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Donor haptoglobin phenotype determines outcome following liver transplantation

Reinhart Speeckaert,[1] Hans Van Vlierberghe,[2] Roberto Troisi,[3] Dirk De Bacquer,[4] Marijn M. Speeckaert,[1] Marc L. De Buyzere,[1] Lutgarde Claeys,[1] Bernard de Hemptinne[3] and Joris R. Delanghe[1]

1 Department of Clinical Chemistry, Ghent University Hospital, Gent, Belgium
2 Department of Gastroenterology and Hepatology, Ghent University Hospital, Gent, Belgium
3 Department of General and Hepatobiliary Surgery, Liver Transplantation Service, Ghent University Hospital, Gent, Belgium
4 Department of Public Health, Faculty of Medicine and Health Sciences, Ghent University, Gent, Belgium

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Correspondence
Prof. Dr. Joris Delanghe, Ghent University Hospital, De Pintelaan 185, B 9000 Gent, Belgium. Tel.: +32 9 332 29 56; fax: +32 9 332 49 85; e-mail: joris.delanghe@ugent.be

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Summary

Haptoglobin (Hp) is a polymorphic plasma protein with multiple functions defined by three major phenotypes (Hp 1-1, Hp 2-1, and Hp 2-2). In this article, the effects of the donor Hp phenotype (determined by starch gel electrophoresis) on the outcome and the iron status after liver transplantation were investigated. A total of 450 liver transplant patients were enrolled in this study with a median follow-up of 37 months. Kaplan–Meier and Cox regression survival analyses showed a significantly worse graft survival for liver transplantation cases with an Hp 2-2 donor phenotype, which was associated with an increased mortality rate in this group. In male patients, the Hp 2-2 phenotype was associated with higher serum ferritin concentrations, which may be linked to the significantly increased likelihood of infectious complications in this phenotype. Liver transplant patients with Hp 1-1 and Hp 2-1 grafts had a better outcome probability than recipients of an Hp 2-2 graft, which may be explained by differences in iron metabolism induced by the Hp genotype of the graft.

Introduction

The clarification of genetic factors involved in the outcome of liver transplant patients is of major importance to predict the likelihood of complications. The influence of genetic polymorphisms has become an important area of research, especially in genes coding for proteins involved in the immune response. Genetic variability may have a major effect on the outcome after organ transplantation. The discovery of their clinical repercussions may enhance individualized treatment strategies [1]. Besides the production of the majority of plasma proteins, the liver plays a key role in the immune system and in the metabolism of nutrients and drugs [2].

Haptoglobin (Hp) is an α-2-glycoprotein with an almost exclusive hepatic synthesis [3]. It is an acute phase protein, elevating rapidly in response to inflammatory stimuli produced during infections, injuries, and malignancies. The best known physiological function of Hp is the capture of free hemoglobin (Hb) released into the circulation by hemolysis of the red blood cells [4]. The Hp–Hb complexes are taken up by the macrophages through binding of the Hb scavenger receptor, CD163, and are converted by heme oxygenase-1 to free iron, carbon monoxide, and biliverdin [5,6]. This mechanism contributes to iron accumulation, especially following inflammation. Hp acts as a natural bacteriostat by preventing the utilization of Hb by pathogenic bacteria, which require iron for their growth [7]. Furthermore, Hp plays a role in the cell-mediated immunity, as it is an alternative ligand for the CD11b/CD18 integrin dimers on granulocytes and monocytes [8]. These integrins are involved in cell–cell and cell–matrix interactions, including binding to fibrinogen and inter-cellular adhesion

molecule 1 (ICAM-1). Hp has also been shown to inhibit cathepsin B activity, protecting against active proteolysis during inflammatory processes [9].

The genetic Hp polymorphism is characterized by three major phenotypes (Hp 1-1, Hp 2-1 and Hp 2-2), which have important structural and functional differences [10]. In comparison with Hp 2-2 subjects, higher plasma Hp concentrations are reported in Hp 1-1 and Hp 2-1 individuals [11]. The Hp phenotype is a determinant factor in iron homeostasis. In male patients, the Hp 2-2 phenotype is associated with a higher iron load [12], which has also been observed in pathological conditions such as hereditary hemochromatosis [13] and human immunodeficiency virus (HIV) infection [14]. Complexes of Hb and multimeric Hp 2-2 exhibit a higher affinity for CD163 than Hb–Hp 1-1 complexes, which may explain an increased iron accumulation in this phenotype [15]. However, some other studies detected no or only a modest association [16,17]. The immunomodulatory capacity of Hp is phenotype dependent, which is illustrated by a lower production rate of Interleukin (IL)-6 and IL-10 by Hb–Hp 2-2 complexes phagocytosed by macrophages, compared with the Hb–Hp 1-1 complexes [4]. In addition, pro-inflammatory cytokines (e.g. IL-6) promote the transcription of the hepcidin gene through a STAT3 binding motif. Hepcidin is a principal iron-regulatory hormone, which reduces iron levels in plasma by degrading the iron export protein, ferroportin, in enterocytes, hepatocytes, and macrophages [18].

