SUPPLEMENTARY APPENDIX

Revisiting the immune microenvironment of diffuse large B-cell lymphoma using a tissue microarray and immunohistochemistry: robust semi-automated analysis reveals CD3 and FoxP3 as potential predictors of response to R-CHOP

Rita Coutinho,[1] Andrew J. Clear,[1] Emanuele Mazzola,[2] Andrew Owen,[1,3] Paul Greaves,[1] Andrew Wilson,[1] Janet Matthews,[1] Abigail Lee,[1,3] Rute Alvarez,[4] Maria Gomes da Silva,[4] José Cabeçadas,[5] Donna Neuberg,[3] Maria Calaminici,[1,3] and John G. Gribben[1]

[1]Department of Hemato-Oncology, Barts Cancer Institute, Queen Mary University, London, UK; [2]Department of Biostatistics and Computational Biology, Dana-Farber Cancer Institute, Boston, MA, USA; [3]Department of Histopathology, Barts Health NHS Trust, Royal London Hospital, UK; [4]Department of Hematology, Portuguese Institute of Oncology, Lisbon, Portugal; and [5]Department of Pathology, Portuguese Institute of Oncology, Lisbon, Portugal

 doi:10.3324/haematol.2014.110189
The online version of this article has a Supplementary Appendix.
Manuscript received on August 3, 2014. Manuscript accepted November 24, 2014.
Correspondence: rittakoutinho@gmail.com

Supplementary data

Supplementary Methods

Tissue Microarrays construction

Each original block was cut and stained with Haematoxylin and Eosin (H&E). Well-represented tumour areas were marked with a pen on the slide. Donor blocks were aligned with the marked slides. A semi-automated arrayer (Beecher Scientific, Silver Spring, MD) was used. Reactive tonsils were used as controls and heart cores as orientation. A standard microtome technique was used for sectioning 3 µm sections into slides.

Immunohistochemistry

TMA slides were placed in plastic racks at 60^{o}C overnight. For paraffin removal slides were incubate in xylene for 2 consecutive periods of 5 min. Subsequently, slides were incubate for 3 consecutive periods of 2min in Industrial Methylated Spirits (IMS) and further 2 periods of 2min in hydrogen peroxide in order to dehydrate tissue and reduce non-specific staining from the action of endogenous peroxidises on the chromogen. A final incubation of 2min in IMS is required prior to antigen retrieval. While performing first incubation steps, 3000ml of a working solution of antigen unmasking solution was warmed up in a pressure cooker. When boiling, the plastic racks with slides were immersed and left for 10min at high heat (120-130^{o}C). When finished, the pressure cooker was left to cool down under cold tap water for 5min and the slides quickly transferred to a wash buffer pot.

Slides were marked using hydrophobic pen around the edge of the array field and kept wet with wash buffer throughout the remaining procedure.

The DAKO Autostainer System is composed of a robot arm with nozzles and a pump system, which allows for timed dispensing of reagents into the slides. Before using, the software (Dako Autostainer Plus) is programmed for the number of slides, reagents and incubation times and rinse steps. The Super Sensitive™ Polymer-HRP IHC Detection System (Biogenex) was used for signal detection. The Autostainer was run for 2-3 hours as specified. After finishing all slides were replaced in plastic racks and rinsed in tap water for 5min. As a counterstain, the slides were suspended in haematoxylin solution for 5min, rinsed for 2min in running water and plunged into acid alcohol solution, quickly, for 5 times, after which were transferred into tap water wash. Finally the tissue is re-hydrated using IMS and clarified by incubation in xylene baths. Using DPX mounting media, which provide a high

quality durable mounting with refractive properties, cover slips were applied leaving no trapped air bubbles. Finally, slides were left to dry and labelled appropriately.

