Impact of age on genetics and treatment efficacy in follicular lymphoma

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SUPPLEMENTARY APPENDIX

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SUPPLEMENTARY METHODS

Study cohort for clinical analyses: The clinical study cohort was derived from the GLSG2000 trial of the *German Low Grade Lymphoma Study Group.*[1] We included patients with grade 1-3A FL with stage III/IV disease or localized disease considered ineligible for curative radiotherapy, who received Rituximab (R)-CHOP as frontline treatment in our analysis (Figure S3).[1] Patients who received consolidative autologous stem cell transplantation were censored at the time of transplant. All patients were in need of systemic treatment as defined by the presence of B symptoms, bulky disease (mediastinal lymphomas >7.5 cm or other lymphomas >5 cm), impairment of normal hematopoiesis or rapidly progressive disease (>50% increase of lymphoma manifestations within 6 months). Patients with Eastern Cooperative Oncology Group (ECOG) performance status > 2 (unless related to lymphoma) and severe comorbidities were not eligible to be enrolled into this trial.[1] The trial was approved by the institutional review board and all participants gave written informed consent.

Treatment of patients within the GLSG2000 trial: Patients in the GLSG2000 trial were initially randomized to either CHOP (cyclophosphamide 750 mg/m^2, day 1; doxorubicin 50 mg/m^2, day 1; vincristine 1.4 mg/m^2, day 1; prednisone 100 mg/day, days 1–5; every three weeks) or immunochemotherapy with CHOP plus Rituximab (375 mg/m², day 0). After randomization was stopped, additional patients were allowed to register and assigned to R-CHOP treatment. The use of granulocyte colony-stimulating factor (G-CSF) or antiinfective prophylaxis was not recommended as part of the induction treatment. Induction therapy was ended after 4 courses if patients had achieved a complete remission (CR). Patients with less than CR received two additional courses of induction therapy. Patients up to 60 years with at least partial remission (PR) could participate in a second randomization comparing myeloablative radiochemotherapy followed by autologous stem cell transplantation (ASCT) with interferon-a (IFNα) maintenance. Patients randomized to IFNα maintenance received two additional courses of induction therapy (in total 6-8 courses). IFNα was applied 5 x 10^6 U s.c. 3 times weekly until progression or the occurrence of intolerable side-effects.

Patients in the ASCT arm received the mobilization chemotherapy Dexa-BEAM (dexamethasone 3 x 8 mg p.o., days 1 to 10, BCNU 60 mg/m2 i.v., day 2, melphalan 20 mg/m2 i.v., day 3, etoposide 75 mg/m2 i.v., days 4 to 7, cytarabine 2 x 100 mg/m2 i.v., days 4 to 7, G-CSF initiated day 11) within 4 to 8 weeks after end of induction therapy and subsequent peripheral hematopoietic stem cell harvest. Myeloablative therapy was performed within 2 months after mobilization and consisted of fractionated total body irradiation (TBI, 12 Gy; days –6 to –4) and high-dose cyclophosphamide (60 mg/kg body weight i.v., days –3 and –2). Peripheral blood stem cells were reinfused on day 0. R-maintenance was not part of the study treatment.

Treatment of patients from the BCCA: Patients with symptomatic FL in need of systemic treatment received R-CVP (rituximab 375 mg/m², cyclophosphamide 1000 mg/m², vincristine 1.4 mg/m² on day 1; and prednisone 100 mg/day, days 1–5) every 3 weeks for 6 to 8 cycles. From 2006 onwards, patients achieving at least a partial response were scheduled for rituximab maintenance 375 mg/m^2 given every 3 months for a total of eight doses (n=93/107, 87%). The BCCA cohort was not used for any analysis of clinical outcome.

