor	*Levels*	*N (%)*	*%CR*	*%OR*	*TTF (mo)*	*OS (mo)*
All evaluable		280 (100)	31	75	21	46
Age	0-60	154 (55)	39	74	27	58
	61-70	87 (31)	24*	80	21	42*
	>70	39 (14)	13*	69	14*	22*
Rai stage	Stage 0 to II	152 (55)	41	86	28	66
	Stage III	34 (12)	26	76	28	48*
	Stage IV	94 (34)	16**	59**	12*	31*
Bulky disease+	No	209 (75)	35	78	23	50
	Yes	69 (25)	17*	68	17	39
Cytogenetic	Chromosome 17 abnormal	19 (11)	0*	37**	5**	9**
hierarchy°	Complex (≥3 abn.)	22 (12)	9*	64	9*	26
	Deletion 11q (ATM)	13 (7)	15*	69	12*	33
	Other abnormalities	13 (7)	31	77	21	43
	Trisomy 12	16 (9)	38	69	28	79
	Normal or deletion 13q	97 (54)	34*	82*	28*	52*
	No Result	100				
Number of prior	1	116(41)	44	82	30	61
treatments	2	78(28)	33	77	28	53
	3	44(16)	18*	73	21*	39*
	≥4	42 (15)	2**	57*	9*	25*
Treatment hierarchy	Antibody +/- steroids only	25 (9)	52*	92	47*	NR*
	Alkylator, no PA (+/- R)	36 (13)	28	78	20	44
	PA, no alkylator (+/- R)	61 (22)	48*	80	37*	90*
	Alk and PA (separate)	55 (20)	25	76	17	45
	FC, FCR, FND, FM	77 (28)	25	74	19	42
	Multiagent§/SCT	26 (9)	4*	46*	6**	20*
Fludarabine Refractory	No	227(81)	36	80	27	51
	Yes	53(19)	8**	57*	7**	37*
Alkylator refractory	No	214(76)	37	78	26	56
	Yes	66(24)	11**	67	10**	37*
Prior rituximab exposure	No	182 (65)	30	76	21	48
	Yes	98 (35)	32	73	20	45
β2-microglobulin	<4 mg/dL	111 (41)	45**	86*	32	67
	-6.5 mg/dL	100 (37)	29*	74**	20	49*
	6.5 mg/dL	62 (23)	6**	60**	7	22*

*Level of statistical significance: * p<0.05, ** p<0.001. +Bulk defined as lymph node >6cm, spleen >5cm, liver >8cm. °Cytogenetic hierarchical categories grouped according to highest risk abnormality. Treatment hierarchy according to most intensive therapy. §Multiagent includes combination therapies with 3 or more agents (eg. CHOP, ESHAP...), SCT stem cell transplant.*

Methods. We administered FCR between 1999 and 2008 to 280 pts with relapsed CLL. Pts received F, 25 mg/m^2, and C, 250 mg/m^2, both daily for 3 days and R, 375-500 mg/m^2, each course for up to 6 courses. Pretreatment characteristics were assessed including refractoriness to alkylator (Alk) or fludarabine-based therapy. Outcomes included response, time to progression (TTP), time to failure (TTF) and overall survival (OS). RESULTS. Pts entered into the study had a median age of 60 years (31-84y); median number of prior treatments was 2 (1 to 10) including 51% of pts with both prior F and Alk therapy. 35% had received prior rituximab. 46% of pts had Rai III-IV disease and 60% had a β2-M greater than 4mg/l. High-risk cytogenetics by karyotype (abnormal chr 17, 11q-, or complex) was present in 31% of 183 pts. Responses included 31% CR, 15% NPR and 30% PR (Table 1). Prior exposure to rituximab therapy did not impact on CR or survival parameters. Factors which predicted for higher CR/nPR were younger age, low β2-M, high Hgb or Plt, less prior treatments and lack of F refractoriness. Median OS is 46 months [95% CI: 41-54 months], 103 pts remain alive. Median OS has not been reached for pts achieving CR or NPR with a median follow-up of 69 months (Table 1). Among the 211 pts achieving a response, the median TTP was 32 months [95% CI, 28-39 months]. On multivariate analysis, variables independently associated with shorter TTP were: chr17 abnormality and higher number of prior treatments. Six full dose cycles were administered to 36% of pts. Patients over 70y were less likely to receive 6 cycles of FCR (13% v. 47%, p<0.0001). 113 of 160 pts did not complete 6 cycles due to myelosuppression (n=65), infection (n=33) or hemolytic anemia (n=9). The most common hematological toxicity was G3/4 neutropenia complicating 22% and 34% of treatment courses. Forty-six pts (16%) experienced pneumonia or sepsis during or treatment. CONCLUSION. FCR is an effective regimen in relapsed CLL with high response rates and good remission duration and survival. The majority of pts over 70 years did not complete six cycles of therapy due to myelosuppression or infections. FCR should be considered as salvage therapy in younger fludarabine sensitive pts with three or less prior treatments as this group demonstrated very good treatment outcomes. Refractory pts beyond their third salvage regimen and patients with abnormal chr 17 should be considered for alternative investigational therapies.

