The genotype of *MLH1* identifies a subgroup of follicular lymphoma patients who do not benefit from doxorubicin: FIL-FOLL study

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SUPPLEMENTARY APPENDIX CONTENTS

THE GENOTYPE OF *MLH1* IDENTIFIES A SUBGROUP OF FOLLICULAR LYMPHOMA PATIENTS THAT DO NOT BENEFIT FROM DOXORUBICIN: FIL-FOLL05 STUDY

Davide Rossi,[1] Alessio Bruscaggin,[1] Piera La Cava,[2] Sara Galimberti,[3] Elena Ciabatti,[3] Stefano Luminari,[4] Luigi Rigacci,[5] Alessandra Tucci,[6] Alessandro Pulsoni,[7] Giovanni Bertoldero,[8] Daniele Vallisa,[9] Chiara Rusconi,[10] Michele Spina,[11] Luca Arcaini,[12] Francesco Angrilli,[13] Caterina Stelitano,[14] Francesco Merli,[15] Gianluca Gaidano,[1*] Massimo Federico,[4*] Giuseppe A. Palumbo[2*]

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Supplementary Methods

Figure S1. Kaplan-Meier estimates of time to treatment failure stratified according to the *FCGR2A rs1801274* genotype, clinical features at presentation and treatment randomization.

Figure S2. Kaplan-Meier estimates of time to treatment failure stratified according to the *FCGR3A rs396991* genotype, clinical features at presentation and treatment randomization.

Figure S3. Kaplan-Meier estimates of time to treatment failure stratified according to the genotypes of *FCGR3A* rs396991 and FCGR3A *rs396991* and patients' gender.

Figure S4. Kaplan-Meier estimates of time to treatment failure stratified according to the genotypes of *MLH1* rs1799977, *GSTA1* rs3957357, and *CYBA* rs4673, and treatment randomization.

Table S1. Distribution of the alleles and Hardy-Weinberg equilibrium

Table S2. Clinical features of the whole study cohort by *FCGR2A* rs1801274 genotype

Table S3. Clinical features of the whole study cohort by *FCGR3A* rs396991 genotype

Table S4. Clinical features of R-CHOP arm cohort by *MLH1* rs1799977 genotype

Supplementary Methods

Patients

Patients were enrolled in the FOLL05 study between March 2006 and September 2010. The clinical database was locked on May 2012. The FLIPI score was a pre-specified criterion of patient stratification and was assessed at the time of patient enrollment and before treatment. The three study arms included eight doses of rituximab combined with eight courses of CVP, or six cycles of CHOP or six cycles of FM, every 3 weeks. Doses and administration schedules for each regimen were as follows: CVP (cyclophosphamide 750 mg/m^2 on day 1, vincristine 1.4 mg/m^2 [capped at 2 mg] on day 1, prednisone 40 mg/m^2 on days 1-5), CHOP (cyclophosphamide 750 mg/m^2 on day 1, vincristine 1.4 mg/m^2 [capped at 2 mg] on day 1, doxorubicin 50 mg/m^2 on day 1, prednisone 100 mg on days 1-5,), and FM (fludarabine 25 mg/m^2 on days 1-3, mitoxantrone 10 mg/m^2 on day 1). Rituximab (375 mg/m^2 at each infusion) was administered on day 1 of each chemotherapy course. Growth factors were administered at physicians' discretion. Dose reduction/treatment delay rules were pre-specified in the protocol. Median overall delivered dose-intensities were 0.956, 0.964, and 0.918 for R-CVP, R-CHOP, and R-FM, respectively. Final response was assessed within one month after last rituximab infusion. Response assessment was performed by physical examination, laboratory tests, and total-body CT scan. Bone marrow biopsy was required only for assessment of final response if positive at baseline. Patients showing progressive (PD) or stable disease (SD) were coded as experiencing treatment failure and shifted to salvage treatment. According to study protocol, maintenance therapy was not allowed. During follow-up, disease status was to be assessed at months +3, +6, +12, +18, +24, and +36 with CT scan and with bone marrow biopsy if positive at baseline.

Study design

Sample size calculation of this biologically ancillary study was performed according to the assumption of a proportion of patients with the genotype at risk $\geq$10%. By pooled analysis of the three treatment arms, we estimated that 428 patients would allow detecting at least a 20% difference in 3-year TTF between patients harboring the genotype at risk (3-year-TTF=35%) and patients harboring the common genotype (3-year-TTF=56%) (power=82%; alpha=0.05). In the R-CVP arm, we estimated that 135 patients would allow detecting at least a 30% difference in 3-year TTF between patients harboring the genotype at risk (3-year-TTF=15%) and patients harboring the common genotype (3-year-TTF=45%) (power=80%; alpha=0.05). In the R-CHOP arm, we estimated that 143 patients would allow detecting at least a 30% difference in 3-year TTF between patients harboring the genotype at risk (3-year-TTF=28%) and patients harboring the common genotype (3-year-TTF=63%) (power=80%; alpha=0.05). In the R-FM arm, we estimated that 143 patients would allow detecting at least a 30% difference in 3-year TTF between patients harboring the genotype at risk (3-year-TTF=28%) and patients harboring the common genotype (3-year-TTF=58%) (power=84%; alpha=0.05).

