SUPPLEMENTARY APPENDIX

The complex karyotype landscape in chronic lymphocytic leukemia allows the refinement of the risk of Richter syndrome transformation

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SUPPLEMENTARY METHODS

IGHV mutational status

Analysis of the IGHV mutational status was performed within 12 months from diagnosis on peripheral blood CLL cells from fresh samples or frozen purified CLL cells harvested in DMSO. RNA was extracted from 2x106 B cells using the RNeasy™ Total RNA kit (Qiagen, Hilgen, Germany) and reverse transcribed using the SuperScript™ Preamplification System for first-strand cDNA synthesis (Life Technologies, Carlsbad, CA). The CLL B-cell HV gene family was assigned as previously described(25, 26). HV gene sequences were determined by amplifying 5µl of the original cDNA using the appropriate HV leader and HC primers. PCR products were directly sequenced after purification with the Wizard PCR Preps (Promega, Madison, WI) using an automated genetic analyzer (3130 ABI Applied Biosystems, Foster City, CA, USA). Sequences were analyzed using the IMGT/VQUEST and BLAST softwares(27) to detect VDJ junction. Cases with a sequence homology <98 from the corresponding germline gene were considered as mutated (M-IGHV), and those with a homology ≥98% as unmutated (U-IGHV)(28, 29). Stereotyped B-cell receptor (BCR) was assessed with ARResT(30, 31).

Cytogenetics by fluorescence in situ hybridization (FISH) and mutations

FISH was performed on standard cytogenetic preparations from peripheral blood(26). The slides were hybridized with the multicolor probe set LSI p53/LSI ATM and LSI D13S319/LSI 13q34/ CEP12 and RP11-177O8 according to the manufacturer's instructions(32). Three hundred interphase nuclei were analyzed for each probe and the cut-off for positive value was 10% for deletion of 11q22.3 (ATM), 17p13.1 (TP53) loci and 13q14.3 (D13S319), and 5% for trisomy 12. TP53 gene sequencing was performed according to ERIC guideline assessing exons 4-10; if negative exons 2, 3 and 11 were also investigated(22). NOTCH1 c.7544_7545delCT mutation was performed according to Rossi D et al (33) following local policy.

LEGENDS TO FIGURES

Figure S1. Prevalence of CK subtypes and RS. In the upper panel (A) there are apple-pie graphics of the complex karyotype (CK) rate in the whole population, on the left, and CK qualitative and quantitative subtypes on the right. The percentage of CK subtypes refers to 20% of all patients. In the middle panel (B), there is an apple pie-graphic showing the prevalence of Richter syndrome (RS) in our study population. In the bottom panel, there is a bar graph comparing clinico-biological features of patients who developed RS and those who did not transform (no RS). Fisher exact-T test, * = $p<0.05$, ** = $p<0.005$, *** = $p<0.0005$, **** = $p<0.0001$

Figure S2. Bar graph and Kaplan-Meier curves according the presence of complex karyotype and its subsets. In the upper part of the figure we report a bar graph (A) showing the prevalence of chromosome abnormalities in the whole population and according to the qualitative classification of complex karyotype [i.e. no complex karyotype (no-CK), type-1 CK (CK1), type-2 CK (CK2)]. The presence of at least 5 chromosome abnormalities (high-CK) is rare in the whole CLL population, it is common in patients with CK2 ($p<0.0001$). In the middle and bottom there are Kaplan-Meier curves for overall survival from CLL diagnosis according to the IGHV mutational status (B, mutated vs unmutated IGHV genes), qualitative CK subtypes (C, type 2 CK vs type 1 CK vs no CK), quantitative CK subtypes (D, 0 vs 1-2 vs 3-4 vs ≥5 chromosome abnormalities).

Figure S3. Kaplan-Meier curves of time to Richter syndrome according to clinico-biological variables. We reported time to Richter syndrome according to gender (A, male vs female), age (B, ≥65 years vs <65 years), Binet stage (C, stage B-C vs stage A), deletion 11q22-23 (11q-) by FISH (D, 11q- vs +12 vs other FISH results (i.e. 13q- and normal FISH)), IGHV mutational status [E, M-IGHV (mutated IGHV gene) vs U-IGHV (unmutated IGHV gene)], TP53 abnormalities (TP53 abn, including deletions and mutations) [F, TP53 abn vs TP53 wild-type], qualitative CK subtypes (G, CK2 vs CK1 vs no CK) and quantitative CK subtypes (0 vs 1-2 vs 3-4 vs ≥5 chromosome abnormalities).

