Cyclosporine and methotrexate-related pharmacogenomic predictors of acute graft-*versus*-host disease

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ABSTRACT

Effective immunosuppression is mandatory to prevent graft-*versus*-host disease and to achieve a successful clinical outcome of hematopoietic stem cell transplantation. Here we tested whether germline single nucleotide polymorphisms in 20 candidate genes related to methotrexate and cyclosporine metabolism and activity influence the incidence of graft-*versus*-host disease in patients who undergo stem cell transplantation for hematologic disorders. Recipient genetic status of the adenosine triphosphate-binding cassette sub-family C1 and adenosine triphosphate-binding cassette sub-family C2 transporters, 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/inosine monophosphate cyclohydrolase within the methotrexate pathway, and nuclear factor of activated T cells (cytoplasmic 1) loci exhibit a remarkable influence on severe acute graft-*versus*-host disease prevalence. Indeed, an increased risk of acute graft-*versus*-host disease was observed in association with single nucleotide polymorphisms located in 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/inosine monophosphate cyclohydrolase (hazard ratio=3.04; *P*=0.002), nuclear factor of activated T cells (cytoplasmic 1) (hazard ratio=2.69; *P*=0.004), adenosine triphosphate-binding cassette sub-family C2 (hazard ratio=3.53; *P*=0.0018) and adenosine triphosphate-binding cassette sub-family C1 (hazard ratio=3.67; *P*=0.0005). While donor single nucleotide polymorphisms of dihydrofolate reductase and solute carrier family 19 (member 1) genes are associated with a reduced risk of acute graft-*versus*-host disease (hazard ratio=0.32-0.41; *P*=0.0009-0.008), those of nuclear factor of activated T cells (cytoplasmic 2) are found to increase such risk (hazard ratio=3.85; *P*=0.0004). None of the tested single nucleotide polymorphisms was associated with the occurrence of chronic graft-*versus*-host disease. In conclusion, by targeting drug-related biologically relevant genes, this work emphasizes the potential role of germline biomarkers in predicting acute graft-*versus*-host disease. Further investigations are warranted to improve our understanding of these relationships to personalize immunosuppressive therapy and optimize outcomes.

Introduction

One of the main obstacles to the success of allogeneic hematopoietic stem cell transplantation (HSCT) is related to immunological complications resulting from allogeneic reactions, including graft-*versus*-host disease (GvHD). Following transplantation procedures, effective immunosuppression is mandatory to prevent deleterious GvHD given its high rate of morbidity/mortality in the post-transplant period.[1-3] Despite prophylactic treatment, a significant proportion of patients still develop GvHD clearly suggesting that other factors such as yet to be characterized, inter-individual genetic susceptibility could be involved in the development of acute and chronic GvHD.[4,5] Besides the mandatory human leukocyte antigen (HLA) matching, identifying inherited genetic factors associated with outcome would represent a major advance in preventing severe cases of acute GvHD and in improving patients' survival.[6-11] Indeed, most of the progress in this regard is related to better selection of donor/patient pairs through the use of high-resolution HLA genotyping, to the use of new immunosuppressive agents and to better prevention and treatment of severe infections.[1,12-15] Despite these improvements, in the absence of biologically relevant biomarkers, it is not possible to predict which patients are at high risk of developing GvHD. The identification and validation of such preventive/diagnostic/prognostic tools will certainly improve the transplant procedure.

In this context, it is recognized that the genetic diversity of xenobiotic/drug metabolizing enzyme genes together with clinical factors could partly predict the development of GvHD and several candidates have been identified from hypothesis-driven studies,[7,9-11,16-24] such as donor/recipient differences in

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copy-number variations of the UDP-glucuronosyltransferase 2B17 loci, which were associated with increased risk of GvHD following HLA-matched HSCT.[25] To date, the influence of genetic polymorphisms related to the methotrexate and cyclosporine A pharmacological pathways have not attracted extensive attention despite the routine use of short-course methotrexate therapy at low doses in combination with cyclosporine A in post-HSCT settings.[26-28] So far, only a few studies have addressed such an important phenotype-genotype relationship, although genetic variations in the gene coding for methylenetrahydrofolate reductase (MTHFR) enzyme have received great attention.[18,22-24,29-33]

As the inter-individual variability in GvHD occurrence among HSCT patients may be, at least partly, related to the genetic diversity of genes involved in the bioavailability and in the metabolism of methotrexate/cyclosporine A, we tested in this study whether selected single nucleotide polymorphisms (SNP) located in loci encoding molecules implicated in the methotrexate/cyclosporine A metabolic and transport pathways could influence the incidence of GvHD in HSCT recipients.

Methods

Selection of polymorphisms

The design of the present study involved two consecutive steps. An initial set of 219 haplotype-tagging SNP (htSNP) scattered along 20 candidate genes related to the methotrexate/cyclosporine

