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Post-transplant events

The hemochromatosis C282Y allele: a risk factor for hepatic veno-occlusive disease after hematopoietic stem cell transplantation

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Summary:

Hepatic veno-occlusive disease (HVOD) is a serious complication of hematopoietic stem cell transplantation (HSCT). Since the liver is a major site of iron deposition in *HFE*-associated hemochromatosis, and iron has oxidative toxicity, we hypothesized that *HFE* genotype might influence the risk of HVOD after myeloablative HSCT. We determined *HFE* genotypes in 166 HSCT recipients who were evaluated prospectively for HVOD. We also tested whether a common variant of the rate-limiting urea cycle enzyme, carbamyl-phosphate synthetase (CPS), previously observed to protect against HVOD in this cohort, modified the effect of *HFE* genotype. Risk of HVOD was significantly higher in carriers of at least one C282Y allele (RR = 3.7, 95% CI 1.2–12.1) and increased progressively with C282Y allelic dose (RR = 1.7, 95% CI 0.4–6.8 in heterozygotes; RR = 8.6, 95% CI 1.5–48.5 in homozygotes). The *CPS A* allele, which encodes a more efficient urea cycle enzyme, reduced the risk of HVOD associated with *HFE* C282Y. We conclude that *HFE C282Y* is a risk factor for HVOD and that *CPS* polymorphisms may counteract its adverse effects. Knowledge of these genotypes and monitoring of iron stores may facilitate risk-stratification and testing of strategies to prevent HVOD, such as iron chelation and pharmacologic support of the urea cycle.

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Hematopoietic stem cell transplantation (HSCT) is the treatment of choice for many malignant and some nonmalignant blood diseases.[1–4] The effectiveness of high-intensity conditioning regimens used in HSCT is limited, however, by toxicity to vital organs, most commonly hepatic veno-occlusive disease (HVOD) and acute lung injury.[5] The clinical syndrome of HVOD is manifested by hyperbilirubinemia (serum bilirubin level >2.0 mg/dl), painful hepatomegaly, and fluid retention that usually occur by post transplant day 21.[6,7] Damage to hepatic venules and sinusoidal endothelium and to zone 3 hepatocytes, leading to microvascular obliteration, are the histological hallmarks of HVOD (also called sinusoidal obstruction syndrome). The reported incidence of HVOD in adults undergoing myeloablative SCT has ranged from 6 to 54%.[8,9] Although the results of treatment with antithrombotic agents like defibrotide have been encouraging, prevention of HVOD is important, because it causes considerable morbidity and mortality and is a frequent harbinger of multiorgan dysfunction.[8,10] The pathophysiology of HVOD is not well understood. Risk factors for HVOD include pre-existing liver dysfunction, vancomycin therapy during cytoreduction, prior abdominal radiation or HSCT, a reduced pulmonary diffusing-capacity for carbon monoxide (D_LCO), and the use of a mismatched or matched-unrelated donor. High-intensity conditioning regimens that contain busulfan or total body irradiation (TBI) in combination with cyclophosphamide are generally considered to carry an increased risk of HVOD. The more frequent use of these regimens for allogeneic HSCT conditioning may explain why some (but not all) studies report a higher risk of HVOD in recipients of allogeneic as compared to autologous HSCT.[11–13] Nonmyeloablative regimens cause considerably less acute organ toxicity, but this benefit may be offset by higher disease relapse rates.[10,14]

High-dose chemoradiotherapy rapidly depletes intracellular antioxidants and represents a significant oxidative challenge to the liver.[9,15–17] Increased liver iron content is

known to augment oxidative liver injury and fibrosis due to other hepatotoxins such as alcohol and the hepatitis C virus.[18,19] Iron, a potent catalyst for free-radical reactions, generates highly reactive oxygen species (ROS) such as the hydroxyl free radical from ubiquitous, weakly reactive species such as hydrogen peroxide and the superoxide radical via Fenton chemistry (Figure 1). Since these ROS irreversibly damage cellular constituents, an excess of iron available for these reactions is highly toxic.[20] In individuals with hereditary hemochromatosis, a common genetic disorder caused by hyperabsorption of dietary iron, iron is abnormally deposited in the parenchyma of the liver and other organs. This iron overload disease occurs predominantly in individuals homozygous for the major mutation in the hemochromatosis (*HFE*) gene on chromosome 6*p*, in which a tyrosine residue is substituted for a cysteine residue at position 282 (C282Y).[21,22] The HFE protein regulates intestinal iron absorption and reticuloendothelial cell iron uptake by mechanisms that are incompletely understood, and the C282Y mutation disrupts these critical functions.[23,24] Other single-nucleotide polymorphisms (SNPs) in *HFE* increase the propensity to iron overload in C282Y heterozygotes.[25,26] However, one in 10 persons of Northern European descent is heterozygous for the C282Y mutation. Both homozygotes and heterozygotes for *HFE* C282Y have elevated plasma levels of reactive iron and mean liver iron content, potentially increasing hepatic vulnerability to an oxidative challenge.[27,28]