Automated Image Analysis

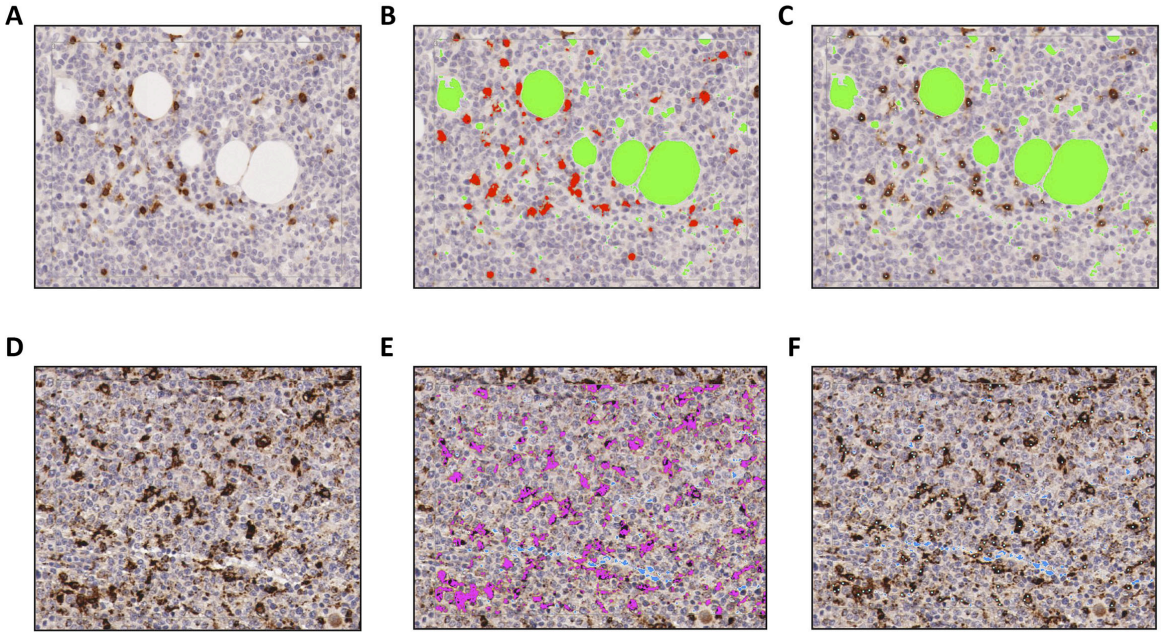
Automated Image analysis using the Ariol SL-50 visual analysis software

Slides were scanned using an Olympus BX61 microscope with an automated platform (Prior). A review of all cores was performed manually. Whole cores with less than 50% of tumor representation were excluded from analysis. Fibrotic and necrotic areas were also excluded. Representative regions were selected for training. Positive stained cells acquire a brown/black colour characteristic of DAB. Colour hue, saturation and intensity were manipulated to allow contrast with the background. This was achieved by selection of individual pixels from positive events and avoiding the negative cells and the non-specific stained areas. Training is improved by limiting the size and the shape of the areas considered positively stained with DAB. In order to estimate areas of representative tumour tissue, a second colour training for non-cellular areas was included (Supplementary Figure 1). The area of viable tissue was calculated by subtracting the non-cellular areas from the total area analysed. Using this system we calculated the number of positive cells as well as the area of DAB stained per area of lymphoma tissue. The values obtained were corrected to a $1mm^2$ area and a mean for each patient was calculated.