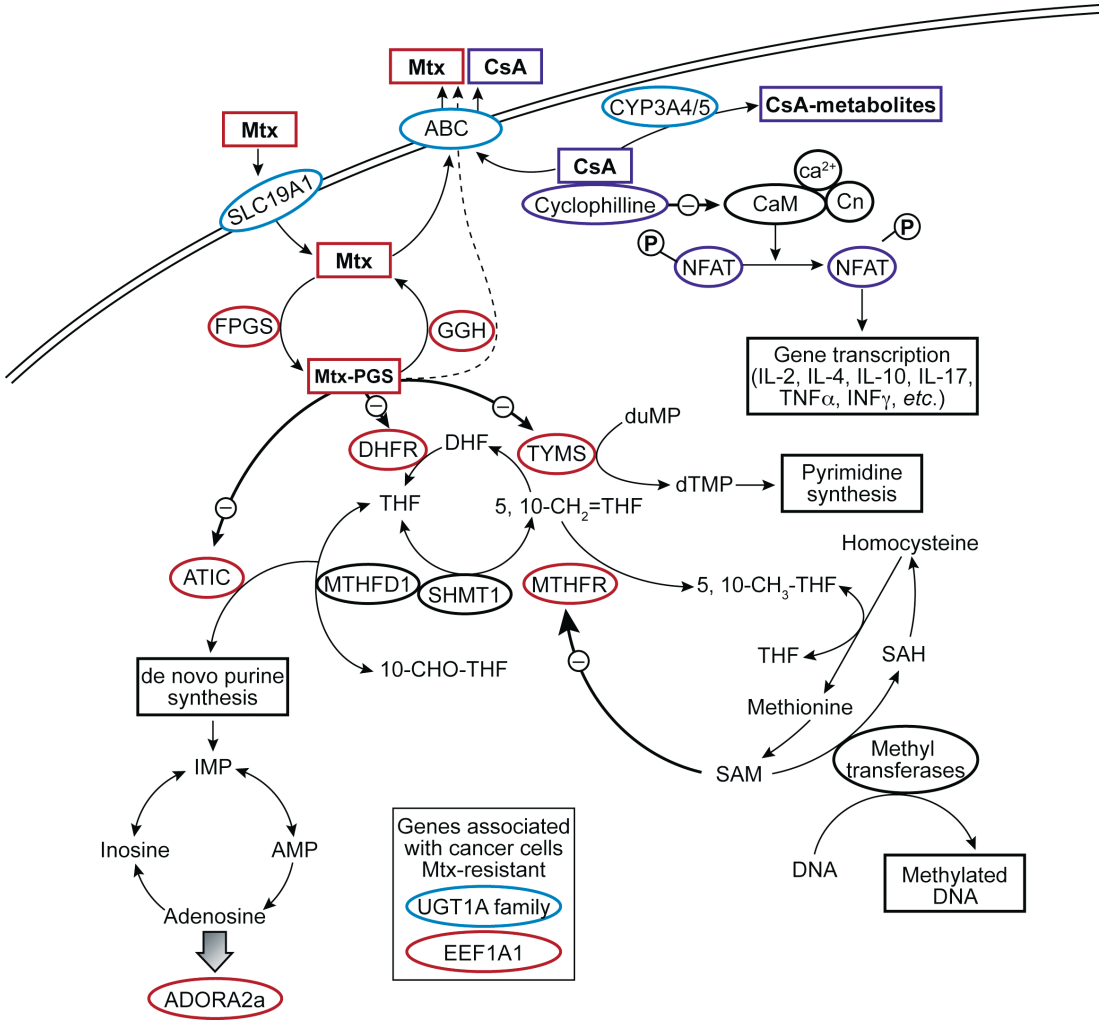

Figure 1. Schematic representation of the biologically relevant candidate genes selected in this pharmacogenomic study. Candidate genes screened in the exploratory cohort are circled: red, genes of the pharmacodynamic pathways of methotrexate; purple: genes of the pharmacodynamic pathway of cyclosporine A. Genes that are involved in pharmacokinetics pathways of methotrexate and/or cyclosporine A are circled in blue. Mtx: methotrexate; Mtx-PGS: methotrexate polyglutamates; CsA: cyclosporine A; AMP: adenosine monophosphate; ADORA2a: adenosine receptors; ATIC: aminoimidazole carboxamide ribonucleotide transformylase/inosine monophosphate cyclohydrolase; ABC: ATP-binding cassette (including ABCC1, ABCC2, ABCB1, ABCG2); DHFR: dihydrofolate reductase; EEF1A1: eukaryotic translation elongation factor 1 alpha 1; FPGS: folylpolyglutamyl synthase; GGF: γ-glutamylhydrolase; IMP: inosine monophosphate; MTHFD1: methylenetetrahydrofolate dehydrogenase; MTHFR: methylenetrahydrofolate reductase; SHMT1: serine hydroxymethyltransferase; TYMS: thymidylate synthase; SAH: S-adenosylhomocysteine; SAM: S-adenosylmethionine; SLC19A1: solute carrier 19A1; THF: tetrahydrofolate; UGT1A: UDP-glucuronosyltransferase 1A; Cn: calcineurin; CaM: calmodulin; IL: interleukin; NFAT: nuclear factor of activated T cells; TNF-α: tumor necrosis factor-alpha; INF-γ: interferon-gamma; CYP3A4/5: CYP3A4 cytochrome P450, family 3, subfamily A, polypeptide 4 and 5.

A pathways were initially selected in order to capture 935 allelic variations covering ≥80% of the genetic diversity in all genes, except for *NFATC1*, *NFATC2* and UDP-glucuronosyltransferase 1A (*UGT1A*) (coverage of 47%, 79% and 69%, respectively) (Figure 1). In order to test the pertinence of the future SNP to analyze in a large cohort of 420 HSCT recipient/donor pairs, we first assessed this set of 219 htSNP in an independent group of 104 HSCT recipient/donor pairs (*Online Supplementary Tables S1 and S2*). Fifty nine htSNP found to be associated with GvHD or risk of death with *P* values <0.10 were then genotyped in the cohort of patients described below.