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IMMUNOPHENOTYPIC PATTERNS OF CORD BLOOD MONONUCLEAR CELLS IN A COHORT OF SUDANESE NEONATES: SUBTLE MARKERS OF HEALTH AND DISEASE

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Immunophenotyping of cord blood mononuclear cell is an important tool in the diagnosis and follow up of Newborn with congenital immunodeciencies, HIV infection, or other immune disorders. Infection is still an important cause of neonatal morbidity and mortality, despite the development of broad spectrum antibiotics and advanced life support machines. Diagnosis of congenital or neonatal infections are often based on clinical symptoms and signs, that may not be specific, while routine laboratory tests, may not be adequate. Serological tests and isolation of microorganisms do not give immediate results and haematological tests that are currently used can be difficult to interpret. Leucocytosis is difficult to interpret due to the wide 'normal' range in the newborn (8-32.5 103/μL) and an increase in neutrophil immature forms is subjective, with different morphologists giving variable results. The expression of surface functional antigens on neutrophils, and their up-regulation in infection is difficult to interpret due to the effects on these surface antigens by a number of variables such as anticoagulants, type of fixative used and cell separation procedures. In addition, such tests require immediate processingand and are time-consuming. Following institutional ethics approval and parental consent, cord blood was collected into K3-EDTA for full blood counts, peripheral blood films examined and immunophenotyping by flowcytometry. The study population divided in to possibly infected (n=99) and non-infected (n=111) depending on the mother's account during pregnancy. There was no different in the lymphocyte absolute count between term and preterm infants (p=0.2), but with statistically significant difference in absolute lymphocyte between infected and non infected neonates (p=0.003). There were significant differences in the immunophenotypic patterns of mononuclear cells types in different newborns groups at the time of birth with respect to history of infection (p<0.005). The proportion of CD3$^+$ cells increased with gestational age and with the history infection. In addition, neonates born to mothers with a history of infection had significantly less CD4$^+$ cells and CD4$^+$/CD8$^+$ double-positive cells than neonates with no history of infection. It was also shown that the CD4$^+$/CD8$^+$ ratio was increased in neonates with infection. Cells that were reactive to CD4$^+$/CD45RO$^+$ and CD8$^+$/ CD45RO$^+$ were more in the infected group compared to those with no history of maternal infection. A strong positive correlation in CD45RO$^+$ cells and a negative correlation with CD45RA$^+$ cells were seen with increased gestational age. In conclusion, increased absolute lymphocyte and CD3$^+$ cells and decreased CD4$^+$ cells in neonates could point to early neonatal infection. The incorporation of other early activation markers such as CD69 and CD25 may contribute additional sensitivity in the detection of early infection.

LIVER ONSET OF RICHTER SYNDROME IN PATIENTS TREATED WITH ALEMTUZUMAB

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Background. Richter Syndrome is an uncommon complication of chronic lymphocytic leukemia (CLL). There is little information in the literature about its risk and outcome. In this presentation we addressed the incidence of liver involvement in a cohort of relapsed/refractory CLL patients treated with alemtuzumab subcutaneously at reduced dose. *Material and Methods.* The study initiated in March 2005 have been included 25 pts belonged at two centers and the last patient was included in October 2008.The study was conducted with the approval of local ethics committees according to the principles of the Declaration of Helsinki, and all patients gave written, informed consent prior to enrolment. Alemtuzumab 10 and 20 mg was administered SC day 1 and 2, followed by 30 mg two times per week until lymphocyte count drop 1x10^6/L then it was administrate only if lymphocytes count raised these value. *Results*. Seven out 25 pts (31%) developed RS and are the base of our presentation. Four out 7 pts showed liver infiltration as only feature at once of RS. The other 3 patients developed nodal RS in 2 cases and