SNP genotyping

Genotyping of *FCGR2A* rs1801274, *FCGR3A* rs396991, *CYBA* (rs4673) and *GSTA1* (rs3957357) was analyzed by single base sequencing on a ABI Prism 3100 Genetic Analyzer (Applied Biosystem), using the ABI Prism SNaPshot Multiplex Kit according to manufacturer instructions and the following sequencing primers: GATGGAGAAGGTGGGATCCAAA, TTTTTTTTTCCTACTTCTGCAGGGGGCTT, AAAAAAAACCTCCCCCAGGGGACAGAAG and AAAAAAAAAAAAAAAAAAAAAAAATCTCTCCCACTGAAAGAAG. In brief, 100 ng of genomic DNA were amplyfied in 50 µl, using the same primer couples as above at a final concentration of 0.2 microM, on a GeneAmp 9700 thermal cycler (Applied Biosystem) for 50 cycles (1' at 94°C, 1' at 60°C, 1' at 72°C), using 1.25 units of AmpliTaq Gold (Applied Biosystem), its 1X buffer with MgCl2 and dNTPs (Applied Biosystem) at a final concentration of 100 µM each. Then, to remove unincorporated dNTPs, 5 µl of the PCR product were treated with 2 µl of ExoSAP-IT (USB) at 37°C for 15' and inactivated at 80°C for 15'; 3 µl of the purified template were added to 5 µl of SNaPshot Multiplex Ready Reaction Mix, and 0.2 µM of the sequencing primer, and placed in the thermal cycler for 25 cycles (96°C for 10", 50°C for 5". 60°C for 30"). Post-extension treatment, to remove unincorporated ddNTPs, consisted in adding 1 U of Shrimp Alkaline Phospatase (USB), followed by incubation at 37°C for 1 hour and inactivation at 75°C for 15'. Finally, samples underwent electrophoresis on the Genetic Analyzer at 60°C with POP-4 polymer using the E5 Run Module (injection time: 5"; electrophoresis and EP voltages: 15 kV; collection time 24'; syringe pump time: 150"; preinjection EP: 120"). Collected data were analyzed using the GeneScan software ver. 3.1.2. Primers used for amplification and direct Sanger sequencing of the *MLH1* rs1799977 polymorphism were. 5'-GCCTCAACCGTGGACAATA and 3'- TCACGCCACAGAATCTAGGA.

Table S1. Distribution of the alleles and Hardy-Weinberg equilibrium

SNP	Assessable	Homozygote for the common allele	Heterozygote	Homozygote for the minor allele	HWE p
***FCGR2A* rs1801274**	407	139 (AA)	193 (AG)	75 (GG)	.578
***FCGR3A* rs396991**	406	127 (TT)	183 (GT)	96 (GG)	.060
***MLH1* rs1799977**	411	182 (AA)	189 (AG)	40 (GG)	.368
***NCF4* rs1883112**	417	164 (GG)	202 (AG)	51 (AA)	.351
***CYBA* rs4673**	417	144 (CC)	198 (CT)	75 (TT)	.629
***GSTA1* rs3957357**	416	126 (CC)	217 (CT)	73 (TT)	.271

Table S2. Clinical features of the whole study cohort by *FCGR2A* rs1801274 genotype

	Homozygote for the common allele (AA; n=139)		Heterozygote (AG; n=193)		Homozygote for the minor allele (GG; n=75)		
	n	%	n	%	n	%	p
FLIPI							.517
0-1	27	19.4	34	17.6	13	17.3	
2	83	59.7	102	52.8	43	57.3	
3-5	29	20.9	57	29.5	19	25.3	
Age >60 years	46	33.1	67	34.7	19	25.3	.331
Male	79	56.8	93	48.2	41	54.7	.269
ECOG PS >1	3	2.2	8	4.1	1	1.3	.305
Ann Arbor stage III-IV	126	90.6	176	91.2	69	92.0	.946
Nodal areas >4	88	63.3	121	62.7	51	68.0	.709
Extranodal sites >1	46	33.1	77	39.9	28	37.3	.448
Bone marrow involvement	73	52.5	103	53.4	41	54.7	.956
Largest involved node >6 cm	29	20.9	56	29.0	24	32.0	.134
Hb <12 g/dl	16	11.5	34	17.6	11	14.7	.305
LDH >ULN	26	18.7	29	15.0	18	24.0	.377
Beta-2-microglobulin >ULN	62	44.6	86	44.6	35	46.7	.947
Grading							.133
1	40	28.8	71	36.8	26	34.7	
2	72	51.8	82	42.5	32	42.7	
3	12	8.6	30	15.5	11	14.7	
Unclassified	15	10.8	10	5.2	6	8.0	
Treatment (ITT)							.219
R-CVP	44	31.7	60	31.1	23	30.7	
R-CHOP	37	26.6	72	37.3	28	37.3	
R-FM	58	41.7	61	31.6	24	32.0	
CR	103	76.3	135	71.1	52	69.3	.460
3-years TTF		59.1		57.9		62.1	.742
3-years PFS		65.0		61.8		69.6	.452
3-years OS		95.8		96.2		97.2	.077