Definition of clinical endpoints: Overall response was complete or partial remission (CR/PR) after induction treatment. POD included refractory disease (<PR) at end of induction treatment, progression, or relapse. Failure-free survival (FFS) events consisted of POD or death. FFS and OS were calculated from date of treatment initiation. POD24 was defined as POD within 24 months.[2, 3] Patients with shorter follow-up or death within 24 months were not evaluable. CR30 was defined as CR observed before and after 30 months.[4] No CR30 was not achieving a CR before and after 30 months, or death or POD within 30 months. For patients with at least one disease evaluation between 27 and 33 months, the status closest to 30 months was applied. The remaining patients were not evaluable.

Study cohort for genetic analyses: The study cohort for genetic analyses consisted of 258 patients with advanced stage FL or localized disease considered ineligible for curative radiotherapy in need of treatment (Table S1 and Figure S1). 151 patients were from GLSG2000 trial, and an additional 107 patients from a population-based registry of the *British Columbia Cancer Agency* (*BCCA*) as previously published.[5] Sequencing was done from diagnostic lymphoma biopsies obtained ≤1 year before treatment initiation.

Filtering strategy for somatic mutations: We called variants with allele frequencies (VAF) ≥10% in the 74 genes listed below. We excluded polymorphisms from the Exome Sequencing Project and the dbSNP (build 142) databases. Known sequencing artifacts were manually excluded.

Targeted mutational burden (TMB): The targeted mutational burden reflects the number of non-germline variants at allele frequencies ≥10% within the full coding regions of 74 captured genes.

Prediction of functional impact: Nonsense mutations, frameshift insertions/deletions (InDels), splice-site mutations, and disruption of the start codon were considered disruptive mutations. Computational evolutionary conservation analysis was used to predict the functional impact of non-silent single nucleotide variants (SNV) (mutationassessor.org, release 3).[6] Scores >3.5 were classified as *high functional impact*. Mutations in non-coding regions and synonymous mutations were considered *silent*.

Features of aberrant somatic hypermutation (aSHM): Single nucleotide variants (SNV) were analyzed for features of aSHM (transitions, mutations within 2kb from transcriptional start site (TSS), mutations in WRCY motifs and known targets of aSHM). Fourteen of 74 captured genes are known targets of aSHM [7]: BCL7A, BTG1, BTG2, CIITA, CXCR4, ETS1, IRF4, IRF8, MYC, P2RY8, PAX5, PIM1, SGK1 and BCL2.

Genes with non-silent mutations: Genes were considered non-silently mutated as previously described.[5] We used the MutSigCV algorithm to identify genes that are mutated more often than expected by chance, i.e. above the background mutational rate.[8] Significantly mutated genes in the molecular cohort are listed below.

List of 74 targeted genes included in molecular analysis: *ARID1A, ARID1B, ARID2, ARID3A, ASXL1, ATM, B2M, BCL2, BCL7A, BRAF, BTG1, BTG2, CARD11, CCND1, CCND3, CD58, CD79A, CD79B, CDKN2A, CDKN2B, CHD2, CIITA, CREBBP, CTSS, CXCR4, DNMT3A, EP300, EPHA7, ETS1, ETV6, EZH2, FAS, FBXO11, FOXO1, GNA13, GNB1, IDH1, IDH2, IKZF1, IKZF2, IKZF3, IRF4, IRF8, KDM6A, KLHL6, KMT2D, MALT1, MEF2B, MUM1, MYC, MYD88, NF1, NOTCH1, NOTCH2, NPM1, P2RY8, PAX5, PDGFRA, PDGFRB, PIM1, PTEN, RB1, SF3B1, SGK1, SMARCA2, SMARCA4, SMARCB1, STAT6, TET2, TNFAIP3, TNFRSF14, TP53, TRAF3, TYK2*

List of 25 significantly mutated genes according to MutSigCV from molecular cohort: *ARID1A, ARID1B, BCL7A, CARD11, CD79A, CIITA, CREBBP, CTSS, CXCR4, EP300, ETS1, EZH2, FAS, IKZF3, IRF8, KMT2D, MEF2B, NOTCH2, P2RY8, PAX5, SMARCB1, STAT6, TNFAIP3, TNFRSF14, TP53*