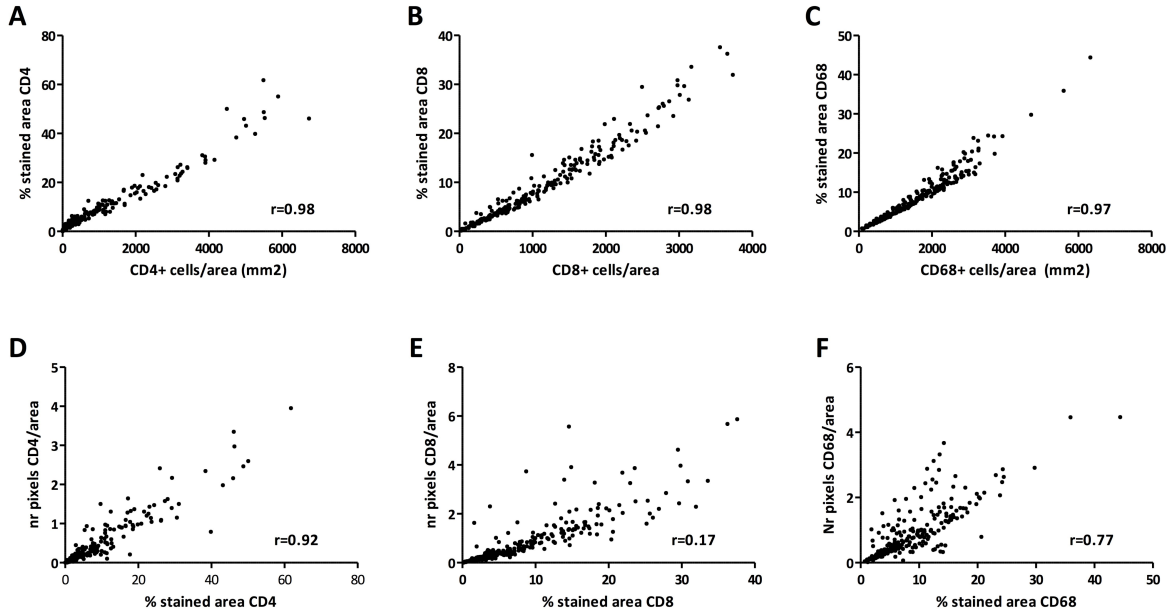
Automated Image analysis using the Pannoramic Viewer System

Slides were scanned using the Pannoramic 250 Flash II scanner (3DHISTECH). Each core was observed on a computer screen using the Pannoramic Viewer computer interface and meticulous bookmarking of representative tumour areas was done and areas quantified. After selection of representative tumour areas, the DensitoQuant module was used to quantify the number of DAB stained pixels. This module distributes pixels to 3 grades of positive classes by their RGB values. We used only the top red and orange levels for identification of stained areas. After adjusting the brown tolerance and the score levels an optimal script was saved for each antibody and applied for analysis in all areas. Finally, the number of brown pixels/selected area was calculated and a mean value was estimated for each patient.

Supplementary Figure 1



Supplementary Figure 2



Supplementary Table 1: Clinical characteristics and outcome of the R-CHOP dataset

	R-CHOP dataset (n= 161)	
Demographic or clinical characteristics	**No.**	**%**
Male	92	57.0
Age, years		
Median	62	
Range	16-86	
> 60 years	84	52.0
Stage		
I	36	22.4
II	37	23.0
III	37	23.0
IV	51	31.7
Stage III-IV	88	54.7
≥ 2 Extranodal sites	29	18.0
High LDH	105	65.2
ECOG performance status		
0	83	51.6
1	55	34.2
2	16	9.9
3	7	4.3
"B-symptoms"	39	26.0
International prognostic index (IPI)		
Low	65	40.4
Low-intermediate	33	21.0
High-intermediate	43	26.7
High	20	12.4
IPI ≥3	63	39.1
Response		
CR/CRu	115	72.3
PR	27	17.0
SD	2	1.3
PD/failure	15	9.4
Relapse rate (from CR/CRu)	27	23.5
Death rate	47	29.2
Causes of death		
Lymphoma	31	66.0
Toxicity	8	17.0
Other	7	14.9
Follow-up (median in months, range)	55.1 (3 - 110)	

No., number; LDH, lactate dehydrogenase; ECOG, Eastern Cooperative Oncology Group; IPI, International Prognostic Index; CR, complete response; CRu, complete response/undetermined; PR, partial response; SD, stable disease; PD, progressive disease

Supplementary Table 2: Primary antibodies and conditions of use

Antibody	Source	Clone	Antigen retrieval	Dilution
CD20	Dako	L26	Citrate buffer (pH 6.0) pressure cooker 10 min	1/2000
CD3	Lab Vision, Thermo Scientific	SP7	Citrate buffer (pH 6.0) pressure cooker 10 min	2/1000
CD4	Novocastra, Leica Biosystems	4B12	Citrate buffer (pH 6.0) pressure cooker 10 min	2/1000
CD8	Dako	C8/144B	Citrate buffer (pH 6.0) pressure cooker 10 min	2,5/1000
CD68	Dako	KP1	Citrate buffer (pH 6.0) pressure cooker 10 min	1/8000
FOXP3	Abcam	263A/E7	Citrate buffer (pH 6.0) pressure cooker 10 min	1/100
TIA1	Immunotech, Beckman Coulter	2G9A10F5	Citrate buffer (pH 6.0) pressure cooker 10 min	1/400