Fenton reaction: $Fe^{3+} + O_2\bullet- \longrightarrow Fe^{2+} + O_2$

Haber-Weiss reaction: $Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH- + \bullet OH$

(Sum) $\{O_2\bullet- + H_2O_2 \xrightarrow{(Fe)} OH- + \bullet OH + O_2\}$

Figure 1 Mechanism of *in vivo* free radical generation by iron – Only trace amounts of iron (Fe) are necessary to produce abundant quantities of the hydroxyl radical (•OH) from less reactive oxygen species (eg, the superoxide anion, $O_2^{\bullet-}$). The hydroxyl radical causes irreversible oxidant damage to DNA, lipids, and proteins.

The urea cycle is the exclusive source of nitric oxide (NO) in the hepatocyte and the only nondietary source of essential substrates (arginine or citrulline) for endothelial NO synthesis (Figure 2). Accumulating data suggest that NO regulates cellular iron metabolism during stress and inflammation. In addition, this molecule plays a critical role in maintaining vascular integrity, because it acts as a local antioxidant, vasodilator, and platelet antagonist.[29–31] The enzyme carbamyl-phosphate synthetase (CPS) catalyzes the rate-limiting step of the hepatic urea cycle, thereby controlling the availability of NO precursors. A *C*-to-*A* nucleotide transversion in exon 36 of the *CPS* gene results in the substitution of asparagine (*Asn*) for threonine (*Thr*) at position 1405 (T1405N) in the critical *N*-acetylglutamate-binding domain of the enzyme.[32] We previously observed that patients undergoing HSCT who had the *Asn1405* CPS variant (*AA* genotype) had significantly higher plasma levels of citrulline and NO metabolites at base-line (ie, superior urea cycle function) than those with the *Thr1405* variant of CPS; they also had significantly lower rates of HVOD and fatal lung injury.[10,33,34]

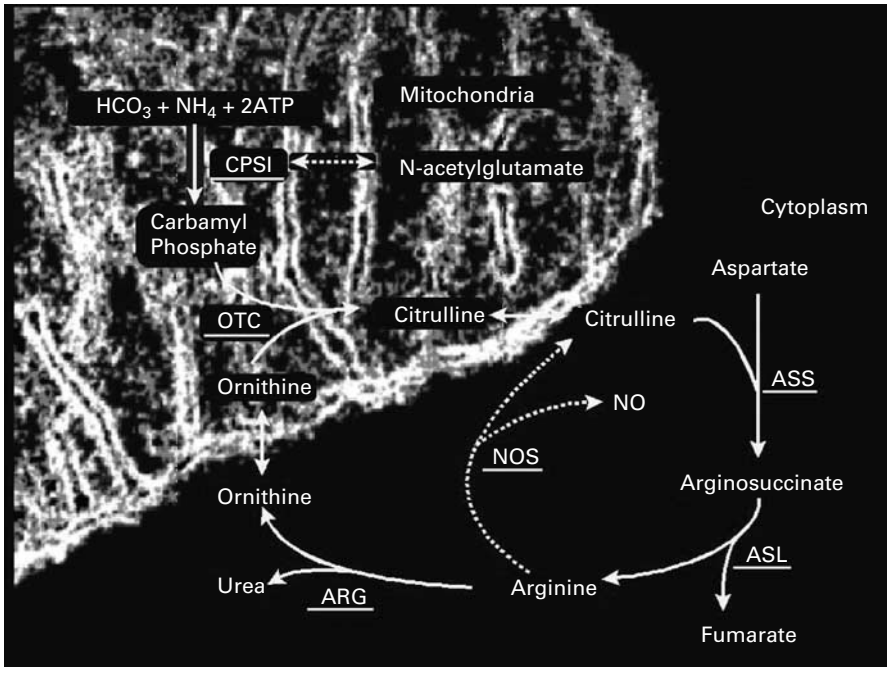

Figure 2 The hepatic urea cycle – Dysfunction of either CPS-1 or OTC results in a fall in citrulline and a rise in ornithine levels. CPS-1 = carbamyl phosphate synthetase 1; OTC = ornithine transcarbamylase; ASS = arginosuccinate synthetase; ASL = arginosuccinate lyase; ARG = arginase; NOS = nitric oxide synthetase.

We therefore hypothesized that patients with at least one *HFE* C282Y allele have compromised antioxidant defenses, placing them at increased risk of HVOD following high-dose chemotherapy and HSCT, and that the effects of *HFE* C282Y may differ by *CPS* genotype.