Characteristics of the patients, donors and transplants

The population of 420 recipient/donor pairs consisted of patients recruited from Saint-Louis Hospital (Paris, France) between 1994 and 2012 who underwent allogeneic HSCT for hematologic disorders (Table 1). Each participant provided written informed consent and the institutional review board approved the research protocol. Acute GvHD and chronic GvHD were diagnosed and graded according to standard criteria.[34,35] The severity of acute GvHD was recorded as grade 0 (no GvHD), I, II, II or IV, while chronic GvHD was classified as absent or present, regardless of the extent. The HLA-matched score was based on high-resolution HLA-A*, -B*, -C*, -DRB1* and DQB1* genotyping. All patients, donors and transplant characteristics are summarized in Table 1.

Statistical analysis

The incidence of GvHD was estimated by applying a standard regression method with competing risks, using a proportional cause-specific hazard model, death being treated as a competing event (acute or chronic GvHD was the event of interest). In the absence of a competing hazard, the proportional cause-specific hazard model is reduced to a standard Cox survival model. In the multivariate model, we further adjusted for relevant clinical factors found to be associated with the risk of GvHD (*Online Supplementary Table S3*), namely recipient and donor age (<20, 20-50 and >50 years), recipient-donor incompatibility for gender (female donor to male recipient), stem cell source (bone marrow *versus* peripheral blood stem cells), hematologic disease (malignant *versus* non-malignant), conditioning regimen (myeloablative *versus* reduced intensity regimen) and HLA disparity (matched related donor, matched and mismatched unrelated donor). The inclusion of anti-thymocyte globulin and total body irradiation in the multivariate model was considered and generated similar results. For acute GvHD, we explored the association between SNP and two clinical sub-phenotypes, namely grade II-IV *versus* grade 0-I, and grade III-IV *versus* grade 0-II. The associations of SNP with clinical outcomes were evaluated for genomic modes of transmission (additive, dominant and recessive). Statistical analyses were conducted using SAS Statistical Software version 9.2 (SAS Institute) and the following R packages: etm, compeer, survival, and cmprsk. *P* values were considered statistically significant if <0.05. False-discovery rates (*q* values) were calculated to determine the degree to which the tests were prone to false-positives, using the R QVALUE package (*http://genomics.princeton.edu /storeylab/qvalue/*). To account for multiple comparison testing, results were considered positive only if both *P* and *q* values were <0.05.

Results

In this study, 76.7% of the patients in the cohort were transplanted for hematologic malignancies. Overall, the percentage of relapses in our cohort was 20.8%, and of these, 85% have died from their diseases. The mean follow-up of survivors was 5.1 years (range, 0.27-15.8 years). The relative frequencies of the associated SNP and their corresponding hazard ratios [HR; 95% confidence interval (CI)] as well as *P* and *q* values are summarized in Tables 2-5. The observed frequencies of major and minor alleles are similar to those reported in the CEU HapMap population (*Online Supplementary Table S2*).

Table 1. Characteristics of patients (n=420), donors and transplants.

Variables	Donors/Patients (n=420)	
Patients' age, n. (%)		
<20 years	146	(34.8)
20-50 years	253	(60.2)
>50 years	21	(5.0)
Donors' age, n. (%)		
<20 years	90	(21.4)
20-50 years	304	(72.4)
>50 years	26	(6.2)
Disease, n. (%)		
Non-malignant hematologic disorder	98	(23.3)
Hematologic malignancy	322	(76.7)
Donor type, n. (%)		
Matched related	241	(57.4)
Matched unrelated	125	(29.8)
Mismatched unrelated	54	(12.9)
Sex match (donor/patient), n. (%)		
Male/male	138	(32.9)
Male /female	102	(24.3)
Female/male	104	(24.8)
Female/female	76	(18.1)
Cytomegalovirus match (donor/patient)		
Negative/negative	130	(31.0)
Negative/positive	93	(22.1)
Positive/negative	51	(12.1)
Positive/positive	146	(34.8)
Stem cell source, n. (%)		
Bone marrow	269	(64)
Peripheral blood	151	(36)
Conditioning, n. (%)		
Myeloablative	394	(93.8)
Non-myeloablative	26	(6.2)
Total body irradiation, n. (%)		
No	244	(58.1)
Yes	176	(41.9)
Cyclosporine A prophylaxis, n. (%)		
Cyclosporine A+methotrexate	420	(100)
Acute GVHD grade II-IV, n. (%)	212	(50.5)
Time of occurrence, day (mean, range)	20	(4-111)
Missing data	1	0.2
Acute GVHD grade III-IV, n. (%)	63	(15.0)
Time of occurrence, day (mean, range)	16	(4-50)
Missing data	1	0.2
Chronic GVHD, n. (%)	162	(38.6)
Time of occurrence, day (mean, range)	302	(53-2318)
Survival status		
Alive	259	(61.7)
Dead	152	(36.2)
Missing data	9	(2.1)
Follow-up of survivors, year (mean, range)	5.1	(0.27-15.8)

Acute graft-versus-host disease grade II-IV and the competing risk of death

Half of the study cohort experienced at least a grade II to IV episode of acute GVHD (n=212, 50.5%). A total of five recipients' genetic variations were significantly associated with the risk of acute GvHD after correction for multiple testing. In particular, the risk was associated with genetic variations related to pharmacodynamic pathways of methotrexate and cyclosporine A including methylenetetrahydrofolate reductase (*MTHFR*) *rs2274976* and *rs3737967*, and 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/inosine monophosphate cyclohydrolase (*ATIC*) *rs*17514110 (HR=1.94-2.36; *P*=0.002-0.003, *q*=0.031). Similarly, polymorphisms associated with worse prognosis were also found in nuclear factor of activated T-cells, cytoplasmic calcineurin-dependent (*NFATC1 rs*1017860 and *NFATC2 rs*6123048), the molecular target of cyclosporine A in the dominant model (HR=1.59; *P*=0.003, *q*=0.036 and HR=2.23; *P*=0.001, *q*=0.034) (Table 2). Donor *MTHFR* rs1801133 status was also significantly associated with a high risk of acute GvHD and death but the association did not reach statistical significance after correction for multiple testing (HR=2,19; *P*=0.003, *q*=0.062 and HR=4.69; *P*=0.005, *q*=0.065, respectively) (*Online Supplementary Table S4*). None of the other genetic variations associated for the competing risk of death prior to the occurrence of developing acute GvHD displayed a significant association after correction for multiple testing (Table 3 and *Online Supplementary Table S4*).