bone marrow onset in another one. Presenting features of liver RS included B-symptoms (100%), progressive hepatomegaly (100%) and DHL more than 1500 UI/L (100%). The median age at diagnosis of CLL and RS were 63 y (range 48-76 y) and 69 y (range 52-79 y) respectively. The median time to transformation from CLL diagnosis was 73 months (range 26-240 m). All patients had been previously treated for CLL with fludarabine base therapy. Fourteen patients (57%) had received >1 prior therapy. The median lymphocyte count at diagnosis was $18x10^9$/L (range $13-90x10^9$/L). All patients with liver RS dead with a median follow up of 2 m (range:1-6 m). *Conclusions.* We are unable for find predictive factor for liver onset of RS but the patients with RS had increased frequency of DAT + test during disease follow up. The unexpected frequency of liver localization bares in mind the possibility of special distribution of alemtuzumab with scarce liver concentration and sanctuary homing for CLL cells.

Table. Liver onset of Richter Syndrome (RS) in patients treated with alemtuzumab.

Parameter	*Richter syndrome*	*No richter syndrome*
Number	7	18
Sex M/F (ratio)	4/3 (1.33)	11/7 (1.55)
Age at diagnosis CLL Median (range)	61 (47-67)	59 (42-77)
Stage at Diagnosis		
Binet A, B, C	1,5,1	0, 14, 3
Median Lymphocyte count at diagnosis	$58x10^9$/L ($24-72x10^9$/L)	$49x10^9$/L ($26-86x10^9$/L)
DAT positive	4/7	2/17

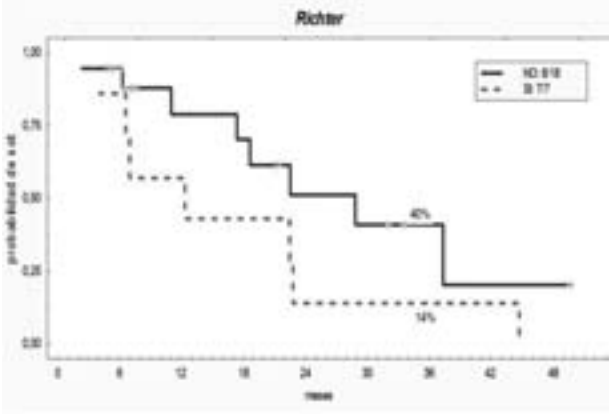

Figure 1. OS in patients with & without RS.

DOES ZAP-70 REALLY STAY UNCHANGED DURING CHRONIC LYMPHOCYTIC LEUKEMIA PROGRESSION?

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Mutational status of the immunoglobulin heavy-chain variable-region (IgVH) genes is a new prognostic factor in CLL patients. ZAP-70 is a 70 kDa protein tyrosine kinase, encoded by a gene located on chromosome 2, normaly expressed in T cells and NK cells and no in B cells. It is variably expressed in CLL. In CLL cases, ZAP-70 is a prognostic marker, selected from a lot of other markers for best distinction between prognostically defined CLL subtypes. ZAP-70 seems to be closely correlated to the IgVH genes mutational status,it is highly expressed in the CLL subtype with unmutated IgVH genes (bad prognosis) and less expressed or absent in the subtype with somatically mutated IgVH genes (good prognosis). Thus, ZAP-70 is a surogate marker for IgVH mutation status (and indirect for time to disease progression and overall survival). IgVH mutation status is expensive and difficult to detect in laboratory, hence the usefulness of ZAP-70 assay. The presence of somatic mutation in the Ig VH gene is a marker of B-cell differentiation and is a fixed and unique characteristic of the leukemic cells. Is it ZAP-70 stable over time, as is presumed to be? In some cases ZAP-70 and IgVH mutation status analysis yield discordant results. ZAP-70 correctly predicts mutational status in 70-90% of patients. Could help testing for ZAP-70 (at diagnosis time) identify patients likely to have progressive disease in the future? We present a case of a 60 year old man, diagnosed as B-CLL stage 1/A in 08/2004 according to flow – cytometry findings; ZAP-70 was 10% at this time. ZAP-70 increases progressively, parallel with advancement of the disease, until 95% (when the patient was in stage 4/C). The findings in this case raise the question of whether the value of of ZAP-70 is constant during the disease, like mutational status (hence the importance as prognostic factor at diagnosis), or whether it is progressive together with the advancement of the disease (in this case the prognostic significance is decreased).