Methods

Patients

A total of 168 patients who underwent HSCT at Vanderbilt University Medical Center or the Veterans Affairs Medical Center in Nashville, Tennessee, between 1995 and 1999, were eligible for this study. They provided informed consent for biochemical and genetic analyses to explore risk factors for organ injury, and the Institutional Review Boards of both institutions approved these studies. Candidates for HSCT were 19–64 years of age (under age 51 years for allogeneic HSCT) with hematological malignancies, aplastic anemia, breast cancer, and germ cell tumors. Eligibility criteria for HSCT and for this study included: serum creatinine <1.5 mg/dl, serum bilirubin <2.0 mg/dl, pulmonary D_LCO (corrected for hemoglobin) $>50\%$ of predicted value, and left ventricular ejection fraction $>50\%$. Patients with pretransplant cytomegalovirus ($N=1$), hepatitis C, or HIV infection, and patients undergoing a second transplant ($N=1$) were excluded from the study, because they were deemed to be at high risk for early complications and not representative of the cohort.[7]

Conditioning therapy and source of stem cells

The conditioning regimens were: busulfan 1 mg/kg orally every 6 h, days -7 through -4 (total dose 16 mg/kg) and cyclophosphamide 120 mg/kg by intravenous (i.v.) infusion over 3 days, starting on day -3 (BuCy); etoposide 1800 mg/m^2 i.v. on day -7, cyclophosphamide 50 mg/kg/day i.v., days -6, -5, and -4, and total body irradiation in 5 or 6 fractions (200 cGy each), days -3, -2 and -1 (CyVPTBI); and cyclophosphamide 1800 mg/m^2/day i.v., days -6 through -3, etoposide 2400 mg/m^2 i.v. on day -7, BCNU 400 mg/m^2 i.v. on day -2 (CBV). Patients with metastatic breast cancer received a slightly modified STAMP-V regimen (cyclophosphamide, thiotepa, and carboplatin, or CTCb); carboplatin was generally omitted in patients with stage I–III disease.[35] A few patients received other myeloablative drug combinations.

All unrelated donors were serologically identical at the HLA-A, B, and DR loci, and T-cell depletion of the graft was not performed. Marrow and/or growth-factor-mobilized, peripheral blood stem cells collected by apheresis were infused on the day of transplant (day 0).

Supportive care and GVHD prophylaxis

Patients received treatment on a myelosuppression unit equipped with high-efficiency particulate-air (HEPA)-filtered, positive-pressure ventilation rooms. Monitoring for cytomegalovirus and the administration of antimicrobial prophylaxis, blood products, immunoglobulin, and growth factors was standard.[36,37] Vancomycin was routinely started with or without other antibiotics in febrile, neutropenic patients or in febrile non-neutropenic patients with a suspected skin or catheter source. Graft-*versus*-host-disease (GVHD) prophylaxis generally consisted of cyclosporine 3 mg/kg/day i.v. in divided doses, starting on day -1, methotrexate 10 mg/m^2 i.v. bolus on days 1, 3, and 6, and leucovorin 10 mg i.v. bolus every 6 h, on days 2, 4, and 7.[36]

Ascertainment of early complications

Cases of HVOD were identified on the first day that patients satisfied strict Baltimore criteria: serum bilirubin level >2 mg/dl before day 21 and either weight gain $>5\%$ of base-line, or new-onset, tender hepatomegaly.[7,38] Acute lung injury was defined as bilateral infiltrates on chest roentgenograms on three consecutive dates combined with a ratio of partial pressure of oxygen in arterial blood to the fraction of inspired oxygen (PaO_2/FiO_2) of less than 300 in the absence of clinical cardiac dysfunction.[39] In all suspected cases of acute lung injury/idiopathic pneumonia syndrome, an infectious etiology was excluded by fiberoptic bronchoscopy with pan-microbial cultures and stains of bronchoalveolar lavage fluid. Patients alive through day 60 were considered early survivors; patients discharged before day 60 were similarly evaluated on a daily basis as outpatients. All regimen-related toxicities were determined before performing genetic analyses.

Detection of HFE and CPS alleles

Genomic DNA was isolated from the circulating cell pellet that was obtained by bedside centrifugation of whole blood before starting conditioning therapy (Qiagen, Valencia, CA, USA). All samples were stored at -70°C. Genotypes at the *HFE* C282Y and *CPS* T1405N loci were determined by single-strand conformation polymorphism (SSCP) analysis. Based on the published *HFE* gene sequence, the polymerase chain reaction (PCR) was used to amplify a 158-base-pair fragment encompassing the *G*-to-*A* nucleotide transition at position 845 in exon 4 of the *HFE* gene (oligonucleotide primers 5′TGG ATG CCA AGG AGT TCG A 3′ and 5′ACC CCA GAT CAC AAT GAG GG 3′).[21] Oligonucleotide primers constructed from within exon 36 (U4295: 5′CGG AAG CCA CAT CAG ACT GG 3′) and intron 36 (L136: 5′ GGA GAG TGA AAC TTG ACA ATC ATC 3′) of the *CPS* gene were used to amplify a 253-base-pair fragment containing the T1405N polymorphism.[32] After treatment with formamide, PCR blanks, patient samples, and controls of sequence-confirmed genotype were subjected to nondenaturing, mutation-detection electrophoresis (MDE) in an MDE gel matrix (FMC, Rockland, ME, USA). Electrophoresis conditions were: 9 h at 4°C for *HFE* C282Y and 5 h at 4°C for *CPS* T1405N. Gels were stained with silver nitrate to detect DNA bands, and investigators blinded to clinical outcomes scored the genotypes. Direct sequencing and SSCP in 17 randomly selected patients gave identical results.