The remarkable functional differences between the Hp phenotypes have been linked to incidence and outcome of several infections, malignancies, metabolic disorders, and autoimmune diseases [19–22]. Clinical studies have demonstrated that the Hp polymorphism may play a role in bacterial and viral infections including tuberculosis, *Streptococcus pneumonia*, *Haemophilus influenzae*, *Legionella*, Epstein–Barr virus (EBV), hepatitis C virus (HCV), and HIV [14,19,23–25]. Following hepatitis B vaccination, the immune response is influenced by the Hp phenotype [23].

After liver transplantation, the donor Hp phenotype is expressed in the recipient, reaching normal values at least 2 weeks after transplantation [26–29]. As Hp is almost exclusively synthesized by the liver, and the donor Hp phenotype is expressed by the transplant recipient, it may be postulated that the donor Hp phenotype might have clinical consequences following liver transplantation. The implications of the donor Hp phenotype on the outcome following liver transplantation were investigated. In view of the important effects of Hp polymorphism on the iron status, the effect of the donor Hp phenotype on the post-transplantation iron status and the effect of ferritin concentrations on outcome were also studied.

Patients and methods

Study population

From 1985 to 2010, 450 transplant cases, 246 men and 204 women, with a median transplantation age of 51 years [interquartile range (IQR): 37–59 years] were enrolled in the study. Median follow-up time after transplantation was 37 months (IQR: 5–104 months). A control group consisting of 353 men and 369 women (median age: 36 years, IQR: 22–49 years) from the same region (Flanders, Belgium) was studied for Hp phenotype distribution and iron status. The study was approved by the local Ethical Committee, following the ethical guidelines of the 1975 Declaration of Helsinki. Informed consent was obtained from all participants.

Biochemical determinations

Hp phenotyping was carried out on Hb-supplemented serum using the electrophoretic method of Smithies [30]. Starch gel was prepared using 11.5% hydrolyzed starch (Connaught Laboratories, Connaught Laboratories, Toronto, ON, Canada) in a 0.1 M Tris citrate buffer (pH 8.86). Electrophoresis was performed during 1 h at 200 V in a 0.3 M borate buffer (pH 8.4). The Hb–Hp complexes were visualized by staining the gel using metal-enhanced peroxidase reagents (Pierce, Rockford, IL, USA). In case of low plasma Hp concentration, high performance gel permeation chromatography (HPGPG) was used for confirmation [31]. In 60 cases, the recipient Hp phenotype was compared with the donor Hp phenotype.

To assess the post-transplantation iron status, serum ferritin concentration was determined in the absence of an acute phase reaction [C-reactive protein (CRP) <1 mg/dl]. The serum ferritin concentration was assayed using a latex-enhanced immunonephelometric method on a BN II analyser (Dade-Behring, Marburg, Germany) [32]. Serum ferritin data were available in 314 patients.

Model for end-stage liver disease (MELD) scores

The MELD was included in the survival analysis to adjust for possible differences in disease severity between the Hp phenotype groups at the time of transplantation. As the Eurotransplant allocation policy uses the MELD score since December 2006, MELD scores of patients transplanted since 2006 were available at the Transplantation Coordination Department of Ghent University Hospital. The MELD scores of patients transplanted before 2006 were retrospectively calculated with serum creatinine, bilirubin, and international normalized ratio (INR) as follows: MELD score = $10 \times [0.957 \log_e(\text{creatinine [mg/dl]}) + 0.378 \log_e(\text{bilirubin [mg/dl]}) + [1.120 \log_e(\text{INR}) + 0.643]$.

The lower-limit value of serum creatinine, bilirubin, and INR was set to 1 and serum creatinine values above 4 were set equal to 4. A total of 222 MELD scores were available for statistical analysis.