Figure S4. Kaplan-Meier curves for time to Richter syndrome of CK2 and/or high-CK patients according to the presence of TP53 abnormalities or IGHV mutational status, and time to Richter syndrome according to different prognostic models. In upper panels we report time to Richter syndrome transformation of CK2 and/or high-CK patients according to the presence of TP53 abnormalities (TP53 abn, including deletions and mutations) or not (TP53 wild type) (A), and according to the IGHV mutational status (M-IGHV, mutated, or U-IGHV, unmutated) (B). The increased risk of developing Richter syndrome of CK2 and/or high-CK patients is independent from TP53 abnormalities or U-IGHV status. In the lower panels there are Kaplan-Meier curves according to the CLL-IPI, on the bottom-left (C), and Barcelona-Brno scores, on the bottom-right (D) applied to the whole CLL study population.

Figure S5. Kaplan-Meier curves for overall survival analysis. In the upper-left panel (A) is reported the overall survival from CLL diagnosis for the whole population**.** Panels B and C show overall survival from RS diagnosis according to the presence of CK2 (type-2 complex karyotype), high-CK (≥5 chromosome abnormalities). In the bottom-left panel (D) there is overall survival analysis from RS diagnosis according to our proposed RS scoring system; high-risk defines patients with CK2 and/or high-CK; intermediate risk were patients with U-IGHV/TP53 abnormalities/11q-/Binet B-C; at low-risk were patients with M-IGHV without CK and TP53 wild-type. The very poor prognosis of patients with RS is not influenced by CK2 nor high-CK nor our scoring system predicts survival after RS transformation. In the bottom-right panel (E) we reported the time to Richter

syndrome for patients who were untreated during the follow-up, those treated with chemo-chemoimmunotherapy (CT or CIT), a those treated with BTK or BCL2 inhibitors.

TABLES

Table S1. Univariate and multivariate analysis for overall survival from CLL diagnosis

	UNIVARIATE ANALYSIS			MULTIVARIATE ANALYSIS		
	HR	95% C.I	p values	HR	95% C.I	p values
MALE	1.48	0.97-2.26	0.0711	-	-	-
AGE>65yy	4.87	3.11-7.63	<0.0001	4.06	2.57-6.43	<0.0001
Binet B-C	2.31	1.41-3.78	0.0009	2.06	1.35-3.16	0.0009
B2MG high*	1.83	1.23-2.97	0.0142	1.59	1.02-2.47	0.0389
U-IGHV	2.85	1.85-4.38	<0.0001	2.89	1.82-4.59	<0.0001
11q-	5.04	2.40-10.59	<0.0001	2.62	1.58-4.35	0.0002
TP53 abn	2.14	1.11-4.12	0.0226	1.94	1.14-3.30	0.0138
CK	3.63	2.16-6.11	<0.0001	2.75	1.80-4.20	<0.0001
CK2	5.52	3.04-10.02	<0.0001	3.28	2.12-5.06	<0.0001
HighCK	5.98	2.89-12.37	<0.0001	3.16	1.93-5.15	<0.0001
RS	25.00	10.10-61.91	<0.0001	4.98	3.02-8.20	<0.0001

β2MG high = beta2-microglobulin >3.5mg/L, U-IGHV = unmutated IGHV gene, 11q- = deletion of 11q22-23 by FISH, TP53 abn = TP53 abnormalities include deletions and/or mutations, CK = complex karyotype, CK2 = type-2 CK, highCK = ≥5 chromosome abnormalities, RS = Richter syndrome. * data available from 520 (96%) patients, 26 (93%) who developed a RS and 494 (96%) who did not transform.