CD5 NEGATIVE CHRONIC LYMPHOCYTIC LEUKEMIA

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Typically, chronic lymphocytic leukemia (CLL) expresses CD5 on its clonal B cells. CD5 negative CLL represents less than 2% of all CLLs. these cases are generally associated with shorter survival, more advanced stage of disease, atypical morphologic features and more cytogenetic abnormalities. we report herein 5 cases of CD5- CLL. the results of morphologic, immunophenotypic and immuno-histo-chemical analysis were consistent with CD5- CLL. we studied the epidemiological, clinical , morphologic and biological features and the follow-up of these cases in order to spring their particularities. In conclusion, controversy is often existing wether to consider CD5- CLL as atypical variant of CLL or a distinct entity within the B lymphoproliferatifs syndromes.

THE EFFECTIVENESS OF ALEMTUZUMAB TREATMENT AMONG RESISTANT TO FLUDARABINE B-CHRONIC LYMPHOCYTIC LEUKEMIA PATIENTS

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The aim of this study was to explore the effectiveness of treatment with Alemtuzumab (ALZ) of B-cell Chronic Lymphocytic Leukemia (CLL) patients (pts) resistant to Fludarabine(F). *Meterials and methods.* We observed 6 pts (3 male and 3 female). The median age was 56 years old(52-69). 4 of the pts had Rai stage I desease, 1 patient had Rai stage III, 1 had Rai stage IV. We revealed that 4 pts had 17p deletion with high level, 1 patient did not have del 17p , 1 - was not assessed. High level of the expression of $CD38^+$ was determined, the median number was 37,3%(15-82). All pts were pretreated with Chlorambucil, COP, CHOP, FC for 18 months(7-48). All pts were resistant to F. 5 pts received ALZ monotherapy during median 9 weeks (2-18). Subcutaneous(SQ) ALZ dose escalation: 3 mg-10 mg-30 mg on days 1,3,5, followed by 30mg Monday, Wednesday and Friday for 17 weeks. 1 patient received 5 courses FluCam(ALZ 30 mg 1,2,3 days IV after dose escalation 3mg-10 mg-30 mg, F. 25 mg/m^2 1,2,3 days). All pts received Pneumocystis carinii prophylaxis, herpes and cytomegalovirus(CMV) and fungal prophylaxis as well as CMV viral DNA monitoring. Responses were based on NCI-WG 1996 criteria. Minimal residual disease (MRD) was measured in peripheral blood and bone marrow aspirate using flow cytometry for $CD19^+/CD5^+/CD23^+$ lymphocytes. *Results.* The effectiveness of the treatment of CLL pts was following: 2 pts (33.3%) displayed disease progression(PD) in 2 and 4 weeks of ALZ monotherapy, 2 pts achived (33.3%) CR in 14 and 16 weeks, 2 pts (33.3%), achived PR, among them 1 patient showed PR after 5 FluCam courses, the other - after 14 weeks of ALZ monotherapy. At the completion of the study 4 pts (66,6%) had no evidence of MRD by flow cytometry(<0.01%). *Conclusion.* Obtained results showed high effectiveness of ALZ and acceptable toxity, among resistant to F. CLL pts (66.6% responded), the majoority of them had unfavourable del 17p.