Statistical analyses: Statistical analyses were carried out with the statistical software R (version 3.3.2) using the packages *survival_2.41-3*, *cmprsk_2.2-7, and ggplot2_2.2.1.* Quasi-Poisson regressions, ANOVA, Spearman's rank correlation and univariate logistic regression were used to explore the impact of age on somatic mutations. The Bonferroni-Holm method was used to correct for multiple testing. The Kaplan-Meier method and competing risk analyses using cumulative incidences of POD and death without (w/o) POD were used to describe time-to-event endpoints. Cox proportional hazards regression, competing risk and logistic regression were applied to investigate the impact of age on treatment outcome. Multivariate analyses included the FLIPI risk factors age (either continuous or categorical variable as indicated), hemoglobin <120 g/L, number of nodal areas > 4, increased LDH, and Eastern Cooperative Oncology Group performance status (ECOG-PS) >1.

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SUPPLEMENTARY TABLES

Table S1. Baseline characteristics of the molecular cohort.

Characteristics		GLSG n=151	BCCA n=107	Age (years) 18-40 n=17	>40-50 n=48	>50-60 n=77	>60-70 n=82	>70 n=34	$p^{\#}$
Male gender		78/151 (52 %)	59/107 (55 %)	11/17 (65 %)	33/48 (69 %)	43/77 (56 %)	35/82 (43 %)	15/34 (44 %)	0.032
Clinical risk factors	>2 FLIPI risk-factors*	55/151 (36 %)	22/104 (21 %)	5/17 (29 %)	11/48 (23 %)	21/77 (27 %)	20/82 (24 %)	20/31 (65 %)	<0.001
	Nodal sites >4	106/151 (70 %)	78/107 (73 %)	15/17 (88 %)	36/48 (75 %)	49/77 (64 %)	57/82 (70 %)	27/34 (79 %)	0.19
	LDH elevated	49/151 (32 %)	22/103 (21 %)	4/17 (24 %)	10/47 (21 %)	21/77 (27 %)	20/82 (24 %)	16/31 (52 %)	0.035
	Hb <120g/l	32/151 (21 %)	12/105 (11 %)	4/17 (24 %)	7/48 (15 %)	11/77 (14 %)	11/82 (13 %)	11/32 (34 %)	0.071
	Stage III/IV	151/151 (100 %)	94/107 (88 %)	17/17 (100 %)	45/48 (94 %)	76/77 (99 %)	75/82 (91 %)	32/34 (94 %)	0.24
	ECOG PS >1	8/151 (5 %)	16/107 (15 %)	0/17 (0 %)	5/48 (10 %)	5/77 (6 %)	8/82 (10 %)	6/34 (18 %)	0.25
Histologic FL grade	1/2	135/139 (97 %)	NA	13/14 (93 %)	28/28 (100 %)	44/45 (98 %)	39/40 (98 %)	11/12 (92 %)	0.54
	3	4/139 (3 %)	NA	1/14 (7 %)	0/28 (0 %)	1/45 (2%)	1/40 (3 %)	1/12 (8 %)	

FLIPI: Follicular lymphoma international prognostic index; LDH: lactate dehydrogenase; Hb: hemoglobin; ECOG PS: Eastern Cooperative Oncology Group Performance Score; FL: Follicular lymphoma; GLSG: German Low Grade Lymphoma Study Group (GLSG2000 trial); BCCA: British Columbia Cancer Agency (registry); NA: not available

* excluding age; # Pearson's chi-squared test across all age cohorts

Table S2. Univariate logistic regression analyses for association of non-silently mutated genes with age.