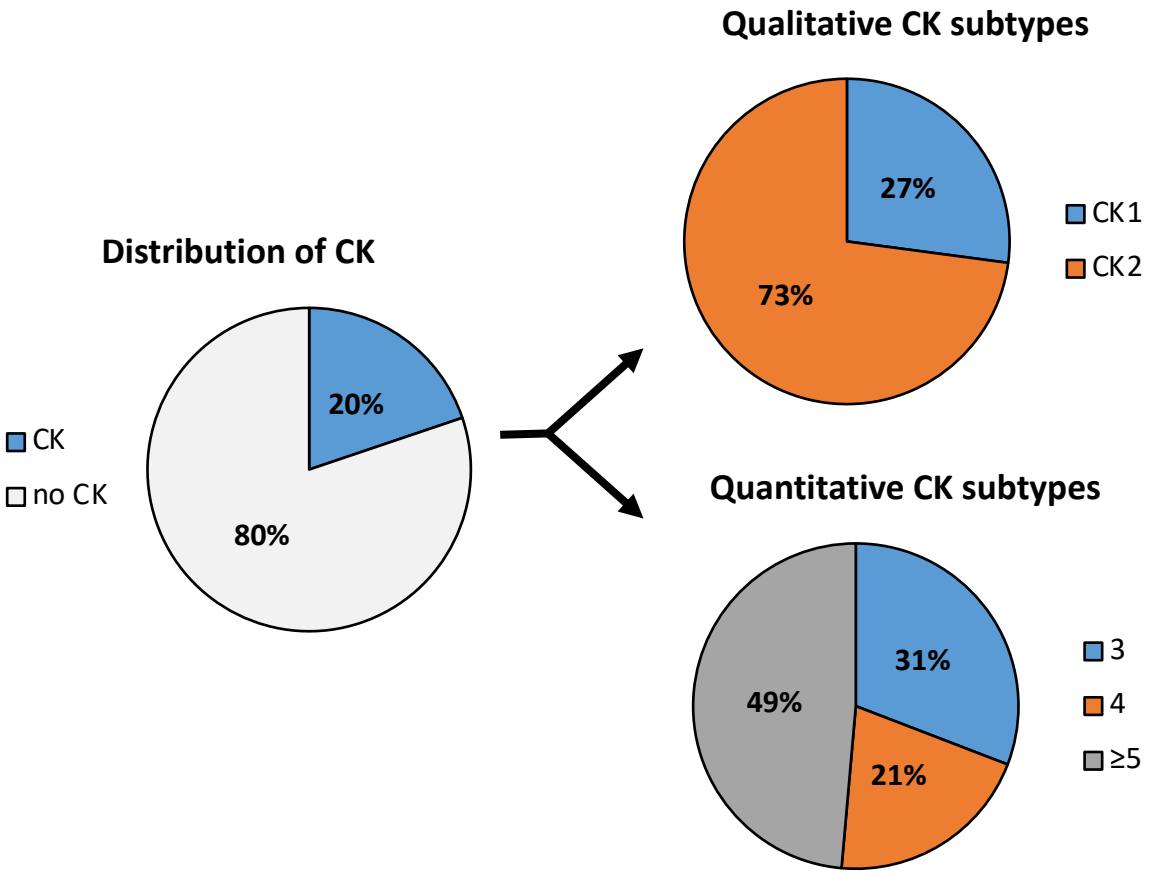
Table S2. Univariate and multivariate analysis for overall survival from RS

	UNIVARIATE ANALYSIS			MULTIVARIATE ANALYSIS		
	HR	95% C.I	p values	HR	95% C.I	p values
MALE	1.34	0.55-3.24	0.5152	-	-	-
AGE>65yy	1.39	0.57-3.39	0.4733	-	-	-
Binet B-C	0.80	0.33-1.95	0.6244	-	-	-
B2MG*	2.39	0.69-8.34	0.1706	-	-	-
U-IGHV	1.07	0.36-3.16	0.8987	-	-	-
PRE. THER.	1.47	0.54-3.95	0.4500	-	-	-
11q-	1.31	0.40-4.35	0.6574	-	-	-
TP53 abn	0.79	0.32-1.93	0.6029	-	-	-
CK	1.47	0.60-3.64	0.3982	-	-	-
CK2	1.56	0.64-3.83	0.3317	-	-	-
HighCK	1.01	0.41-2.48	0.9864	-	-	-

β2MG high = beta2-microglobulin >3.5mg/L, U-IGHV = unmutated IGHV gene, PRE. THER. = previous CLL treatment, 11q- = deletion of 11q22-23 by FISH, TP53 abn = TP53 abnormalities include deletions and/or mutations, CK = complex karyotype, CK2 = type-2 CK, highCK = ≥5 chromosome abnormalities, RS = Richter syndrome. * data available from 520 (96%) patients, 26 (93%) who developed a RS and 494 (96%) who did not transform.