Iron studies

Plasma, available in a subset of patients, had been collected in the presence of EDTA and stored at −70°C and was therefore unsuitable for measurement of iron levels. The soluble transferrin receptor level (STR), which is unaffected by the presence of metal chelators and correlates inversely with body iron stores, was measured in a sample of 30 patients that included 15 randomly selected *HFE* C282Y carriers and 15 individuals without the *HFE* mutation. Plasma STR levels were measured using the Chemiluminescence Immunoassay (Nichols Research Corp., El Segundo, CA, USA).

Statistical analysis

Characteristics of the HSCT cohort at base-line were compared using Student's *t*-test (or Mann–Whitney *U*-test for continuous variables not normally distributed) and the χ^2-test or Fisher's exact test (discrete variables). Known HVOD risk factors and *CPS* T1405N genotype (associated in this cohort with HVOD) were analyzed by multivariable logistic regression to test the hypothesis that carriage of *at least one* C282Y allele (homozygous and heterozygous genotypes combined) increases the risk of HVOD. Covariates in the logistic regression equation were previous radiation, donor type (matched-unrelated donor *vs* all other), pulmonary D_LCO at base-line, and *CPS* T1405N genotype. Pretransplant liver function (serum aspartate aminotransferase level), autologous (*vs* allogeneic) HSCT, and conditioning regimen type were not significant in unadjusted analysis and were therefore not included due to limited power; if included, however, *HFE* C282Y genotype remained significant. Since vancomycin use during cytoreduction may well be in the causal pathway by which *HFE* C282Y influences the risk of HVOD, it was not included in the regression despite being a known risk factor. Adjustment was made for acute lung injury, donor type, and *CPS* T1405N genotype in the analysis of day 60 mortality. The frequencies of *HFE* and *CPS* alleles and the distribution of genotypes in the study population were tested for Hardy–Weinberg equilibrium using the χ^2-test with 2 degrees of freedom. The Hardy–Weinberg principle states that $q^2+2pq+p^2=1$, where p and q are allele frequencies in a two-allele system. Expected values for *HFE* alleles were calculated using allele frequencies determined previously in (1) a sample of adults from Tennessee who attended Vanderbilt-affiliated internal medicine clinics and whose racial/ethnic background (>85% non-Hispanic White) was similar to that of the patients in this study ($N=169$) and (2) phase II of the Third National Health and Nutrition Examination Survey (NHANES II), conducted from 1992 to 1994 ($N=5171$ adults).[40,41] Expected values for *CPS* alleles were previously determined in a general population sample from Tennessee by Summar *et al*.[33,34]

The Cuzick nonparametric test, a modification of the Wilcoxon rank-sum test, was used to assess the trend in risk of HVOD (or other morbid outcomes) across ordered *HFE* C282Y categories.[42] The effect of the *CPS* genotype (*A* allele) on risk of HVOD across C282Y genotypes was assessed by stratified analysis, since an odds ratio for HVOD could not be computed by logistic regression for the *AA* genotype (no HVOD outcomes). All statistical analyses were performed using the STATA statistical software program (version 7.0, Stata Corporation, College Station, TX, USA).

Results

Approximately 75% of the cohort had an underlying hematological malignancy, and breast cancer was the indication for HSCT in 23% of the patients studied (Table 1). There were 92 autologous transplants, three

Table 1 Early complications in the cohort by indication for HSCT

Diagnosis	*Regimen-related early complications no. patients*			
	HVOD (N=30)	*Pretransplant vancomycin (N=81)*	*Acute lung injury (N=31)*	*Deaths by day 60 (N=24)*
Acute leukemia				
ANLL ($n=24$)	6 (25)	12 (50)	8 (33)	5 (21)
ALL ($n=6$)	2 (33)	1 (17)	1 (17)	1 (17)
Chronic leukemia				
CML-CP ($n=22$)	2 (9)	11 (50)	3 (14)	2 (9)
CML-AP ($n=4$)	3 (75)	2 (50)	4 (100)	2 (50)
CLL ($n=4$)	1 (25)	2 (50)	1 (25)	1 (25)
Breast cancer ($n=39$)	6 (15)	23 (59)	6 (15)	5 (13)
Non-Hodgkin lymphoma ($n=31$)	3 (10)	15 (48)	3 (10)	3 (10)
Multiple myeloma ($n=13$)	1 (8)	4 (31)	1 (8)	1 (8)
Hodgkin disease ($n=12$)	2 (17)	6 (50)	2 (17)	2 (17)
Myelodysplasia ($n=8$)	3 (37)	4 (50)	2 (25)	2 (25)
Aplastic anemia ($n=1$)	1 (100)	0 (0)	0 (0)	0 (0)
Germ cell tumor ($n=2$)	0 (0)	1 (50)	0 (0)	0 (0)