Gene	Odds ratio*	*p*	Corrected *p*#
ARID1A	1.32	0.11	1.00
ARID1B	1.47	0.072	1.00
BCL7A	1.28	0.26	1.00
CARD11	1.23	0.24	1.00
CD79A	0.49	0.039	0.89
CIITA	0.88	0.67	1.00
CREBBP	0.99	0.94	1.00
CTSS	0.92	0.72	1.00
CXCR4	0.86	0.65	1.00
EP300	1.01	0.96	1.00
ETS1	1.87	0.091	1.00
EZH2	1.27	0.077	1.00
FAS	1.31	0.40	1.00
IKZF3	0.90	0.66	1.00
IRF8	1.36	0.094	1.00
KMT2D	0.82	0.13	1.00
MEF2B	1.41	0.042	0.93
NOTCH2	3.69	0.0041	0.10
P2RY8	1.50	0.16	1.00
PAX5	0.72	0.27	1.00
SMARCB1	0.78	0.62	1.00
STAT6	1.30	0.14	1.00
TNFAIP3	0.94	0.84	1.00
TNFRSF14	1.19	0.15	1.00
TP53	1.82	0.028	0.68

25 MutSigCV genes included in analysis
* Odds ratio reported per 10 year increase; # Bonferroni-Holm method

Table S3. Univariate logistic regression analysis for association of mutation status of *BCL2 with age.*

Gene	Odds ratio*	*p*
BCL2 (any mutation)	0.99	0.93
BCL2 (coding mutation)	1.14	0.25

* Odds ratio reported per 10 year increase

Table S4. Treatment of the clinical cohort.

Characteristics		Age (years) 18-40 n=65	>40-50 n=163	>50-60 n=261	>60-70 n=208	>70 n=58	$p^{\#}$
Time from diagnosis to treatment (mean [days] (SD))		134 (301)	175 (425)	243 (1376)	180 (406)	100 (233)	0.74
R-CHOP courses received (mean (SD))	No ASCT	6.89 (1.45)	6.93 (1.44)	6.80 (1.46)	6.93 (1.46)	6.05 (2.02)	0.003
	ASCT	5.90 (0.44)	5.37 (1.18)	5.53 (0.89)	6.00 (NA)	-	-

ASCT: autologous stem cell transplantation in first remission; SD: standard deviation

ANOVA test across all age cohorts

Table S5. Treatment response by age.

Outcome		Age (years) 18-40	>40-50	>50-60	>60-70	>70	$p^{\#}$
Response to induction therapy	CR/PR	62/63 (98 %)	154/159 (97 %)	248/258 (96 %)	194/206 (94 %)	47/58 (81 %)	2.9×10^{-5}
	SD	1/63 (2 %)	4/159 (3 %)	9/258 (3 %)	4/206 (2 %)	2/58 (3 %)	0.83
	PD	0/63 (0 %)	1/159 (1 %)	1/258 (0 %)	4/206 (2 %)	0/58 (0 %)	0.29
	Death	0/63 (0 %)	0/159 (0 %)	0/258 (0 %)	2/206 (1 %)	7/58 (12 %)	3.7×10^{-13}
	Stopped	0/63 (0 %)	0/159 (0 %)	0/258 (0 %)	2/206 (1 %)	2/58 (3 %)	0.013
CR30		12/39 (31 %)	10/93 (11 %)	29/143 (20 %)	23/184 (13 %)	7/52 (13 %)	0.016
POD24		5/41 (12 %)	24/97 (25 %)	28/152 (18 %)	45/191 (24 %)	14/46 (30 %)	0.19

CR: complete remission; PR: partial remission; CR30: complete remission at 30 months; POD24: progression of disease within 24 months from induction treatment

[#] Pearson's chi-squared test across all age cohorts

Table S6. Cox-regression analysis for overall survival and failure-free survival, and competing risk regression for POD and death without POD.