Acute graft-versus-host disease grade III-IV and the competing risk of death

Severe acute GvHD (grade III and IV) occurred in 15% of patients. In our study, grade III-IV acute GvHD was associated with non-relapse mortality. Indeed, 59% of patients with grade III-IV acute GvHD have died from this complication. Among the tested SNP, 11 (6 in recipients and 5 in donors) were significantly associated with severe

Table 2. SNP associated with grade II-IV acute GvHD.

Gene	SNP	MAF (%)	A/b	Acute GvHD (grade II-IV) Genomic model[a] Mode	HR[c]	95% CI	*P* value	*q* value	Secondary model[b] Mode	HR[c]	95% CI	*P* value	*q* value
				RECIPIENT									
ATIC	rs17514110	8	C/T	[Ab]	1.94	(1.26-2.99)	**0.003**	**0.031**	Dom.	1.78	(1.17-2.71)	**0.007**	0.069
				[bb]	0.55	(0.07-4.15)	0.561	0.684	Rec.	0.53	(0.07-4.01)	0.540	0.320
MTHFR	rs2274976	4	G/A	[Ab]	2.36	(1.36-4.10)	**0.002**	**0.031**	Dom.	2.36	(1.36-4.10)	**0.002**	**0.035**
				[bb]					Rec.				
MTHFR	rs3737967	4	C/T	[Ab]	2.36	(1.36-4.10)	**0.002**	**0.031**	Dom.	2.36	(1.36-4.10)	**0.002**	**0.035**
				[bb]					Rec.				
NFATC1	rs1017860	18	C/T	[Ab]	1.55	(1.13-2.12)	**0.006**	0.059	Dom.	1.59	(1.17-2.16)	**0.003**	**0.036**
				[bb]	2.17	(0.94-5.02)	0.069	0.307	Rec.	1.85	(0.81-4.24)	0.144	0.261
NFATC2	rs6123048	5	A/G	[Ab]	2.23	(1.40-3.55)	**0.001**	**0.031**	Dom.	2.23	(1.40-3.55)	**0.001**	**0.034**
				[bb]					Rec.				

MAF: minor allele frequency, [A]: major allele, [b]: minor allele, Dom.: dominant mode, Rec.: recessive mode, P *and* q *values ≤ 0.05 are in bold characters.* [a]*Genomic model= [AA] vs. [Ab] and [bb], reference group=[AA] (HR fixed at 1.00).* [b]*Dominant model=[AA] vs. [Ab+bb], reference group=[AA] (HR fixed at 1.00); Recessive model=[AA+Ab] vs. [bb], reference group=[AA+Ab] with HR fixed at 1.00.* [c]*Models adjusted for sex mismatch, diagnosis, source of cells, age of recipient and donor, donor type and conditioning regimen. The incidence of GvHD was estimated by competing-risk analysis with death as a competing risk for GvHD. Data are not shown for bb genotype with low frequency (<2%).*

Table 3. SNP associated with the competing risk of death prior to grade II-IV acute GvHD.

Gene	SNP	MAF (%)	A/b	Death prior to acute GvHD (grade II-IV) Genomic model[a] Mode	HR[c]	95% CI	*P* value	*q* value	Secondary model[b] Mode	HR[c]	95% CI	*P* value	*q* value
				RECIPIENT									
ATIC	rs17514110	8	C/T	[Ab]	0.42	(0.17-1.06)	0.066	0.199	Dom.	0.45	(0.18-1.10)	0.078	0.190
				[bb]					Rec.				
MTHFR	rs2274976	4	G/A	[Ab]	0.41	(0.11-1.62)	0.205	0.290	Dom.	0.56	(0.16-1.90)	0.351	0.376
				[bb]	4.34	(0.34-55.62)	0.260	0.528	Rec.				
MTHFR	rs3737967	4	C/T	[Ab]	0.41	(0.11-1.62)	0.205	0.290	Dom.	0.56	(0.16-1.90)	0.351	0.376
				[bb]	4.34	(0.34-55.62)	0.260	0.528	Rec.				
NFATC1	rs1017860	18	C/T	[Ab]	2.64	(1.22-5.71)	**0.014**	0.199	Dom.	2.16	(1.02-4.56)	**0.044**	0.189
				[bb]	0.89	(0.21-3.71)	0.874	0.813	Rec.	0.61	(0.16-2.32)	0.467	0.713
NFATC2	rs6123048	5	A/G	[Ab]	1.23	(0.48-3.17)	0.663	0.466	Dom.	1.23	(0.48-3.17)	0.663	0.430
				[bb]					Rec.				