Figure S1

A



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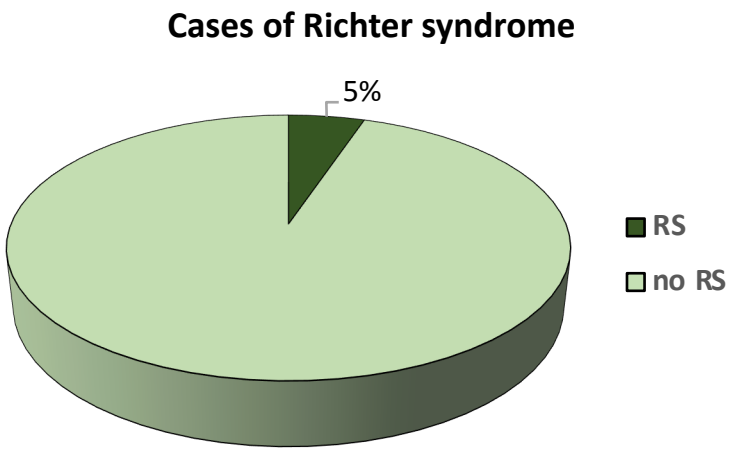

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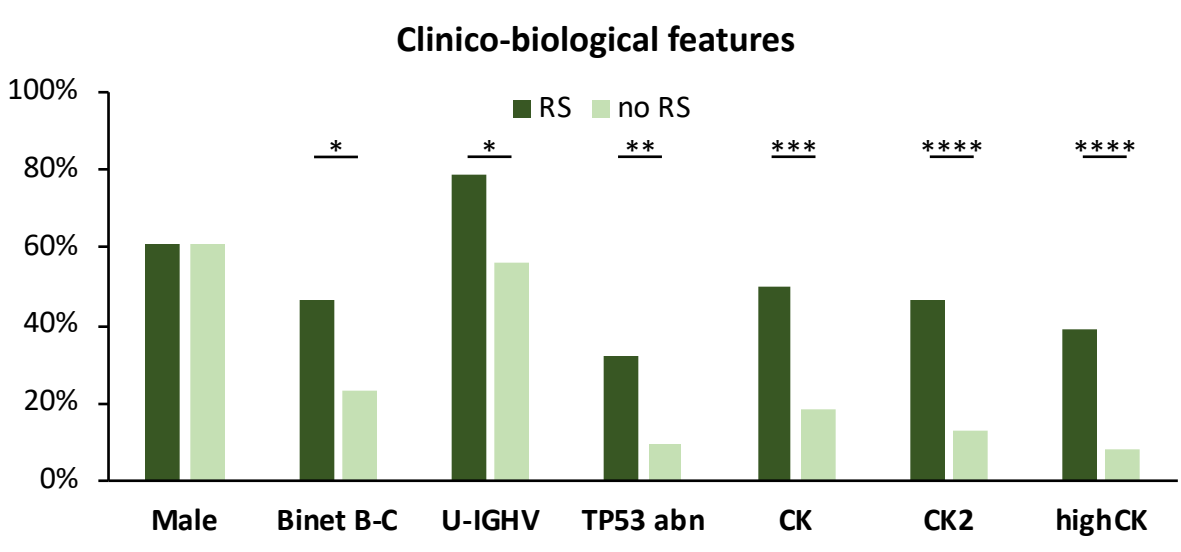

Figure S2

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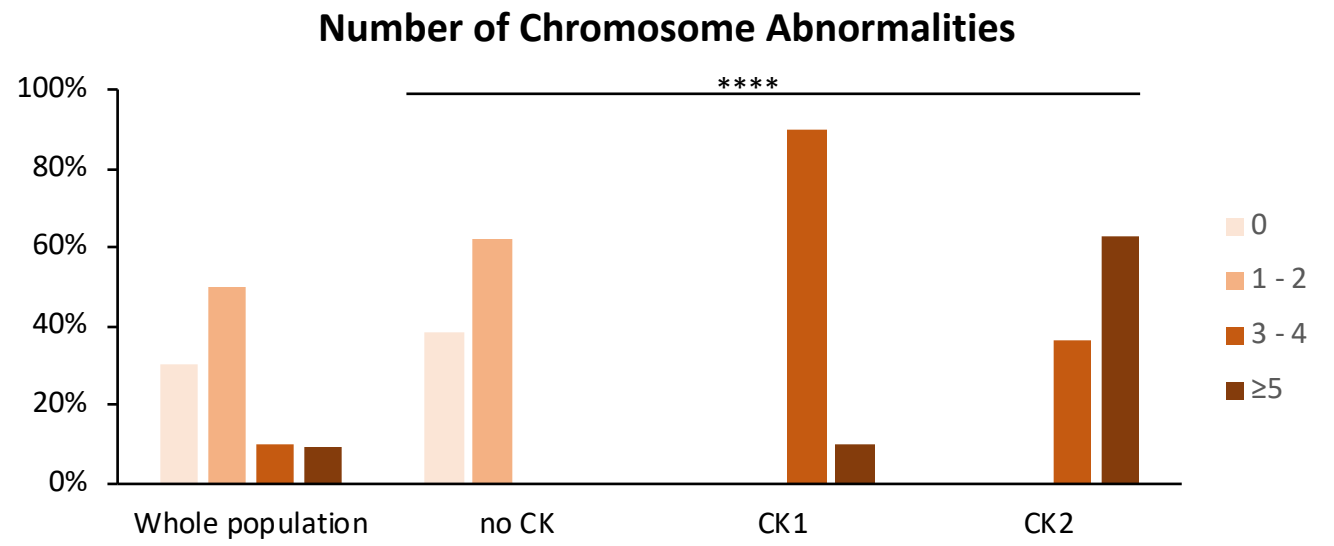