		Univariate analysis[a]			Multivariate analysis*[b]		
	Age (yrs.)	HR	95% CI	*p*	HR	95% CI	*p*
OS	18-40	0.15	[0.02; 1.12]	0.065	0.17	[0.02: 1.29]	0.087
	>40-50	1.01	[0.52; 1.97]	0.98	0.88	[0.42; 1.85]	0.73
	>50-60	Ref.			Ref.		
	>60-70	1.88	[1.14; 3.10]	0.014	1.60	[0.92; 2.79]	0.095
	>70	7.24	[4.21; 12.46]	8.9×10^{-13}	5.68	[3.08; 10.48]	2.6×10^{-8}
	continuous	2.04[c]	[1.67; 2.48]	2.3×10^{-12}	1.82[c]	[1.47; 2.24]	2.6×10^{-8}
FFS	18-40	0.63	[0.35; 1.14]	0.13	0.55	[0.29; 1.04]	0.067
	>40-50	1.03	[0.71; 1.50]	0.88	0.99	[0.66; 1.49]	0.98
	>50-60	Ref.			Ref.		
	>60-70	1.21	[0.89; 1.64]	0.24	1.08	[0.77; 1.50]	0.66
	>70	2.15	[1.44; 3.22]	0.00020	1.81	[1.16; 2.83]	0.0094
	continuous	1.21[c]	[1.07; 1.36]	0.0017	1.16[c]	[1.02: 1.31]	0.020
POD	18-40	0.66	[0.38; 1.16]	0.15	0.58	[0.31; 1.06]	0.077
	>40-50	1.04	[0.71; 1.52]	0.83	1.03	[0.69; 1.56]	0.87
	>50-60	Ref.			Ref.		
	>60-70	1.09	[0.80: 1.50]	0.58	1.05	[0.75; 1.47]	0.78
	>70	1.19	[0.75; 1.89]	0.47	0.90	[0.54; 1.53]	0.71
	continuous	1.05[c]	[0.94; 1.18]	0.39	1.03[c]	[0.91; 1.16]	0.67
Death w/o POD	18-40	N/A	N/A	N/A	N/A	N/A	N/A
	>40-50	0.77	[0.70; 8.52]	0.83	0.72	[0.06; 8.18]	0.79
	>50-60	Ref.			Ref.		
	>60-70	4.58	[0.99; 21.18]	0.052	2.60	[0.57; 11.93]	0.22
	>70	24.65	[5.34; 113.81]	4.0×10^{-5}	16.49	[3.67; 74.11]	0.00026
	continuous	3.72[c]	[2.48; 5.59]	2.2×10^{-10}	3.09[c]	[1.93; 4.94]	2.5×10^{-6}

HR: Hazard ratio; 95% CI: 95% confidence interval; yrs.: years; Ref.: Reference; OS: overall survival; FFS: failure-free survival; POD: progression of disease; Death w/o POD: death without progression of disease; N/A: not available

* adjustment for nodal sites >4, hemoglobin <120 g/l, elevation of lactate dehydrogenase (LDH) and Eastern Cooperative Oncology Group Performance Score (ECOG PS) >1

[a] 755 patients evaluable for overall survival and 747 patients evaluable for failure-free survival, POD and Death w/o POD; [b] 634 patients evaluable for overall survival and 627 patients evaluable for failure-free survival, POD and Death w/o POD

[c] HR reported per 10 year increase

SUPPLEMENTARY FIGURES

Figure S1. Distribution of age in the molecular cohort (n=258).

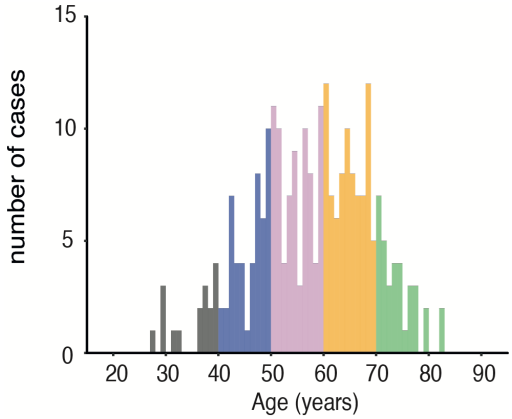

Figure S1

Color legend: gray for 18-40 years; blue for >40-50 years; purple for >50-60 years; orange for >60-70 years; green for >70 years.