MAF: minor allele frequency, [A]: major allele, [b]: minor allele, Dom.: dominant mode, Rec.: recessive mode, P *and* q *values ≤ 0.05 are in bold characters.* [a]*Genomic model= [AA] vs. [Ab] and [bb], reference group=[AA] (HR fixed at 1.00).* [b]*Dominant model=[AA] vs. [Ab+bb], reference group=[AA] (HR fixed at 1.00); Recessive model=[AA+Ab] vs. [bb], reference group=[AA+Ab] with HR fixed at 1.00.* [c]*Models adjusted for sex mismatch, diagnosis, source of cells, age of recipient and donor, donor type and conditioning regimen. The incidence of GvHD was estimated by competing-risk analysis with death as a competing risk for GvHD. Data are not shown for bb genotype with low frequency (<2%).*

acute GvHD using competing risk assessments and correction for multiple testing (*P* and *q* values <0.05) (Table 4 and *Online Supplementary Table S5*). In recipients, SNP positively associated with the occurrence of acute GvHD were localized in genes encoding ATP-binding cassette (ABC) transporters (*ABCC1* and *ABCC2*), in *ATIC* and in *NFATC1*. Variations in *ABCC1 rs*4781712 and *rs*17264736 were associated with a reduced risk of developing acute GvHD (HR: 0.35 and 0.36; *P*=0.003, *q*=0.011), whereas *ABCC1 rs*8058040 and *ABCC2 rs*3740065 were associated with an increased risk of severe acute GvHD (HR 3.67; *P*=0.001, *q*=0.016 and HR 3.53; *P*=0.002, *q*=0.022, respectively) (Table 4). Interestingly, SNP located in *ABCB1* (*rs*4148732 and *rs*6950978), *ABCC1* (*rs*212087) and in *ABCG2* (*rs*12505410 and *rs*13120400) were all associated with the competing risk of death prior to the occurrence of developing acute GvHD (Table 5). A polymorphism associated with worse prognosis was also found in *ATIC*, the rate-limiting enzyme in the *de novo* purine synthesis pathway. Recipients carrying the *rs*2177735 allele in the *ATIC* gene have a higher risk of acute GvHD (HR 3.04; *P*=0.002, *q*=0.022). The recipient variant *NFATC1 rs*8090560 was also significantly associated with an increased risk of acute GvHD (HR 2.69; *P*=0.004, *q*=0.03). In donors, we observed that germline variations in the drug influx solute carrier family 19 (folate transporter) member 1 (*SLC19A1*) were significantly associated with a reduced risk of developing severe acute GvHD (HR 0.29-0.38; *P*=0.005-0.002, *q*=0.048). Two other SNP in donors' genomes appear to modify outcome; the dihydrofolate

Table 4. SNP associated with grade III-IV acute GvHD.

Gene	SNP	MAF (%)	A/b	Acute GvHD (grade III-IV) Genomic model[a] Mode	HR[c]	95% CI	*P* value	*q* value	Secondary model[b] Mode	HR[c]	95% CI	*P* value	*q* value
RECIPIENT													
ABCB1	rs4148732	15	A/G	[Ab]	0.97	(0.50-1.89)	0.931	0.560	Dom.	1.05	(0.54-2.01)	0.895	0.501
				[bb]	5.86	(0.67-51.20)	0.110	0.100	Rec.	5.89	(0.68-51.28)	0.109	0.065
ABCB1	rs6950978	31	A/T	[Ab]	0.97	(0.49-1.91)	0.932	0.560	Dom.	1.05	(0.55-1.98)	0.888	0.501
				[bb]	1.41	(0.51-3.89)	0.507	0.244	Rec.	1.43	(0.55-3.72)	0.462	0.101
ABCC1	rs17264736	48	G/T	[Ab]	0.94	(0.40-2.24)	0.893	0.556	Dom.	0.55	(0.26-1.18)	0.125	0.194
				[bb]	0.35	(0.15-0.84)	**0.019**	0.096	Rec.	0.36	(0.19-0.71)	**0.003**	**0.011**
ABCC1	rs212087	34	C/T	[Ab]	0.80	(0.43-1.49)	0.482	0.423	Dom.	0.88	(0.49-1.57)	0.658	0.418
				[bb]	1.25	(0.51-3.06)	0.630	0.262	Rec.	1.41	(0.61-3.25)	0.426	0.100
ABCC1	rs4781712	50	A/G	[Ab]	1.24	(0.54-2.80)	0.613	0.468	Dom.	0.72	(0.35-1.51)	0.390	0.321
				[bb]	0.40	(0.17-0.97)	**0.042**	0.100	Rec.	0.35	(0.18-0.71)	**0.003**	**0.011**
ABCC1	rs8058040	21	A/G	[Ab]	3.76	(1.73-8.15)	**0.001**	**0.028**	Dom.	3.67	(1.76-7.64)	**0.001**	**0.016**
				[bb]	3.00	(0.27-33.68)	0.374	0.202	Rec.	3.24	(0.29-35.89)	0.338	0.095
ABCC2	rs3740065	13	T/C	[Ab]	3.53	(1.60-7.82)	**0.002**	**0.031**	Dom.	3.53	(1.60-7.82)	**0.002**	**0.022**
				[bb]					Rec.				
ABCG2	rs12505410	31	T/G	[Ab]	1.19	(0.61-2.32)	0.615	0.468	Dom.	0.93	(0.51-1.69)	0.814	0.484
				[bb]	0.47	(0.14-1.60)	0.227	0.165	Rec.	0.46	(0.13-1.55)	0.207	0.077
ABCG2	rs13120400	22	T/C	[Ab]	1.32	(0.69-2.53)	0.409	0.419	Dom.	1.01	(0.55-1.84)	0.984	0.525
				[bb]	0.35	(0.08-1.60)	0.176	0.147	Rec.	0.34	(0.08-1.54)	0.162	0.068
ATIC	rs2177735	42	T/C	[Ab]	2.87	(1.37-6.01)	**0.005**	0.061	Dom.	3.04	(1.49-6.17)	**0.002**	**0.022**
				[bb]	3.84	(1.27-11.63)	**0.017**	0.096	Rec.	2.22	(0.81-6.13)	0.123	0.065
NFATC1	rs8090560	29	G/A	[Ab]	2.59	(1.26-5.33)	**0.010**	0.085	Dom.	2.69	(1.37-5.27)	**0.004**	**0.030**
				[bb]	2.95	(1.21-7.21)	**0.017**	0.096	Rec.	1.87	(0.85-4.11)	0.119	0.065
DONOR													
DHFR	rs34965641	26	C/T	[Ab]	0.32	(0.16-0.63)	**0.001**	**0.037**	Dom.	0.32	(0.16-0.61)	**0.001**	**0.024**
				[bb]	0.29	(0.07-1.17)	0.082	0.233	Rec.	0.60	(0.16-2.18)	0.434	0.556
NFATC2	rs3787186	31	G/A	[Ab]	0.80	(0.40-1.59)	0.518	0.583	Dom.	1.17	(0.63-2.15)	0.624	0.624
				[bb]	3.40	(1.50-7.75)	**0.004**	0.133	Rec.	3.85	(1.84-8.06)	**0.0004**	**0.013**
SLC19A1	rs1051266	47	G/A	[Ab]	0.37	(0.19-0.74)	**0.005**	**0.048**	Dom.	0.39	(0.20-0.74)	**0.004**	0.064
				[bb]	0.42	(0.18-0.97)	**0.043**	0.233	Rec.	0.86	(0.44-1.68)	0.654	0.658
SLC19A1	rs4818789	24	T/G	[Ab]	0.29	(0.13-0.65)	**0.002**	**0.048**	Dom.	0.39	(0.20-0.78)	**0.008**	0.079
				[bb]	0.66	(0.28-1.57)	0.351	0.474	Rec.	1.30	(0.60-2.81)	0.511	0.618
SLC19A1	rs4819128	47	T/C	[Ab]	0.38	(0.19-0.74)	**0.005**	**0.048**	Dom.	0.41	(0.22-0.76)	**0.005**	0.064
				[bb]	0.48	(0.21-1.06)	0.070	0.233	Rec.	0.90	(0.46-1.77)	0.768	0.720