Supplementary Table 3. Descriptive statistics showing heterogeneity of expression of biomarkers between patients

	Ariol			Pannoramic Viewer	
Variable	**Patients assessable, Number (%)**	**Cells/mm^2, Median (Range)**	**% Stained area, Median (Range)**	**Patients assessable, Number (%)**	**Nr pixels/mm^2, Median (Range)**
CD3	258 (84)	2603 (50-7480)	11.8 (0-56.1)	243 (79)	$1.2x10^6$ ($1.5x10^4$-$2.4x10^{12}$)
CD4	206 (67)	438.4 (0-6730)	5.23 (0-61.8)	231 (75)	$2.3x10^5$ (147.4-$4.5x10^6$)
CD8	251 (81)	857.5 (25-3732)	6.44 (0-37.6)	252 (82)	$5x10^5$ (6566-6.6 $x10^7$)
FOXP3	253 (82)	228.6 (0-3197)	0.68 (0-13.6)	246 (80)	3.7 $x10^4$ (25.2-1.3 $x10^6$)
TIA1	249 (81)	1648 (32-5859)	9.6 (0-80.5)	250 (81)	$1.6x10^5$ (1079-2.5 $x10^6$)
CD68	252 (82)	1380 (85-6320)	7.1 (0-44.4)	249 (81)	$6.5x10^5$ ($1.5x10^4$-$6.1x10^6$)

Supplementary Table 4. Survival Analysis

UNIVARIATE ANALYSIS			
Overall Survival			
Variable	**Median survival (m)**	**Hazard ratio (95% CI)**	***P***
Age >60 years	92.1 vs undefined	1.96 (1.13, 3.44)	0.02
Stage III-IV	82.9 vs undefined	3.33 (1.51, 4.54)	0.0005
ECOG PS ≥2	30 vs undefined	3.84 (1.96, 9.09)	0.001
IPI ≥3	81 vs undefined	2.63 (1.44, 4.76)	0.001
Not achieving CR	17.2 vs undefined	6.66 (3.44, 14.3)	<0.0001
Progression-free survival			
Stage III-IV	34.2 vs 89.1	2.27 (1.42, 3.57)	0.0005
ECOG PS ≥2	11.1 vs 78.7	4.16 (1.85, 9.09)	0.0005
≥2 extranodal areas	10.5 vs 78.7	2.78 (1.43, 5.55)	0.003
Centre (Bart's vs IPO)	81 vs 52.9	1.6 (1.03, 2.59)	0.03
IPI ≥3	24.9 vs 82.1	2.43 (1.47 vs 4)	0.0004
CD3 +cells/area	78.7 vs 27.9	0.50 (0.31, 0.83)	0.005
CD3 %stained area	82.1 vs 29.1	0.46 (0.28, 0.75)	0.001
CD3 Nr pixels/area	78.7 vs 30.3	0.52 (0.31, 0.88)	0.01
FoxP3 +cells/area	89.1 vs 26.2	0.40 (0.24, 0.67)	0.0003
FoxP3 %stained area	undefined vs 27.9	0.43 (0.26, 0.71)	0.0008
FoxP3 Nr pixels/area	82.1 vs 27.9	0.45 (0.27, 0.76)	0.002
MULTIVARIATE ANALYSIS FOR PFS			
Variable		**Hazard ratio (95% CI)**	***P***
Stage III-IV		3.4 (1.72, 6.66)	< 0.0001
High % stained area CD3		0.42 (0.24, 0.73)	0.003

For IHC data, all hazard ratios with corresponding 95% confidence interval were calculated considering the HIGH versus LOW density for each variable. Results from backward stepwise (likelihood ratio) based on 120 cases are reported. m: months; CI: confidence interval; Nr: number