HSCT = hematopoietic stem cell transplantation; ANLL = acute non-lymphocytic leukemia; ALL = acute lymphocytic leukemia; CML-CP = chronic myelogenous leukemia in chronic phase; CML-AP = chronic myelogenous leukemia in accelerated phase; CLL = chronic lymphocytic leukemia. (Numbers in parentheses are the percentages of patients with the row diagnosis who developed complications.)

syngeneic transplants, and 54 allogeneic transplants from HLA-identical relatives. A total of 16 patients received unmanipulated grafts from an HLA-matched, unrelated donor. The self-reported race/ethnicity composition of the study population was 96% non-Hispanic white.

Regimen-related complications and day 60 mortality

In total, 30 patients (18%) developed HVOD by the Baltimore criteria, 50% of whom died by day 60. Prior irradiation, the use of an allogeneic donor, unrelated-donor HSCT, and reduced pulmonary D_LCO (<70% of predicted for age, corrected for hemoglobin) were more common in patients who developed HVOD (*P*-values <0.05, data not shown). No significant differences were observed in the incidence of early complications, including HVOD, based on the underlying diagnosis (Table 1). Acute lung injury, which carried a predictably high mortality, occurred in 31 patients (19%). Vancomycin was used empirically to treat infection in 81 patients (48.8%) during the conditioning phase of HSCT. Overall, 24 patients in the cohort (14.5%) died on or before day 60. Donor type (use of an unrelated donor) and the development of acute lung injury were the only factors associated with day 60 mortality in unadjusted analysis (data not shown, *P*-values both <0.0001); vancomycin requirement did not quite reach statistical significance ($P = 0.056$).

Distribution of HFE and CPS genotypes

Genotyping at the *HFE* C282Y locus was successful in all 166 HSCT patients studied. Patients with and without *HFE* C282Y alleles were similar with respect to pretransplant clinical and demographic variables, conditioning regimens, and the types of transplants they received (Table 2). Genotypes at the *CPS* locus on chromosome 2*q* (not linked to *HFE*) were also similar in both groups. Solid tumors (mostly breast cancers) were more prevalent in carriers of *HFE* C282Y ($P = 0.050$), as previously reported.[40]

The distributions of *HFE* C282Y genotypes within the cohort overall as well as among patients without HVOD were significantly different from the distributions expected based on the Tennessee population sample ($P = 0.002$) or on data from a previously published US national sample ($P < 0.0001$), stratified by race/ethnicity (Table 3).[40,41] A total of 24 patients were heterozygous and 10 patients were homozygous for the C282Y mutation (Table 3). In unadjusted analyses, carriage of one or two C282Y alleles (heterozygous or homozygous genotype) was a significant risk factor for HVOD ($P = 0.015$, Table 3). The number of homozygotes was too small to detect a statistically significant association between homozygous C282Y genotype alone and HVOD (Fisher's exact $P = 0.057$). The proportion of patients with one or more *HFE* C282Y alleles was not increased among early deaths and lung injury cases. Vancomycin therapy during the cytoreductive phase of treatment was also required significantly more often in patients with at least one C282Y allele, however ($P < 0.001$). This finding was independent of the diagnosis and conditioning regimen used. Furthermore, the need for pretransplant vancomycin therapy and the risk of HVOD both increased progressively with the number of C282Y alleles (0 *vs* 1 *vs* 2 alleles) (Table 3).

Table 2 Characteristics of the HSCT cohort

Variable	*0 HFE C282Y alleles (N = 132) n (%)*	*1 or 2 HFE C282Y alleles (N = 34) n (%)*	*P-value*[a]
Mean age (years)	44	45	0.51
Men/women	55/77	14/20	0.96
Hematological cancers[b]	104 (79)	21 (62)	0.05
Solid tumors	28 (21)	13 (38)	0.05
Previous irradiation	28 (21)	11 (32)	0.17
Mean pulmonary D_LCO (% of predicted for age)	84.3	87.0	0.62
Abnormal AST level	29 (22)	5 (15)	0.35
CPS T1405N genotype			0.90
AA	14 (11)	4 (12)	
AC	64 (48)	15 (44)	
CC	54 (41)	15 (44)	
Allogeneic-related donor	44 (33)	10 (29)	0.66
HLA-matched unrelated donor	12 (9)	4 (12)	0.64
Autologous or syngeneic donor	75 (57)	20 (59)	0.83
Conditioning regimen			0.07
BuCy	22 (17)	4 (12)	
CyVPTBI	45 (34)	14 (41)	
CTCb	17 (13)	10 (29)	
CT	10 (8)	3 (9)	
CBV	31 (23)	3 (9)	
Other	7 (5)	0 (0)	