Table S3. Clinical features of the whole study cohort by *FCGR3A* rs396991 genotype

	Homozygote for the common allele (TT; n=127)		Heterozygote (GT; n=183)		Homozygote for the minor allele (GG; n=96)		
	n	%	n	%	n	%	p
FLIPI							.139
0-1	18	14.2	43	23.5	13	13.5	
2	74	58.3	94	51.4	60	62.5	
3-5	35	27.6	46	25.1	23	24.0	
Age >60 years	43	33.9	54	29.5	34	35.4	.543
Male	66	52.0	97	53.0	50	52.1	.980
ECOG PS >1	7	5.5	4	2.2	1	1.0	.106
Ann Arbor stage III-IV	119	93.7	166	90.7	85	88.5	.392
Nodal areas >4	80	63.0	118	64.5	61	63.5	.963
Extranodal sites >1	51	40.2	65	35.5	35	36.5	.698
Bone marrow involvement	73	57.5	89	48.6	54	56.3	.243
Largest involved node >6 cm	38	29.9	50	27.3	21	21.9	.398
Hb <12 g/dl	19	15.0	26	14.2	15	15.6	.949
LDH >ULN	23	18.1	30	16.4	20	20.8	.656
Beta-2-microglobulin >ULN	66	52.0	75	41.0	41	42.7	.143
Grading							.104
1	48	37.8	65	35.5	24	25.0	
2	51	40.2	81	44.3	53	55.1	
3	16	12.6	21	11.5	16	16.7	
Unclassified	12	9.4	16	8.7	3	3.1	
Treatment (ITT)							.637
R-CVP	43	33.9	56	30.6	28	29.2	
R-CHOP	40	31.5	67	36.6	29	30.2	
R-FM	44	34.6	60	32.8	39	40.6	
CR	89	71.8	130	71.8	70	74.5	.880
3-years TTF		61.7		55.0		61.5	.252
3-years PFS		64.7		59.9		69.1	.213
3-years OS		97.1		95.4		96.8	.904

Table S4. Clinical features of R-CHOP arm cohort by *MLH1* rs1799977 genotype

	Homozygote for the common allele (AA; n=65)		Heterozygote (AG; n=62)		Homozygote for the minor allele (GG; n=11)		
	n	%	n	%	n	%	p
FLIPI							.543
0-1	14	21.5	10	16.1	1	9.1	
2	37	56.9	35	56.5	5	45.5	
3-5	14	21.5	17	27.4	5	45.5	
Age >60 years	22	33.8	19	30.6	4	36.4	.855
Male	35	53.8	31	50.0	4	36.4	.556
ECOG PS >1	2	3.1	1	1.6	1	9.1	.371
Ann Arbor stage III-IV	60	92.3	54	87.1	10	90.9	.634
Nodal areas >4	40	61.5	36	58.1	8	72.7	.697
Extranodal sites >1	25	38.5	19	30.6	8	72.7	.035
Bone marrow involvement	32	49.2	34	54.8	9	81.8	.129
Largest involved node >6 cm	12	18.5	18	29.0	3	27.3	.328
Hb <12 g/dl	10	15.4	11	17.7	2	18.2	.942
LDH >ULN	11	16.9	13	21.0	2	18.2	.892
Beta-2-microglobulin >ULN	28	43.1	29	46.8	8	72.7	.198
Grading							.227
1	27	41.5	26	41.9	3	27.3	
2	27	41.5	18	29.0	4	36.4	
3	8	12.3	10	16.1	1	9.1	
Unclassified	3	4.6	8	12.9	3	27.3	
CR	17	26.2	18	29.0	2	25.0	.950
3-years TTF		66.2		68.8		30.3	.011
3-years PFS		68.3		75.6		48.0	.088
3-years OS		97.5		100		90.0	.002