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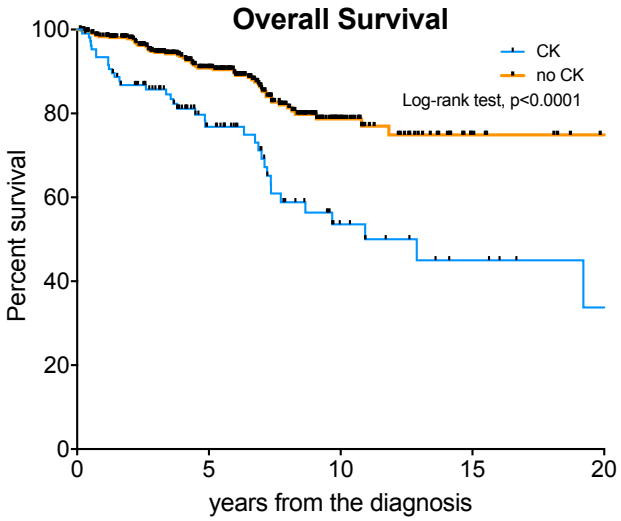

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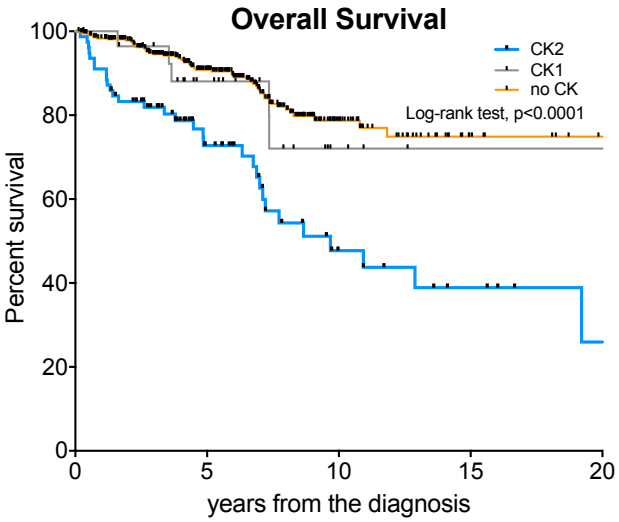

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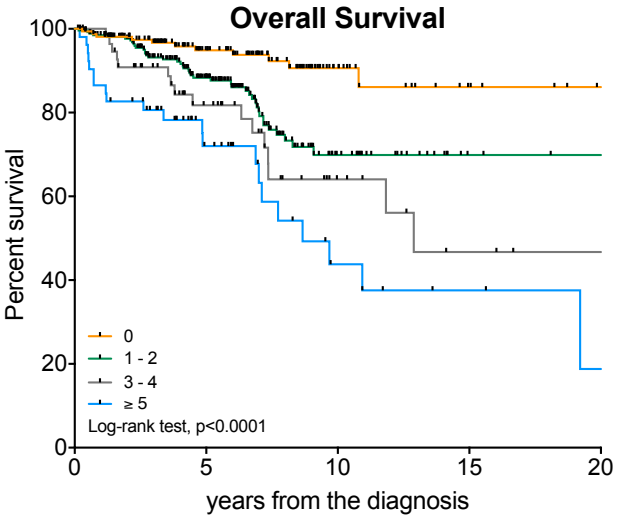