D_LCO = pulmonary diffusing capacity for carbon dioxide (corrected for hemoglobin); AST = aspartate aminotransferase; BuCy = busulfan/cyclophosphamide; CyVPTBI = cyclophosphamide/etoposide/total body irradiation; CTCb = cyclophosphamide/thiotepa/carboplatin; CT = cyclophosphamide/thiotepa; CBV = cyclophosphamide/BCNU/etoposide; *CPS* = carbamyl-phosphate synthetase gene.
[a]Fisher's exact *P*-value is reported when the number of expected observations was <5 in any cell.
[b]Includes one case of aplastic anemia.

The distribution of *CPS* genotypes in the cohort was not significantly different from the distribution in the general population, but it was different in patients with and without HVOD ($P = 0.038$).[10,33] An increasing risk of HVOD was observed as the number of C282Y alleles (allelic dose) increased in patients with the *CPS CC*, but not *AC*, genotype (Table 4). Furthermore, there were *no* cases of HVOD in the 18 patients with the homozygous *AA CPS* genotype (encoding the *Asn1405* variant of CPS), even in one C282Y homozygote.

When we adjusted for other known HVOD risk factors (with the exception of vancomycin use), the association between *HFE* C282Y mutations and HVOD remained significant (RR 3.7 in patients with at least one C282Y allele, 95% CI (1.2–12.1)) (Table 5). Risk of HVOD increased progressively with the number of C282Y alleles (RR 1.7 for heterozygotes, 95% CI (0.4–6.8); RR 8.6 for homozygotes, 95% CI (1.5–48.5)). Day 60 mortality, adjusted for donor type and lung injury, was not

Table 3 *HFE* C282Y genotype frequencies in the general population, the study cohort, and patients who developed early complications after HSCT

Population	*No. of patients*	*No. of HFE C282Y alleles*			*P-values*	
	N	*0*	*1 (heterozygote) n (%)*	*2 (homozygote)*	P_{Trend}	*⩾1 C282Y alleles*[a]
General TN population[b]	169	151 (88.4)	18 (11.6)	0 (0)	—	—
US National sample[c]	5171	1765 (87.5)	245 (12.2)	6 (0.3)	—	—
HSCT cohort	166	132 (80)	24 (14)	10 (6)	—	—[d]
HVOD	30	19 (14)	7 (29)	4 (40)	0.010	0.015[e]
Pretransplant Vancomycin[f]	81	55 (42)	17 (71)	9 (90)	<0.001	<0.0001[e]
Lung injury	31	23 (17)	6 (25)	2 (20)	0.530	0.415[e]
Deaths by day 60	24	17 (13)	6 (25)	1 (10)	0.520	0.277[e]

HSCT = hematopoietic stem cell transplantation; HVOD = hepatic veno-occlusive disease.
[a]Includes both heterozygous and homozygous C282Y genotypes; referent category is 0 C282Y alleles.
[b]Kallianpur *et al*;[40] see Methods.
[c]Data from Steinberg *et al*,[41] frequencies are for non-Hispanic whites.
[d]*P*-values for comparison between genotype distributions in the HSCT cohort and the TN and US population samples were <0.002 and <0.0001, respectively.
[e]*P*-values for HVOD, pretransplant vancomycin, acute lung injury, and day 60 mortality refer to comparisons between the proportions of patients with 1 or 2 C282Y alleles who did *vs* did not develop these complications.
[f]Vancomycin requirement during cytoreduction.

Table 4 Modification of *HFE* C282Y effect on HVOD by the carbamyl-phosphate synthetase (*CPS*) T1405N polymorphism

CPS T1405N genotype	*HVOD cases by no. of HFE C282Y alleles*			*P-value (trend)*
	0 (n/total, %)	*1 (heterozygote) (n/total, %)*	*2 (homozygote) (n/total, %)*	
AA (*N* = 18)	0/14 (0)	0/3 (0)	0/1 (0)	—
AC (*N* = 79)	13/64 (20)	5/13 (38)	1/2 (50)	0.100
CC (*N* = 69)	6/54 (11)	2/8 (25)	3/7 (43)	0.020

HVOD = hepatic veno-occlusive disease. Variants of CPS enzyme: *AA* = *Asn1405*; *AC* = *Asn/Thr1405*; *CC* = *Thr1405*.