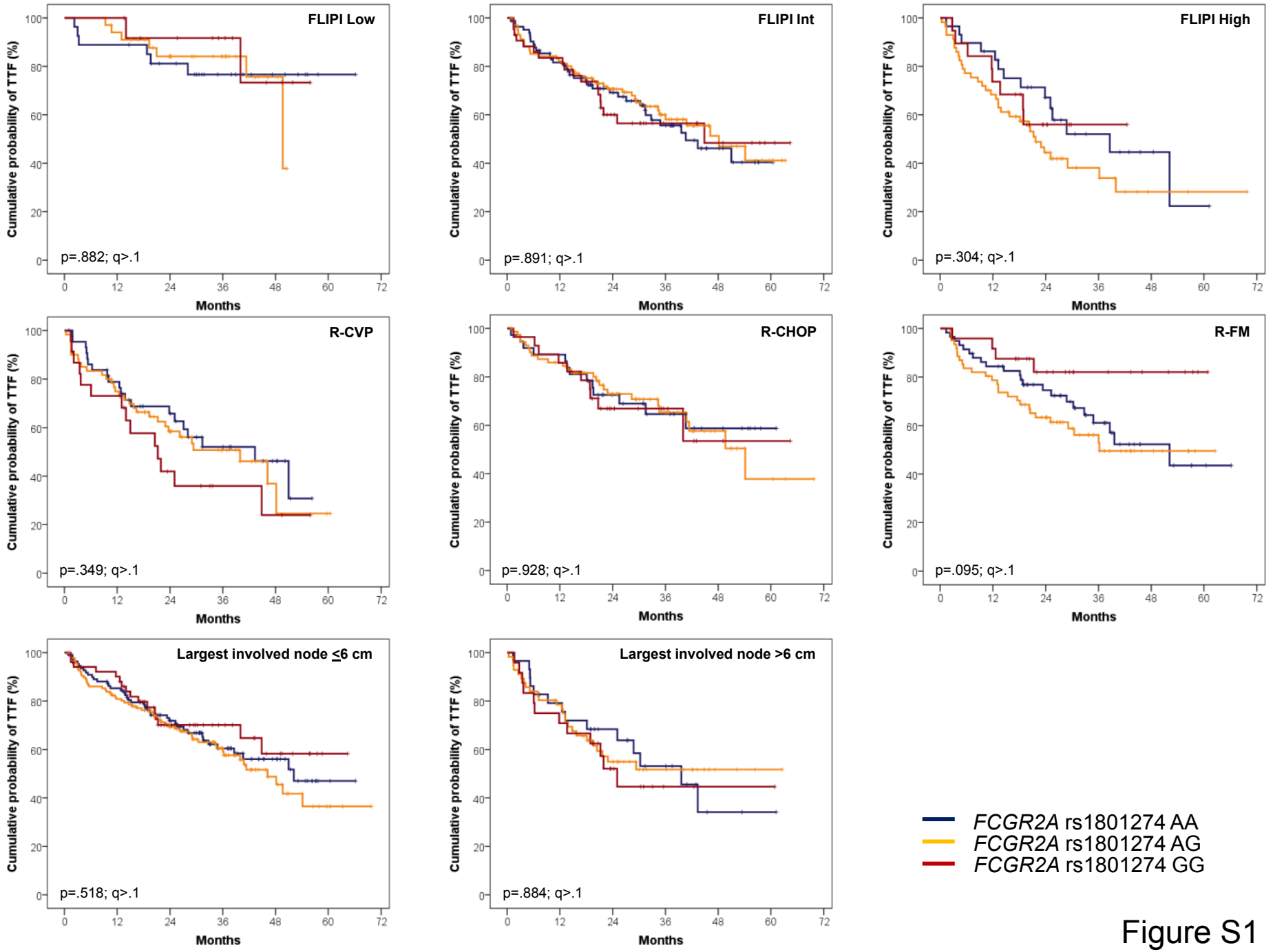

FLIPI Low
p=.882; q>.1
FLIPI Int
p=.891; q>.1
FLIPI High
p=.304; q>.1
R-CVP
p=.349; q>.1
R-CHOP
p=.928; q>.1
R-FM
p=.095; q>.1
Largest involved node ≤6 cm
p=.518; q>.1
Largest involved node >6 cm
p=.884; q>.1
Cumulative probability of TTF (%)
Months
FCGR2A rs1801274 AA
FCGR2A rs1801274 AG
FCGR2A rs1801274 GG