Figure S3

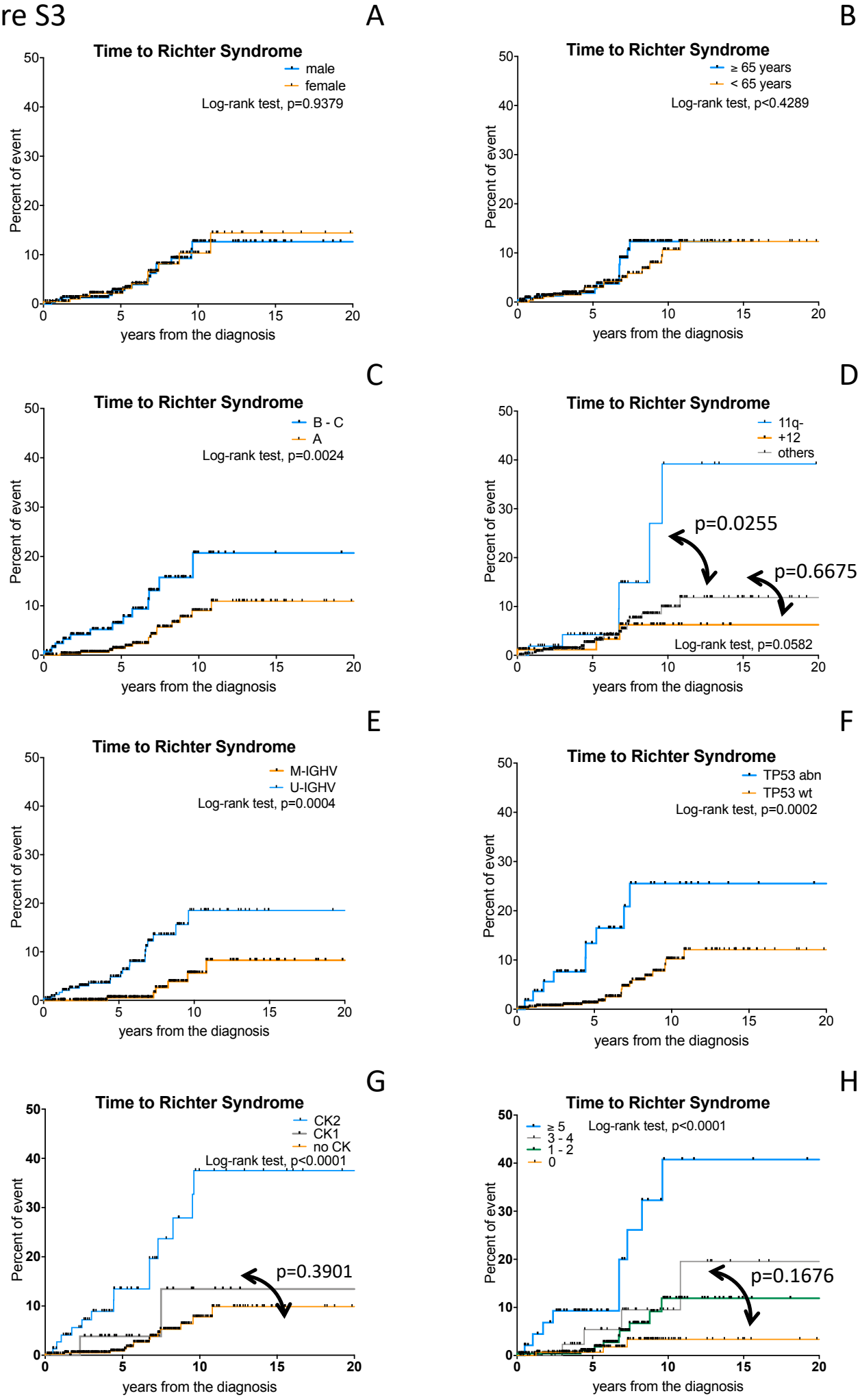
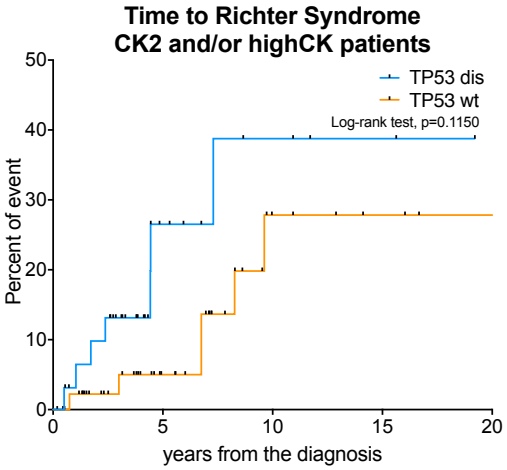