Statistical analysis

Comparison between the Hp phenotype groups was carried out using the Mann–Whitney *U*-test. Agreement of the Hp phenotype distribution with the Hardy–Weinberg equilibrium was investigated using the chi-square test. The effect of Hp polymorphism and ferritin concentration on mortality and graft survival – defined as patients who were retransplanted and patients with a fatal outcome – was examined using Kaplan–Meier survival analysis and Cox regression analysis. In addition, the implications of Hp polymorphism and serum ferritin concentration on infection-related graft failure were investigated. The multivariable Cox proportional hazards regression model was used to analyze the influence of possible confounders. All statistical analyses were performed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). All values are expressed as median (IQR). Statistical significance was set at $P < 0.05$.

Results

Hp phenotype distribution

In the transplanted group, the donor Hp phenotype distribution was as follows: 63 Hp 1-1 cases (14%), 208 Hp 2-1 cases (46%), and in 179 subjects (40%), an Hp 2-2 phenotype was found. The Hp phenotype distribution corresponded to allele frequencies of 0.371 (Hp 1) and 0.629 (Hp 2), which is comparable with the distribution found in a reference population [15% Hp 1-1, 47% Hp 2-1 and 38% Hp 2-2; allele frequencies: 0.382 (Hp-1) and 0.618 (Hp-2)]. The observed Hp phenotype distribution was in close agreement with the Hardy–Weinberg equilibrium. In 60 cases, we additionally typed the donor Hp phenotype on donor serum. The Hp phenotype always corresponded to the post-transplantation recipient phenotype.

Characteristics of the Hp phenotype groups

The reasons for transplantation, the type of immunosuppressive medication, the donor characteristics (donor age and gender), and transplant procedure-related factors (living donor, split liver) were all comparable between the combined group (Hp 1-1 and Hp 2-1) and the Hp 2-2 cases (Table 1). To determine the frequency of rejection episodes according to the Hp phenotype, a total of 253 detailed patient files were reviewed. The frequency of cellular rejection episodes was comparable in both Hp groups (Hp 1-1 or Hp 2-1: 16.4% vs. Hp 2-2: 13.4%).

Table 1. Characteristics of the group of haptoglobin (Hp) 1-1 or Hp 2-1 phenotype vs. the Hp 2-2 cases.

Parameter	Hp 1-1 or Hp 2-1 $n = 271$	Hp 2-2 $n = 179$	Significance†
Recipient characteristics			
Age at transplantation (years)*	52 (40–60)	49 (29–58)	$P = 0.04$
Gender: male (%)	58.3	49.2	NS
MELD Score*	22 (16–29) $n = 151$	24 (17–30) $n = 70$	NS
Indication for liver transplant (%)			
Ethanol abuse	27.6	21.4	NS
Hepatitis B infection	14.4	10.7	NS
Hepatitis C infection	26.0	27.9	NS
Congenital disorders	10.3	12.5	NS
Hepatocellular carcinoma	11.1	10.7	NS
Immunosuppressive medication (%)			
Corticosteroid	18.5	17.4	NS
Calcineurin inhibitor	76.2	67.1	NS
Mycophenolate mofetil	41.1	41.4	NS
mTOR-inhibitor	14.9	10.0	NS
Donor characteristics			
Donor age	41 (27–50)	32 (19–52)	NS
Gender: male (%)	61.3	59.2	NS
Living donor (%)	3.8	4.2	NS
Split liver (%)	6.3	1.8	NS

NS, nonsignificant; mTOR, mammalian target of rapamycin.
*Data expressed as median (interquartile range).
†According to Mann–Whitney *U*-test.

Table 2. Serum ferritin concentrations according to the haptoglobin (Hp) phenotype of transplant patients.

	Serum ferritin concentration (μg/l)*	
	Transplant patients	
	Men, $n = 165$	Women, $n = 149$
Hp 1-1/Hp 2-1	115 (54–290)	74 (28–219)
Hp 2-2	204 (76–511)	84 (26–224)
Significance†	$P = 0.027$	$P = 0.751$

*Data expressed as median (interquartile range).
†According to Mann–Whitney *U*-test.

Iron status and Hp phenotype

The association between the serum ferritin values and the donor Hp phenotype was investigated (Table 2). In male patients, the serum ferritin concentration was associated with the Hp phenotype ($P = 0.027$). The Hp 2-2 pheno-

type was characterized by a higher serum ferritin concentration (median: 204 μg/l; IQR: 76–511 μg/l), compared with the group of Hp 1-1 and Hp 2-1 phenotypes (median: 115 μg/l; IQR: 54–290 μg/l). In female patients, however, no significant differences in post-transplantation serum ferritin concentration were found according to the donor Hp phenotype.