Figure S1

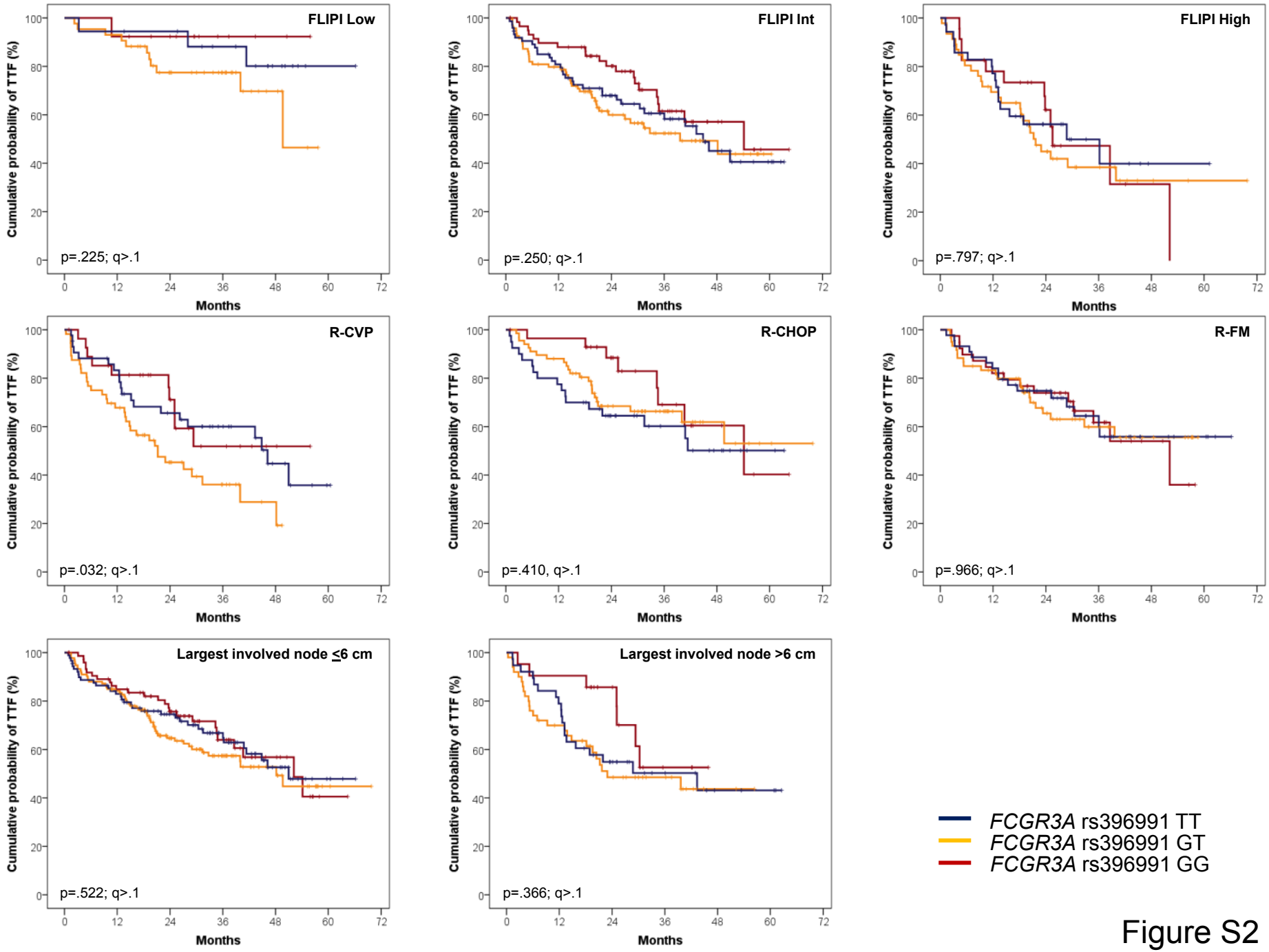
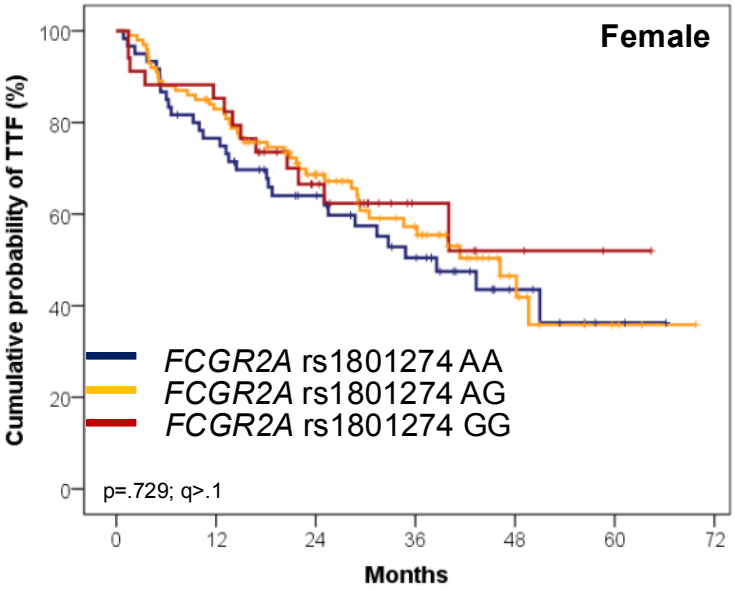
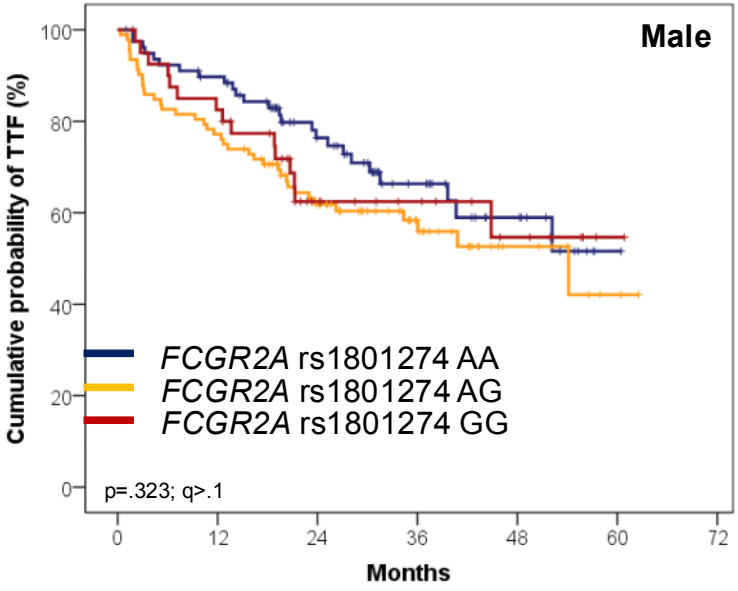
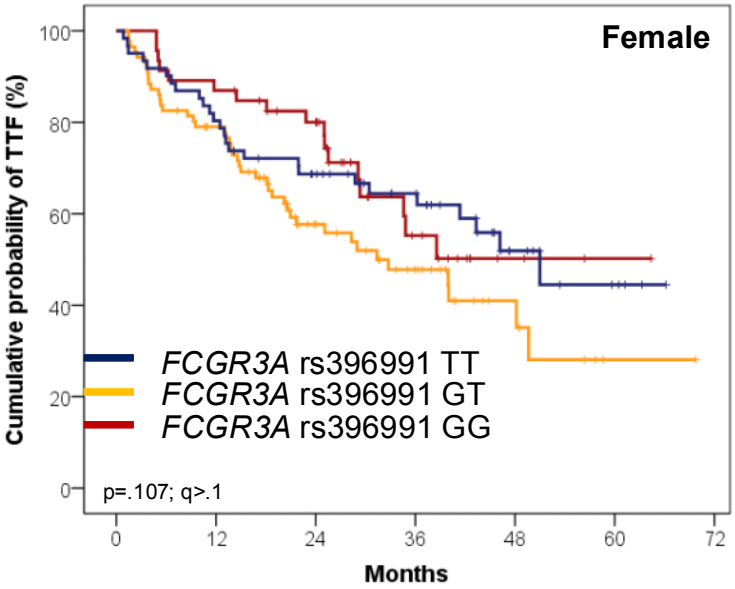