MEASUREMENT OF OXIDATIVE STRESS (OS) IN CLL PATIENTS WITH EARLY STAGE

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Introduction. The overall median survival of patients with chronic lymphocytic leukemia (CLL) is about 10 years. The individual prognosis is however, extremely variable. Whereas in some patients the disease runs an indolent clinical course and life expectancy is not shortened, in others the disease progresses rapidly, has aggressive behaviour, and survival after diagnosis is inferior to 2-3 years. Considering its variable prognosis and the absence of a curative therapy, management of patients with CLL cannot be planned without taking into consideration their prognosis. Clinical staging systems are the mainstay for assessing prognosis in patients with CLL. Patients with low risk disease (Rai stage 0, Binet stage A) have a median survival that is close to 15 years, although a number of biological markers offer important, independent prognostic information. Treatment of patients of CLL should be decided on the basis if classical criteria. An important area of research of CLL prognostication is the identification of markers useful for predicting response to therapy and its duration. Measured oxidative stress (OS), through balance between the prooxidating and antioxidative state of the cells, and makes reference to the imbalance in favour of first of both by the counter-productive effect of the free radicals. *Objective*. To determine the degree of OS in a group of patients with initial stages of CLL (Rai stage 0, Binet stage A), and to relate it with another recognized prognosis factors and to compare with a sex and age matched group control. *Material and Methods.* In a group of 37 patients diagnosed of CLL Rai stage 0, Binet stage A, oxidative stress were the determinated by the measurement of the following parameters: is made in blood: - At erythrocyte level one evaluates the level of Glutation S-transferase (GST, total and termosestable and residual activity) Glutation reduced (GSH) and oxidized (GSSG), Superoxide dismutase (SOD), Catalase (CAT), Glutation peroxidase (GPx), Glutation reductase (GR), reactive substances of thiobarbituric acid (TBARS) and percentage of haemolysis. In plasma it is evaluated: GSH, GSSG and TBARS. To obtain the overall score of oxidative stress, each parameter received a numerical value (zero, -1 or +1) as follows: results within the normal ranges were given zero points (0); results above or below the normal range indicating OS were given one positive point (+1), and results indicating antioxidant status (AS) were given one negative point (-1) (Table 1). The results were processed with the SPSS statistical package.

Table 1. Score of each biomarker to perform the SOS.

Biomarkers	*Value > than upper limit*	*Value < than lower limit*
Antioxidant enzymes		
T-GST	-1	+1
TS-GST	-1	+1
%TS-GST	-1	+1
SOD	-1	+1
CAT	-1	+1
GR	-1	+1
GPx	-1	+1
GSH, GSSH, GSSG/GSH		
GSH erythrocytes	-1	+1
GSSG erythrocytes	+1	-1
GSSG/GSH erythrocytes	+1	-1
GSH plasma	-1	+1
GSSG plasma	+1	-1
GSSG/GSH plasma	+1	-1
Lipid peroxidation products		
TBARS erythrocytes	+1	-1
TBARS plasma	+1	-1
Oxidizability measurements		
HT	+1	-1

Table 2. Score of each biomarker to perform the SOS.

	CONTROL n=37		*PATIENTS n=37*		
PARAMETER	*Mean*	*SD*	*Mean*	*SD*	*p*
TBARSP(nmil/mL)	2.04	1.14	3.21	2.44	NS
TBARSE (nmol/g Hb)	4..06	1.91	3.48	3.33	<0.014
GSHE (umol/g Hb)	4.94	1.57	1.66	1.03	<0.001
GSSGE (umol/g Hb)	0.85	0.37	1.82	0.90	<0.001
QE	0.19	0.11	1.38	1.07	<0.001
SOD (U/g Hb)	1739.55	459.74	1066.39	473.42	<0.001
CAT (mmol/min/g /Hb)	216.52	38.50	173.5	48.0	<0.001
GR (umol/min/g Hb)	3.57	1.32	2.99	2.42	NS
GPx (umol/min/g Hb)	27.03	7.91	19.61	5.40	<0.001
GSHP (nmiol/mL)	21.55	12.39	21.36	15.38	NS
GSSGP (nmol/mL)	25.58	6.67	31.36	15.23	0.039
QP	1.89	1.61	1.99	1.64	NS
POINTS	0.05	0.62	3.00	1.14	<0.001

Results. Once determined the indicative values of activity pro-oxidant as well as of the antioxidative enzyme level, in the group of 10 patients with CLL A/0-1 stage, a level of significantly greater OS is observed in patients group compared with control group (Table 2). Conclusions. A greater Os exists in patients with CLL in initial stages. The determination of the level of this stress in these patients could be considered for its use as prognosis factor of the disease, next to the knowed factors. The antioxidative drug use could limit the degree of OS and be used to limit the possible progression from the disease advanced stages more. However, treatment decisions based on biological parameters are still only justified with clinical trials, but these parameters could help to treat patients in early stage disease.