Table 5 Adjusted relative risks of HVOD and day 60 mortality by *HFE* C282Y genotype

No. of C282Y alleles	*Adjusted relative risk*			
	HVOD (N = 30)	*95% CI*[a]	*Death by day 60 (N = 24)*	*95% CI*[b]
1 (Heterozygote)	1.7	(0.4, 6.8)	2.9	(0.5, 17.3)
1 or 2 C282Y allele(s)	3.7	(1.2, 12.1)	1.7	(0.4, 7.9)
2 (Homozygote)	8.6	(1.5, 48.9)	0.4	(0.03, 6.3)

HVOD = hepatic veno-occlusive disease.
[a]95% confidence interval for HVOD.
[b]95% confidence interval for day 60 mortality.
Referent category = 0 *HFE* C282Y alleles. Variables included in multivariable regression were previous irradiation, donor type, corrected pulmonary D_LCO at base-line, and *CPS* T1405N genotype (see Methods).

Table 6 Estimation of pretransplant iron stores in a random sample of patients in the HSCT cohort with and without *HFE* C282Y alleles

HFE genotype	*Plasma STR level*[a] *(95% CI)*
1 or 2 C282Y alleles (*n* = 15) (12 heterozygotes, three homozygotes)	16.6 (12.0–21.4)
0 C282Y alleles (*n* = 15)	23.5 (11.2–36.8)

[a]STR = soluble transferrin receptor level (nanomoles/l). The STR varies inversely with iron stores.

significantly increased in carriers of at least one C282Y allele (RR 1.7, 95% CI (0.4–7.9)) or in C282Y heterozygotes (RR 2.9, 95% CI (0.5–17.3)) (Table 5).

Genotype–phenotype correlations

Mean STR levels in 15 randomly selected patients with and 15 patients without the C282Y allele (12 heterozygotes, three homozygotes) were 16.6 nmol/l (95% CI 12.0–25.8) and 23.5 nmol/L (95% CI 11.2–36.8), respectively. Although not statistically significant, this difference reflects higher iron stores in carriers of *HFE* C282Y in the cohort than in patients lacking the C282Y allele (Table 6).

Discussion

In this cohort of adults undergoing myeloablative HSCT, the presence of at least one *HFE* C282Y allele (homozygous or heterozygous genotype) was a significant independent risk factor for HVOD. We also observed an association between the presence of C282Y alleles and pretransplant vancomycin therapy, a surrogate marker for infection in

this immunocompromised population and a recognized risk factor for HVOD. Finally, the *CPS AA* genotype attenuated the effect of C282Y on the risk of HVOD, suggesting that the *A* allele is an effect modifier.

Patients undergoing HSCT are particularly vulnerable to iron-mediated oxidative injury. This population tends to be iron-replete due to disease-related dyserythropoiesis and dependence on red cell transfusions.[9,10] The increased prevalence of the *HFE* C282Y allele in our study cohort compared to the general population is consistent with previous reports of its association with blood malignancies.[43–45] This mutation may also be associated with breast cancer, which made up a substantial proportion of the cohort.[40] Despite its increased background prevalence in the study population, however, it was still possible to demonstrate a statistically significant association between *HFE* C282Y and an increased incidence of HVOD. Higher liver iron content and serum reactive iron levels may predispose C282Y heterozygotes and homozygotes to HVOD due to greater oxidative stress and depletion of hepatic antioxidants.[27,28]

We previously showed that the plasma level of F_2-isoprostanes, an extremely sensitive and specific measure of lipid peroxidation *in vivo*, is highest around the time of engraftment (eg, between days 14–21 in allogeneic HSCT recipients).[10,33] The *HFE* C282Y allele may potentiate HVOD by optimizing conditions for infection and the release of proinflammatory cytokines that compound peri-engraftment oxidative stress.[8,10] Otherwise healthy individuals with increased iron stores are at increased risk for some types of infections, due to phagocyte dysfunction and impairment of cellular immunity.[9,46–48] In this cohort, 90% of C282Y homozygotes and 76 percent of all patients with C282Y mutations required early vancomycin therapy. Increased free iron combined with macrophage dysfunction may explain this observation. The cessation of hematopoiesis and erythroid cell death following chemotherapy has been shown to result in a dramatic rise (within 72 h) in the levels of both transferrin-bound and nontransferrin-bound (catalytically reactive) iron in the serum. Nontransferrin-bound iron is normally taken up rapidly by the liver.[49–53] Hepatocytes damaged by the conditioning regimen may also release stored iron.[10] Iron sequestration by reticuloendothelial cells is a physiological mechanism of antimicrobial defense; normal macrophage responses to infection include increased nontransferrin-bound iron uptake, NO production, and preferential iron retention.[54,55] Some of these responses are abnormal in cultured macrophages from *HFE*-hemochromatosis patients.[56,57] Since macrophages from individuals with C282Y mutations exhibit impaired iron transport, one might speculate that scavenging of the excess available iron is defective, further depleting endogenous antioxidants. Finally, iron is probably a radiosensitizing agent; increased hepatic iron may impair tissue responses to total-body irradiation, particularly when irradiation is administered *after* systemic chemotherapy.[58]

There is strong linkage disequilibrium between the hemochromatosis allele and specific HLA-A and HLA-B alleles.[26] Since HLA haplotypes were not analyzed in this study, it is conceivable that the observed association between HVOD and the *HFE* C282Y allele is really due to another tightly linked locus. We believe this possibility is less likely, because this was not purely a linkage study, and *HFE* C282Y genotypes were determined with a physiologically plausible, *a priori* hypothesis in mind.