FLIPI Low
p=.225; q>.1
FLIPI Int
p=.250; q>.1
FLIPI High
p=.797; q>.1
R-CVP
p=.032; q>.1
R-CHOP
p=.410, q>.1
R-FM
p=.966; q>.1
Largest involved node ≤6 cm
p=.522; q>.1
Largest involved node >6 cm
p=.366; q>.1
Cumulative probability of TTF (%)
Months
FCGR3A rs396991 TT
FCGR3A rs396991 GT
FCGR3A rs396991 GG

Figure S2


Female
Cumulative probability of TTF (%)
100
80
60
40
20
0
FCGR2A rs1801274 AA
FCGR2A rs1801274 AG
FCGR2A rs1801274 GG
p=.729; q>.1
0
12
24
36
48
60
72
Months


Male
Cumulative probability of TTF (%)
100
80
60
40
20
0
FCGR2A rs1801274 AA
FCGR2A rs1801274 AG
FCGR2A rs1801274 GG
p=.323; q>.1
0
12
24
36
48
60
72
Months


Female
Cumulative probability of TTF (%)
100
80
60
40
20
0
FCGR3A rs396991 TT
FCGR3A rs396991 GT
FCGR3A rs396991 GG
p=.107; q>.1
0
12
24
36
48
60
72
Months