FULMINANT COMPLICATION OF TREATMENT IN A PATIENT WITH CLL

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Introduction. We present a patient with long standing CLL, who was treated with alemtuzumab due to resistant disease. His peripheral blood count responded promptly to the treatment. Due to reactivation of CMV he was treated with ganciclovir and later developed fulminant acute liver failure, which might be attributed to the treatment with ganciclovir. Case report. A 72 year old patient was diagnosed with CLL stage A in 2001. His bone marrow biopsy showed diffuse infiltration by CLL. 3 years later he was treated with 3 courses of leukeran and prednisone with no significant response and his WBC count remained high. There was no response to a short course of oral cyclophosphamide. In September 2008 his WBC count increased to 227, 000/mm^3 and a large mass of mesenterial and retroperitoneal lymph nodes was observed on CT. His disease was also refractory to 3 courses of oral fludarabine and cyclophosphamide and his WBC increased to 388, 000/mm^3. On 1.2.2009 alemtuzumab treatment was commenced. Zovirax was given as prophylactic therapy for CMV infection. He responded promptly to the chemotherapy; after two weeks of therapy his WBC count dropped to 2000/mm^3 and alemtuzumab was stopped. His urea, creatinine and liver enzymes were normal during the treatment. PCR of CMV load was done prior to therapy and every week during the therapy. He had <1000 copies of DNA at the start of the treatment; however one month later, while off alemtuzumab therapy, the viral load of CMV increased to 218 000 copies of DNA. At the same time he developed fever and was hospitalized with a clinical picture of sepsis. Treatment with tazocin and ganciclovir was initiated. His fever resolved on the second day of the hospital stay. No signs of systemic CMV were present; however, the patient was somnolent during treatment. On day 21 his fever rose to 39o C. Imipenem was substituted for tazocin. After two doses the patient suffered from generalized convulsions and the drug was stopped. Brain CT showed an old infarction and his LP was normal. On day 18 of the treatment with ganciclovir, abnormal liver function tests were first reported; GOT was 913-1616 units, GPT 533-1480 units, LDH 727-3123 units, bilirubin 2.2-16.6 mg%, alkaline phosphatase 137-160 units, GGT 90-180 units and albumin decreased to 1.9 gr/dL. Ganciclovir was stopped because of neutropenia (PMN 470/mm) and foscarnet was started. The last PCR for CMV showed 5900 copies of DNA. Liver biopsy was performed one month after his admission to the hospital. However, the patient died 3 days later due to hepatic failure. The liver biopsy showed massive liver necrosis, which in the pathologist's opinion might be com-

patible with drug induced toxic hepatic injury. No evidence of CMV or liver involvement by CLL was seen. Conclusions. We presume that in the absence of infiltration by CLL or involvement by CMV, the hepatic necrosis in our patient was caused by drug toxicity. Alemtuzumab was stopped at least 3 weeks prior to deterioration of LFT and foscarnet was started in the presence of impaired LFT. Therefore, it is most likely that the fatal complication of acute liver necrosis in our patient was caused by ganciclovir, which was given for 23 days.

SPONTANEOUS TUMOR LYSIS SYNDROME IN A CASE OF CHRONIC LYMPHOCYTIC LEUKEMIA

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Background. CLL is a low grade lymphoproliferative disorder.Tumor lysis syndrome is a metabolic emergency and usually occurs after chemotherapy for Leukemia and lymphomas.the cases of spontaneous Tumor lysis syndrome has been reported in high-grade non-Hodgkin's lymphoma cases with high mitotic rates. *Case Report.* A 60 Y/o male admitted with Nausea and vomiting and anuria.physical examination revealed splenomegaly.and laboratory findings included: Urea=100 , Cr=7.8, K=6.5, uric acid=13, Phos=8.8, Ca=8, LLDH=925; WBC=100,000, Hb=9.3, PLt=103,000, Coomb's Direct&Indirect were negative. Peripheral blood smear revealed many smudge cells and many basket cells and more than 90% of WBCs were small sized mature lymphocytes compatible with CLL. Bone marrow aspiration and biopsy confirmed CLL diagnosis.After 3 times daily dialysis we startedAlloporinol and forced diuresis and urine alkalization with lasix,and hydration with half saline according to 6 hour urine volume plus 300CC and we added 50 meq HCO3Na in each liter of serum.Urineray out put was rised graduately to 3000-4500 CC daily and Cr was decreased from 6 to 1.3 over 10 days. After recovery of acute renal failure we started chemotherapy for CLL. After 3 cycle of chemotherapy (including fludarabine- based chemotherapy)WBC count declined significantly and PLT count was normalized and Hb rised to 12 and he acheived Complete remission. *Results.* Although CLL is a low grade lymphoproliferative disorder and tumor lysis syndrome was reported as a complication of chemotherapy in patients with CLL ,in this case we found spontaneous tumor lysis syndromein an untreated case of CLL. *Conclusions.* Spontaneous tumor lysis syndrome may be seen in patients with CLL before starting chemotherapy.

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