A
Time to Richter Syndrome
male
female
Log-rank test, p=0.9379
Percent of event
years from the diagnosis
B
Time to Richter Syndrome
≥ 65 years
< 65 years
Log-rank test, p<0.4289
Percent of event
years from the diagnosis
C
Time to Richter Syndrome
B - C
A
Log-rank test, p=0.0024
Percent of event
years from the diagnosis
D
Time to Richter Syndrome
11q-
+12
others
p=0.0255
p=0.6675
Log-rank test, p=0.0582
Percent of event
years from the diagnosis
E
Time to Richter Syndrome
M-IGHV
U-IGHV
Log-rank test, p=0.0004
Percent of event
years from the diagnosis
F
Time to Richter Syndrome
TP53 abn
TP53 wt
Log-rank test, p=0.0002
Percent of event
years from the diagnosis
G
Time to Richter Syndrome
CK2
CK1
no CK
Log-rank test, p<0.0001
p=0.3901
Percent of event
years from the diagnosis
H
Time to Richter Syndrome
≥ 5
3 - 4
1 - 2
0
Log-rank test, p<0.0001
p=0.1676
Percent of event
years from the diagnosis

Figure S4

A



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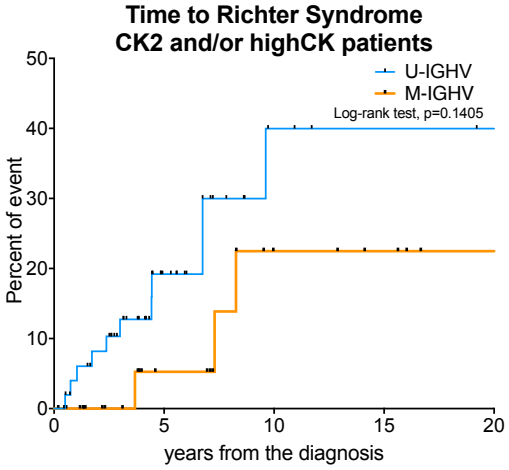

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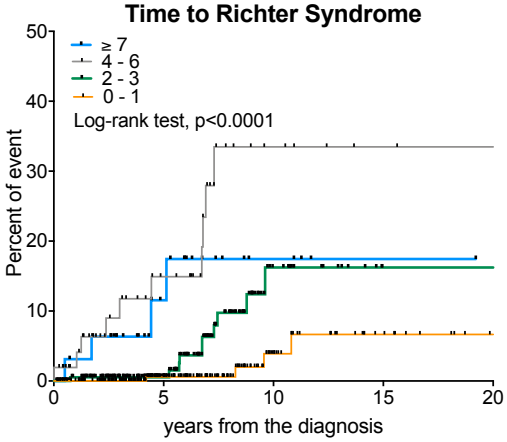

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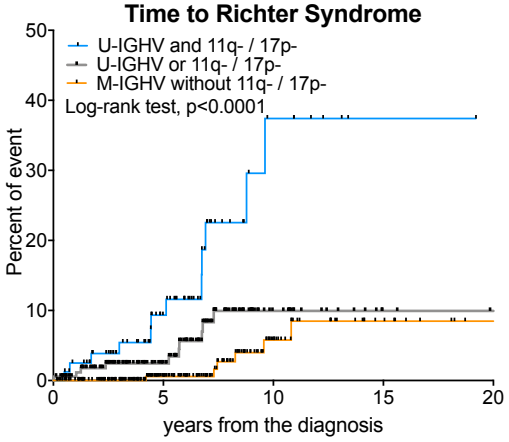

Figure S5

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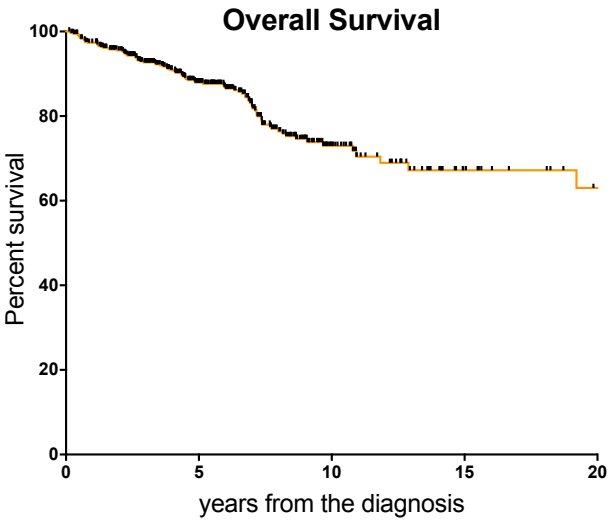