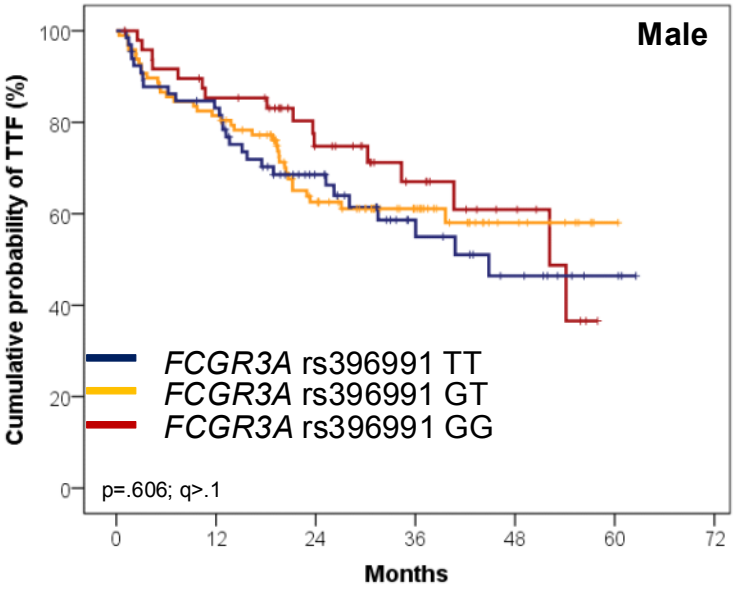
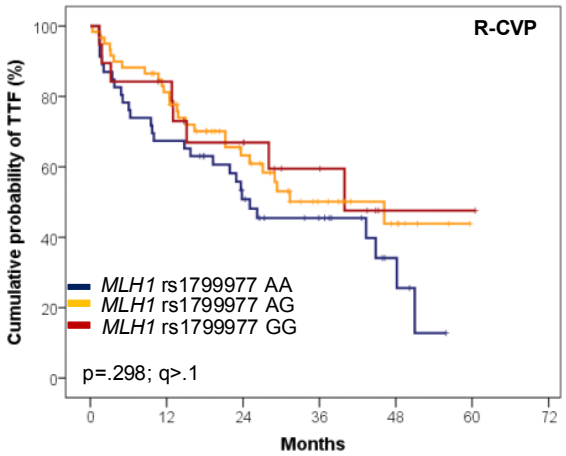
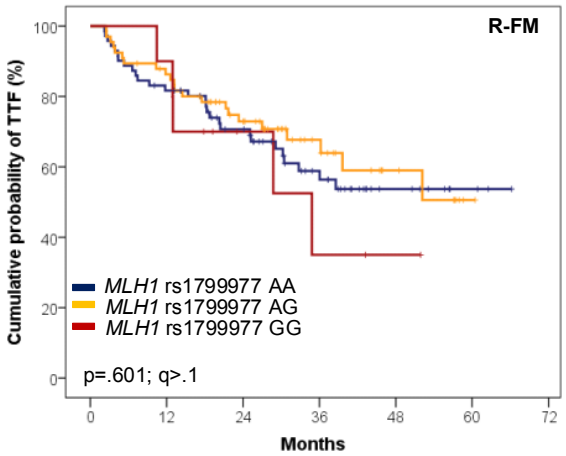
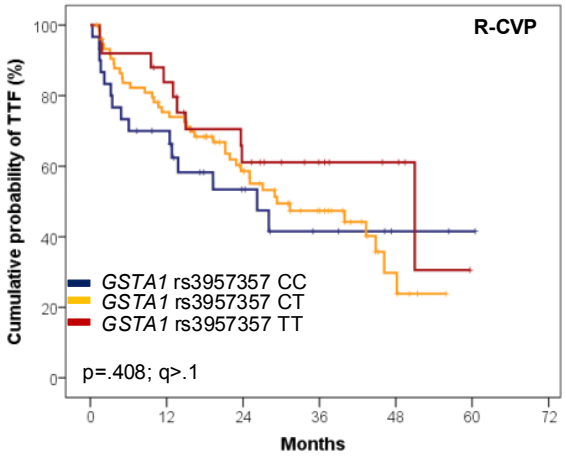

Male
Cumulative probability of TTF (%)
100
80
60
40
20
0
FCGR3A rs396991 TT
FCGR3A rs396991 GT
FCGR3A rs396991 GG
p=.606; q>.1
0
12
24
36
48
60
72
Months