Early mortality was not significantly increased in C282Y carriers. However, nonfatal HVOD contributes to substantial morbidity, complicates the management of GVHD, and may impact quality of life due to prolonged liver dysfunction.[9,59,60] Patients with HVOD frequently develop associated lung toxicity and multiorgan dysfunction. In all, 58% of lung injury cases and 63% of premature deaths in our cohort occurred in patients with HVOD. We believe that a significant increase in mortality may be detectable in a larger patient sample due to iron-related increases in the rates of infection, HVOD, and possibly acute lung injury.[9,61,62] In an uncontrolled study, Strasser *et al*[63] found that HSCT recipients who died between days 50 and 100 post-transplant frequently had liver iron content in the hemochromatosis range. An association between HVOD and high pretransplant serum ferritin levels, which may reflect elevated body iron stores but may also be a marker of disease activity, has been reported.[64]

The observation that the *CPS AA* genotype offsets the risk of HVOD in patients with C282Y mutations is consistent with prior *in vitro* studies of recombinant CPS enzyme kinetics. The *A*-encoded *Asn1405* variant is 20–30% more efficient than the *C*-encoded *Thr1405* variant.[33,65] Biochemical measurement of urea cycle intermediates, ornithine/citrulline ratios, and *in vivo* NO metabolites in this cohort have also supported the hypothesis that intensive chemoradiotherapy impairs both urea cycle function and NO production, predisposing patients to oxidative organ injury and HVOD.[10,32,33] Sustained endothelial NO production, which depends upon substrates generated by the urea cycle, may be important in limiting hepatocyte iron uptake and endothelial injury.[15,66] Individuals with *AC* or *CC CPS* genotypes may benefit from the administration of oral citrulline, which supports urea cycle function and NO production in hepatocytes and endothelial cells.[29,33,67] A randomized, double-blind, placebo-controlled trial of oral citrulline administration during conditioning chemotherapy to prevent regimen-related toxicity in patients undergoing myeloablative HSCT is currently in progress at our institution.

Our study is one of few studies to explore underlying genetic risk factors for HVOD and, based on our findings, to suggest possible strategies for prevention of some of the toxic complications of HSCT.[9,10] Both iron stores and urea cycle function are potentially modifiable. Screening of transplant candidates for *HFE* C282Y and *CPS* mutations is likely to be cost-effective, because these mutations are very common, and prior knowledge of genotypes at these loci may facilitate risk assessment and the prevention of regimen-related complications. In patients who are iron-replete, phlebotomy or treatment with chelating agents such as deferoxamine may decrease the risk of infection and/or HVOD and significantly improve liver function.[68] Chelation therapy has been shown to be safe during HSCT, as it appears to have no adverse effects on engraftment, erythropoiesis, or the development of GVHD. Oral or

parenteral antioxidants and apotransferrin may also be helpful.[9,10]

Clearly, further studies are needed to determine the precise nature of the relationship between *HFE* C282Y, iron stores, and complications such as HVOD and infection in this patient population. Not all individuals homozygous for *HFE* C282Y have elevated iron stores. It will also be important to determine what optimal iron stores are in patients undergoing HSCT, in whom the risk of early organ injury must be balanced with the need for robust erythropoiesis. Differences in systemic iron stores or liver iron content between patients with and without C282Y mutations could not be established with certainty in this study, because neither serum iron studies nor liver biopsies were obtained in HSCT recipients. Quantitative assessment of iron stores has not been a routine part of the pretransplant evaluation. In our cohort, the mean STR level was lower (reflecting higher iron stores) in patients with C282Y alleles, providing evidence of a genotype–phenotype correlation; the STR is not generally affected by disease activity, recent transfusions, inflammation, or the presence of metal chelators.[69,70]

In summary, our data suggest that the *HFE* C282Y mutation is an independent risk factor for HVOD. Furthermore, the risk of HVOD is higher in C282Y homozygotes than in heterozygotes, implicating increased iron stores and/or defective iron transport as the mechanism underlying this relationship. Common polymorphisms in the *CPS* gene that enhance urea cycle function attenuate this risk. Larger studies are needed to address the relative importance of *HFE* C282Y in different HSCT subpopulations. These results have potentially important implications for the prediction and prevention of HVOD following myeloablative HSCT.

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