MAF: minor allele frequency, [A]: major allele, [b]: minor allele, Dom.: dominant mode, Rec.: recessive mode, P *and* q *values ≤ 0.05 are in bold characters. [a]Genomic model= [AA] vs. [Ab] and [bb], reference group=[AA] (HR fixed at 1.00). [b]Dominant model=[AA] vs. [Ab+bb], reference group=[AA] (HR fixed at 1.00); Recessive model=[AA+Ab] vs. [bb], reference group=[AA+Ab] with HR fixed at 1.00. [c]Models adjusted for sex mismatch, diagnosis, source of cells, age of recipient and donor, donor type and conditioning regimen. The incidence of GvHD was estimated by competing-risk analysis with death as a competing risk for GvHD. Data are not shown for bb genotype with low frequency (<2%).*

reductase (*DHFR*) *rs*34965641 and *NFATC2* *rs*3787186 were respectively associated with a reduced and increased risk of acute GvHD in recipients (Table 4). Polymorphisms in *MTHFR*, including the coding *rs*1801131 variant, were not significantly associated with severe acute GvHD after correction for multiple testing (*Online Supplementary Table S5*). A detailed assessment of the association between positive SNP and clinical variables is also outlined in *Online Supplementary Table S6*. Finally, excluding patients who had received mismatched unrelated transplants from the analyses did not modify the results significantly (*Online Supplementary Tables S7-S10*).

Cumulative association of adverse genotypes in recipients

We postulated that the association of biomarkers associated with grade III-IV acute GvHD might be stronger in the case of an additive effect of the associated SNP. Thus, the cumulative effects of SNP in *ABCC1* (*rs*8058040), *ABCC2* (*rs*3740065), *ATIC* (*rs*2177735), and nuclear factor of activated T cells (*NFATC1*) (*rs*8090560) were evaluated for significant association with the occurrence of grade III/IV acute GvHD. As expected, the combination of two or more of these markers had an important cumulative association with grade III-IV acute GvHD in recipients (HR=20.03, 95% CI 6.11-65.68; $P=7.59 \times 10^{-7}$).

Chronic graft-versus-host disease and the competing risk of death

Seven polymorphisms in methotrexate/cyclosporine A pharmacogenes were associated with chronic GvHD with *P* values <0.05. However, all these SNP were associated

Table 5. SNP associated with the competing risk of death prior to grade III-IV acute GvHD.