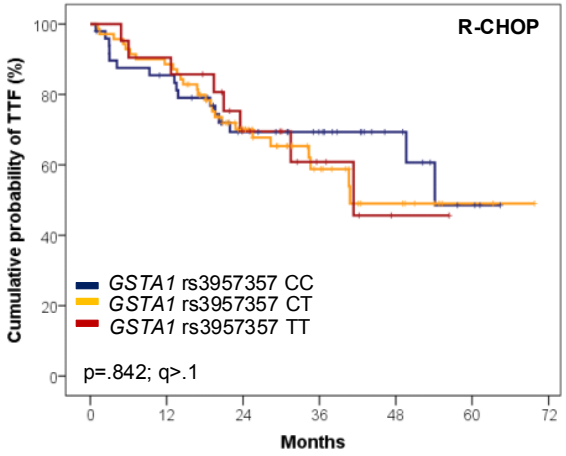
Figure S3

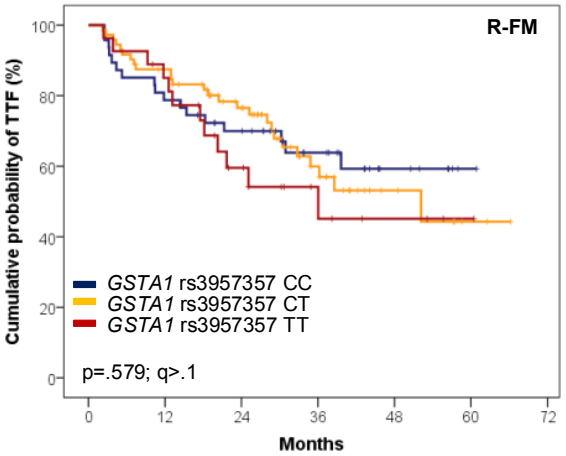
Figure S4

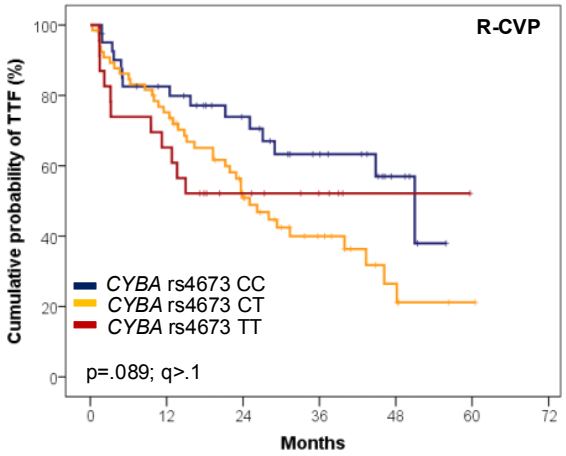

R-CVP
Cumulative probability of TTF (%)
MLH1 rs1799977 AA
MLH1 rs1799977 AG
MLH1 rs1799977 GG
p=.298; q>.1
Months

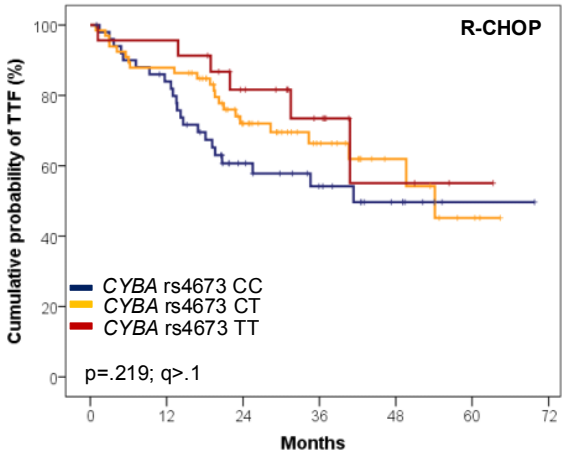

R-FM
Cumulative probability of TTF (%)
MLH1 rs1799977 AA
MLH1 rs1799977 AG
MLH1 rs1799977 GG
p=.601; q>.1
Months

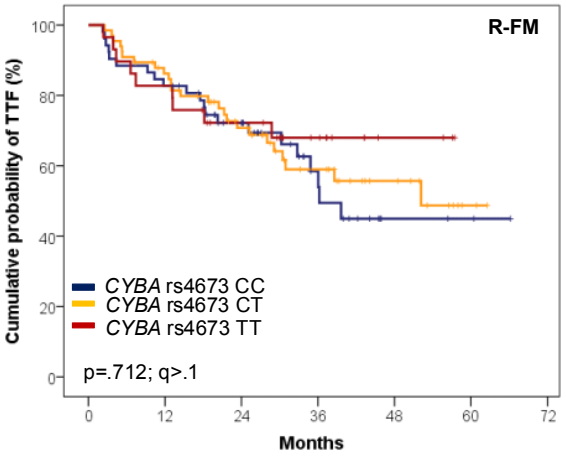

R-CVP
Cumulative probability of TTF (%)
GSTA1 rs3957357 CC
GSTA1 rs3957357 CT
GSTA1 rs3957357 TT
p=.408; q>.1
Months


R-CHOP
Cumulative probability of TTF (%)
GSTA1 rs3957357 CC
GSTA1 rs3957357 CT
GSTA1 rs3957357 TT
p=.842; q>.1
Months


R-FM
Cumulative probability of TTF (%)
GSTA1 rs3957357 CC
GSTA1 rs3957357 CT
GSTA1 rs3957357 TT
p=.579; q>.1
Months


R-CVP
Cumulative probability of TTF (%)
CYBA rs4673 CC
CYBA rs4673 CT
CYBA rs4673 TT
p=.089; q>.1
Months


R-CHOP
Cumulative probability of TTF (%)
CYBA rs4673 CC
CYBA rs4673 CT
CYBA rs4673 TT
p=.219; q>.1
Months


R-FM
Cumulative probability of TTF (%)
CYBA rs4673 CC
CYBA rs4673 CT
CYBA rs4673 TT
p=.712; q>.1
Months