Figure S2. Non-silently mutated genes by age. (A) The number of non-silently mutated genes of 73 targeted genes (*BCL2* excluded) as dotplot by age (quasi-Poisson regression). (B) Impact of age on the molecular component of m7-FLIPI, i.e. the sum of individual gene mutations weighted by their predictor values. Colored boxplots indicate median values with 1st and 3rd quartiles, whiskers represent 1.5x interquartile range.

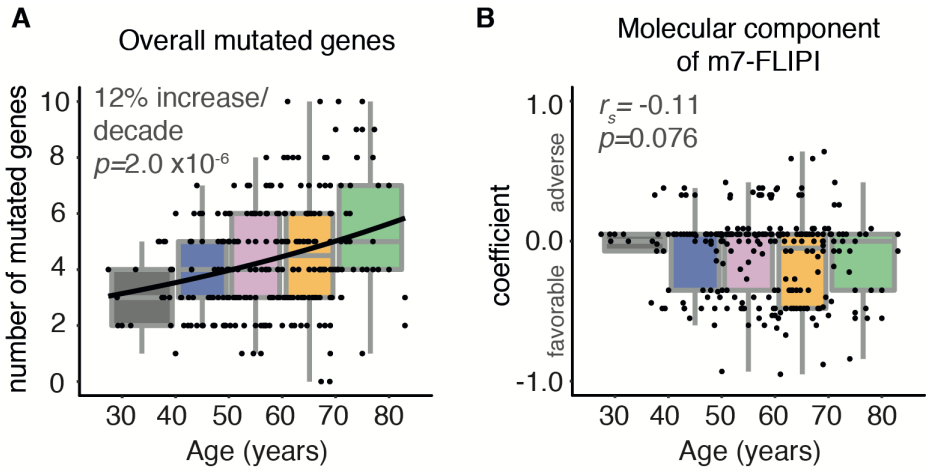

Figure S2

Color legend: gray for 18-40 years; blue for >40-50 years; purple for >50-60 years; orange for >60-70 years; green for >70 years.

Figure S3. CONSORT diagram for patient selection of the clinical cohort.