Gene	SNP	MAF (%)	A/b	Death prior to acute GvHD (grade III-IV) Genomic model[a] Mode	HR[c]	95% CI	*P* value	*q* value	Secondary model[b] Mode	HR[c]	95% CI	*P* value	*q* value
RECIPIENT													
ABCB1	rs4148732	15	A/G	[Ab]	0.52	(0.32-0.85)	**0.009**	**0.043**	Dom.	0.57	(0.36-0.90)	**0.016**	0.186
				[bb]	1.55	(0.46-5.23)	0.481	0.620	Rec.	1.91	(0.57-6.42)	0.294	0.617
ABCB1	rs6950978	31	A/T	[Ab]	0.52	(0.33-0.82)	**0.005**	**0.036**	Dom.	0.55	(0.35-0.85)	**0.007**	0.155
				[bb]	0.88	(0.37-2.10)	0.781	0.679	Rec.	1.39	(0.61-3.16)	0.432	0.705
ABCC1	rs17264736	48	G/T	[Ab]	0.59	(0.35-1.00)	0.052	0.104	Dom.	0.59	(0.35-1.00)	**0.048**	0.293
				[bb]	0.61	(0.32-1.15)	0.125	0.357	Rec.	0.93	(0.57-1.51)	0.755	0.857
ABCC1	rs212087	34	C/T	[Ab]	1.83	(1.15-2.92)	**0.011**	**0.043**	Dom.	1.51	(0.99-2.31)	0.055	0.293
				[bb]	0.95	(0.48-1.89)	0.893	0.723	Rec.	0.75	(0.39-1.45)	0.392	0.673
ABCC1	rs4781712	50	A/G	[Ab]	0.62	(0.35-1.09)	0.096	0.159	Dom.	0.64	(0.37-1.10)	0.107	0.356
				[bb]	0.69	(0.37-1.30)	0.252	0.481	Rec.	1.00	(0.62-1.61)	0.999	0.857
ABCC1	rs8058040	21	A/G	[Ab]	1.24	(0.80-1.93)	0.334	0.257	Dom.	1.28	(0.83-1.97)	0.265	0.504
				[bb]	2.26	(0.75-6.86)	0.148	0.357	Rec.	1.99	(0.68-5.81)	0.210	0.495
ABCC2	rs3740065	13	T/C	[Ab]	0.90	(0.54-1.49)	0.680	0.303	Dom.	0.95	(0.58-1.55)	0.838	0.752
				[bb]	2.73	(0.54-13.86)	0.226	0.481	Rec.				
ABCG2	rs12505410	31	T/G	[Ab]	1.90	(1.21-2.98)	**0.005**	**0.036**	Dom.	1.74	(1.14-2.66)	**0.010**	0.155
				[bb]	1.26	(0.58-2.76)	0.563	0.662	Rec.	1.01	(0.47-2.16)	0.973	0.857
ABCG2	rs13120400	22	T/C	[Ab]	2.16	(1.35-3.44)	**0.001**	**0.024**	Dom.	1.90	(1.22-2.94)	**0.004**	0.155
				[bb]	1.04	(0.40-2.68)	0.941	0.723	Rec.	0.84	(0.34-2.11)	0.715	0.857
ATIC	rs2177735	42	T/C	[Ab]	0.71	(0.45-1.12)	0.144	0.159	Dom.	0.65	(0.42-1.01)	0.054	0.293
				[bb]	0.49	(0.26-0.92)	**0.026**	0.210	Rec.	0.61	(0.35-1.07)	0.085	0.355
NFATC1	rs8090560	29	G/A	[Ab]	0.80	(0.51-1.26)	0.333	0.257	Dom.	0.88	(0.57-1.35)	0.564	0.679
				[bb]	1.67	(0.70-3.97)	0.250	0.481	Rec.	1.81	(0.77-4.24)	0.174	0.483
DONOR													
DHFR	rs34965641	26	C/T	[Ab]	1.26	(0.82-1.94)	0.292	0.592	Dom.	1.15	(0.76-1.75)	0.500	0.538
				[bb]	0.72	(0.29-1.75)	0.465	0.924	Rec.	0.66	(0.27-1.57)	0.343	0.921
NFATC2	rs3787186	31	G/A	[Ab]	0.70	(0.46-1.08)	0.107	0.427	Dom.	0.78	(0.52-1.17)	0.233	0.500
				[bb]	1.35	(0.67-2.71)	0.407	0.924	Rec.	1.59	(0.80-3.12)	0.183	0.921
SLC19A1	rs1051266	47	G/A	[Ab]	0.88	(0.54-1.44)	0.605	0.649	Dom.	0.98	(0.63-1.53)	0.932	0.666
				[bb]	1.17	(0.68-2.00)	0.576	0.924	Rec.	1.25	(0.79-2.00)	0.340	0.921
SLC19A1	rs4818789	24	T/G	[Ab]	0.67	(0.42-1.08)	0.103	0.427	Dom.	0.79	(0.52-1.22)	0.294	0.500
				[bb]	1.39	(0.67-2.86)	0.373	0.924	Rec.	1.54	(0.75-3.16)	0.238	0.921
SLC19A1	rs4819128	47	T/C	[Ab]	0.86	(0.53-1.40)	0.541	0.647	Dom.	0.97	(0.63-1.50)	0.891	0.666
				[bb]	1.17	(0.68-2.00)	0.571	0.924	Rec.	1.26	(0.78-2.04)	0.340	0.921

MAF: minor allele frequency; [A]: major allele; [b]: minor allele; Dom.: dominant mode; Rec.: recessive mode; P and q values ≤ 0.05 are in bold characters. [a]Genomic model= [AA] vs. [Ab] and [bb], reference group=[AA] (HR fixed at 1.00). [b]Dominant model=[AA] vs. [Ab+bb], reference group=[AA] (HR fixed at 1.00); Recessive model=[AA+Ab] vs. [bb], reference group=[AA+Ab] with HR fixed at 1.00. [c]Models adjusted for sex mismatch, diagnosis, source of cells, age of recipient and donor, donor type and conditioning regimen. The incidence of GvHD was estimated by competing-risk analysis with death as a competing risk for GvHD. Data are not shown for bb genotype with low frequency (<2%).

with false discovery rates above the threshold value of 0.05. Of interest, in competing risk analyses, the *ABCC1 rs*8058040 SNP, associated with a higher rate of acute GvHD, was correlated with an increased risk of death prior to the occurrence of chronic GvHD although the association was not statistically significant after correction for multiple testing (HR 6.11; P=0.005, q=0.173). Data are presented in *Online Supplementary Table S11*.

Discussion

Despite prophylactic measures, the fact that a significant proportion of patients still develop GvHD suggests that additional, uncharacterized, inter-individual genetic factors may contribute to the development of acute and chronic forms of GvHD. Reliable and relevant biomarkers that predict severe acute GvHD, beyond HLA matching, are urgently needed to improve and personalize treatment approaches. Our findings support that germline polymorphisms in genes encoding drug transporters and targets may underlie part of the heterogeneity in GvHD development.