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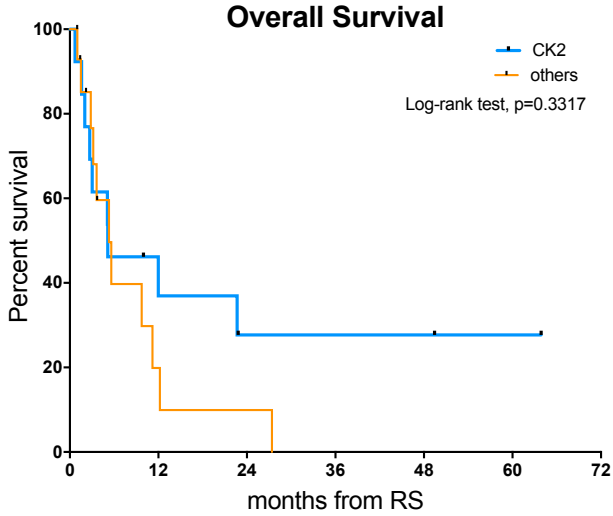

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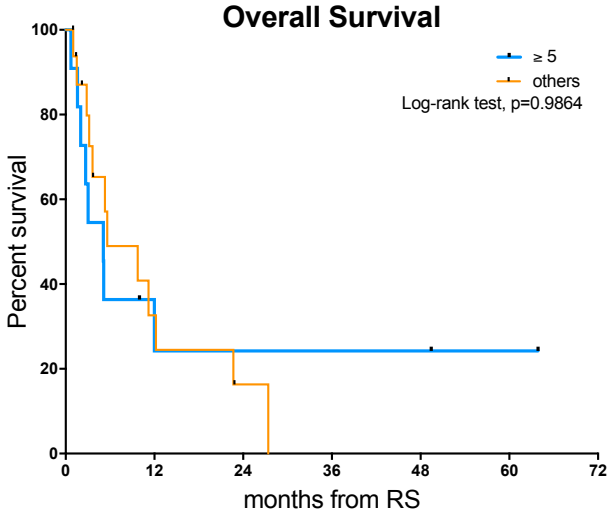

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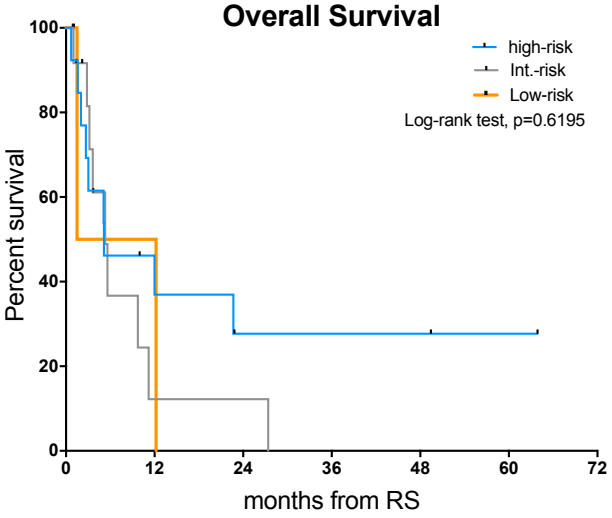
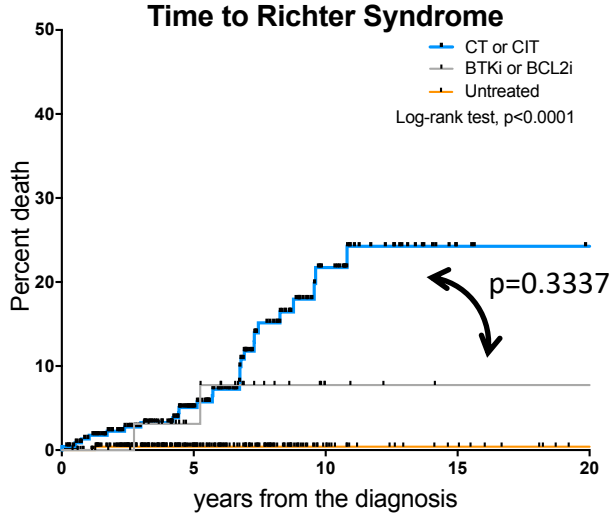
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