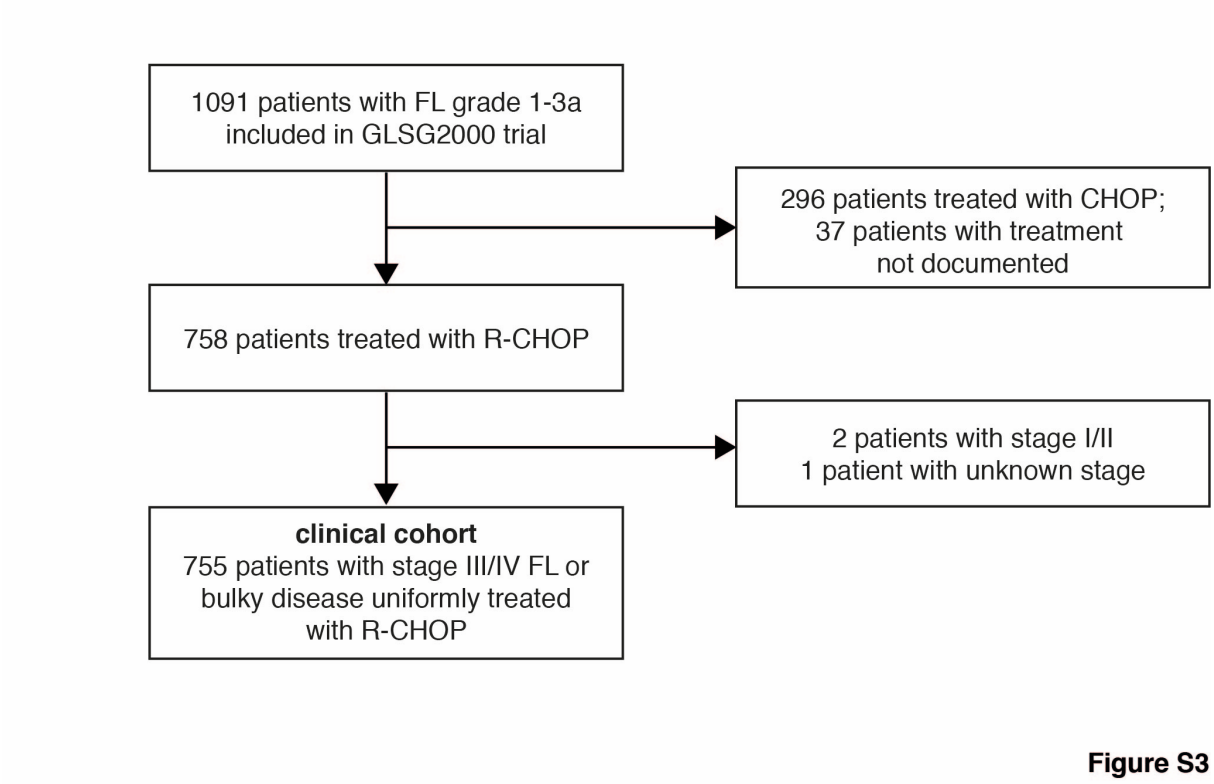

Figure S3

FL: follicular lymphoma; R: rituximab; CHOP: cyclophosphamide, doxorubicin, vincristine and prednisone

Figure S4. Progression-free survival of R-CHOP treated patients according to age cohorts. (A) Kaplan–Meier curve for progression-free survival (PFS). (B) Cumulative incidence analysis of PFS with PFS event without (w/o) Death (dashed lines) as primary and death (solid lines) as competing event.

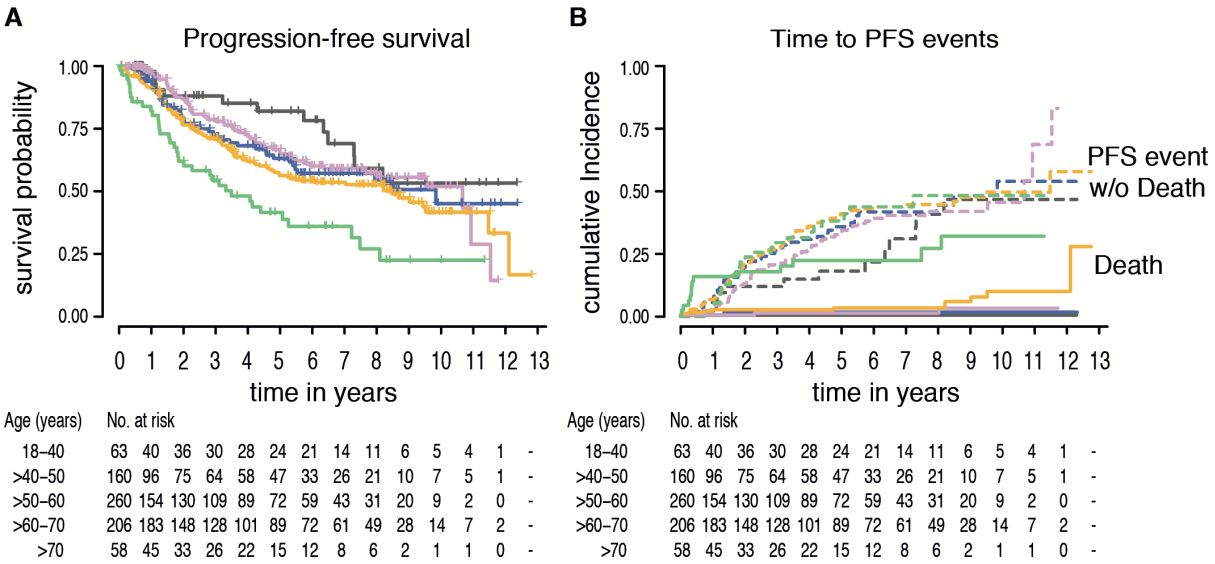

Figure S4

Color legend: gray for 18-40 years; blue for >40-50 years; purple for >50-60 years; orange for >60-70 years; green for >70 years.

PFS: progression-free survival; w/o: without