Common inherited variations in *ABC* genes, *SLC19A1*, *ATIC*, *DHFR* and *NFATC* were significantly associated with either grade III-IV acute GvHD or risk of death. Based on our findings, *ABCB1*, *ABCC1*, *ABCC2* and *ABCG2* subfamily drug-transporter members are likely to play an important role in clinical outcome following transplantation, particularly with regards to the development of severe, acute GvHD. Indeed, the presence of genetic variations in four major efflux transporters (*ABCB1*, *ABCC1*, *ABCC2* and *ABCG2*) and one influx pump (*SLC19A1*) is associated with adverse clinical outcomes. Physiologically, SCL19A1 and ABC transporters mediate an opposite effect on intracellular drug levels; while SCL19A1 imports methotrexate into the cells, the ABC transporters are efflux pumps that export cyclosporine A and methotrexate drugs outside target cells.[36-38] Of major significance in our study, variations in *ABC* genes were positively associated with grade III-IV acute GvHD and with the competing risk of death. Methotrexate and cyclosporine A are both major substrates of ABC transporters and it is postulated that the identified SNP, or those in close linkage, may reflect in part changes in bioavailability, intracellular levels or hepatic/renal clearance of these two drugs (Figure 1). Our findings are in agreement with those of two other studies sustaining a role of *ABCB1* C3435T genetic polymorphism on methotrexate and cyclosporine A pharmacokinetic profiles in HSCT patients.[39,40]

Moreover, the donor' genotype for the influx transporter *SCL19A1* 80GA (rs1051266) was associated with a 2.7-fold lower risk of severe acute GvHD as compared to the 80GG genotype (P=0.005, q=0.044). This polymorphism has been associated with a more effective response to methotrexate therapy in the context of rheumatoid arthritis, with a 3-fold higher rate of remission, suggesting higher drug exposure at the target cell level.[41] The molecular targets of cyclosporine A, *NFATC1* and *NFATC2*, representing essential steps for cytokine gene expression in activated T cells,[42-45] might also influence the development of severe acute GvHD as variations in *NFATC* genes could potentially increase cytokine synthesis, release and T-cell stimulation, thereby contributing to acute GvHD. Of all methotrexate's candidate genes previously examined, *MTHFR*, in particular the non-synonymous C677T (rs1801133) and the A1298C (rs1801131) polymorphisms, has received great attention but yielded conflicting results.[18,23,24,29-31,46] In this study, associations were found between *MTHFR* genetic status and grade II-IV acute GvHD; none, however, remained positive for severe acute GvHD after correction for multiple testing. Based on our data, it is suggested that the SNP associated with grade II-IV acute GvHD herein are either: (i) associated with a less severe form of the disease (grade II), and/or (ii) the lower MAF (<20%) of these variants did not allow us to demonstrate their associations with grade III-IV acute GvHD occurring at a frequency of 15% in our cohort. Indeed, within the methotrexate pathways tested, polymorphisms in *ATIC* and *DHFR*, with higher MAF (>25%), were associated with severe acute GvHD and thus deserve further attention in future studies.

The strengths of this study include a biologically relevant candidate gene approach, thorough coverage of the genetic variability of these genes, the plausibility of the biological associations observed and the conservative adjustments made for multiple comparisons. However, a limitation of our study is related to the fact that the functional consequences of the genetic variants remain unknown. Our findings also require validation in larger, independent, inter-ethnic cohorts of patients undergoing HSCT. The information, which can be gained from a single blood sample before transplantation, may help to identify individuals at higher risk of developing grade III-IV acute GvHD. If our findings are replicated, these novel biomarkers could eventually provide important clinical prognostic, predictive and therapeutic information beyond HLA loci genomics. Identification of useful biomarkers may also lead to the selection of the most appropriate immunosuppressive regimens and/or optimization of drug pharmacokinetics and pharmacodynamics in these patients. Before such clinical translation, it will be essential to establish the precise combinations and cumulative impact of germline variations leading to adverse clinical outcomes and assess their biological impact on drug exposure and activity. Here, we observed that germline genetic markers could potentially increase the risk of severe GvHD (grade III-IV) by an order of magnitude similar to that observed by Lee and colleagues.[47] Their work revealed that a single mismatch detected at HLA-A, B or C loci is associated with a higher relative risk of grade III-IV GvHD (relative risk=1.60-1.62; $P \leq 0.02$).[47]

In conclusion, besides full HLA-matching, optimization of the immunosuppressive regimen is certainly among the leading factors determining the occurrence of acute GvHD and fatal complications. In addition to HLA-matching, germline variations in genes involved in pharmacokinetics and pharmacodynamics of immunosuppressive drugs are likely related to the occurrence of GvHD. Our data support a potential role of membrane transporters in the risk of developing grade III-IV acute GvHD, mostly related to inter-individual variations in drug transport capacity in carriers of these genetic variants. Indeed, variations in drug transport may theoretically alter effective drug delivery at the target cell level and, modify *per se*, the level of immunosuppression achieved and thus influence the risk of severe complications. However, the latter hypothesis will require in-depth molecular and functional studies to address the role of these SNP in GvHD before the information can be exploited clinically. Based on our data, it

seems evident that significant gaps exist in our understanding of the processes involved in drug metabolism as well as in the availability of reliable markers to help predict drug efficacy and delivery. Larger series are warranted to better understand the precise role of inherited germline variations in host and donor genomes to improve the clinical fate of this unique population of patients.

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Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

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