Survival analysis

Graft failure was observed in 139 transplanted patients (102 cases of mortality, 37 patients needed a retransplantation). The survival curves in the transplantation group were compared according to the donor Hp phenotype (Figs 1 and 2). Kaplan–Meier survival analysis demonstrated a significantly worse graft survival ($P = 0.013$) in patients with an Hp 2-2 graft because of higher mortality ($P = 0.0045$) in this group. The higher rate of mortality in the Hp 2-2 group increased gradually starting after the first post-transplant month reaching statistical significance at 6 months after transplantation ($P = 0.015$). In male patients, the effect of the donor Hp phenotype was far more pronounced ($P = 0.005$) than in female patients, where no statistical significance was reached ($P = 0.186$). We used a Cox proportional hazard regression model to adjust for potentially confounding variables. The Cox regression model demonstrated that after adjusting for age and gender, the Hp phenotype remained significantly associated with graft failure ($P = 0.015$) and survival ($P = 0.003$) (Table 3). After additional adjustment for the MELD score, the *P*-value became borderline significant in both endpoints ($P = 0.041$) because of loss of power, illustrated by an increase in the hazard ratio. Cox regression models with additional adjustment for donor- (donor age, donor gender, and donor bilirubin values) and transplantation-related factors (cold ischemia time, living donor, and split liver) confirmed all the significantly higher graft failure and mortality associated with the Hp 2-2 phenotype ($P = 0.039$ and $P = 0.009$ respectively). The influence of the Hp 2-2 phenotype on outcome was most marked in the early post-transplant period, which was significant for both graft failure and survival [$P = 0.011$ and $P = 0.015$ respectively (adjusted for age and gender)].

A Cox regression model was also used to investigate the influence of the serum ferritin concentration on graft failure. After adjusting for age and gender, increased

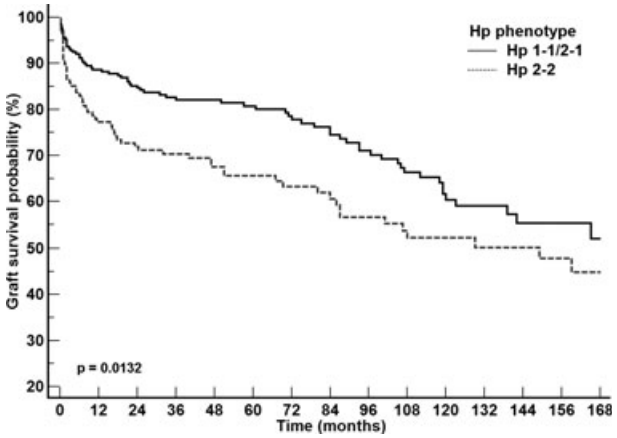

Figure 1 Kaplan–Meier graft survival curve according to the donor haptoglobin (Hp) phenotype.

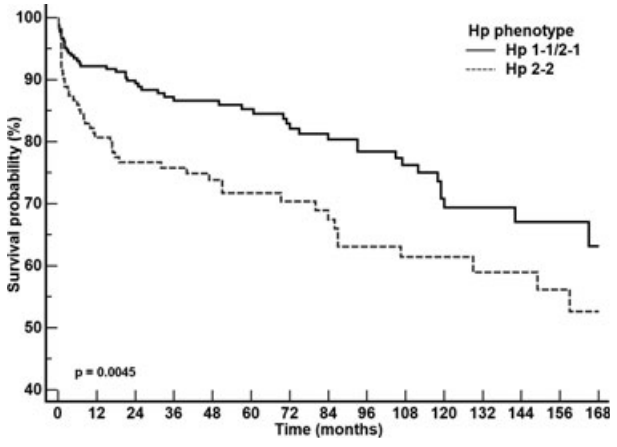

Figure 2 Kaplan–Meier survival curve according to the donor haptoglobin (Hp) phenotype.

Table 3. Cox regression model predicting outcome.

Hp phenotype post-transplantation: Hp 2-2 vs. Hp 1-1 or Hp 2-1	Hazard ratio (95% CI)	Wald χ^2	Significance
Model A* ($n = 450$)			
Graft survival	1.54 (1.09–2.17)	5.92	$P = 0.015$
Survival	1.82 (1.21–2.71)	8.75	$P = 0.003$
Model B† ($n = 222$)			
Graft survival	1.76 (1.02–3.03)	4.16	$P = 0.041$
Survival	1.94 (1.03–3.66)	4.18	$P = 0.041$
Model C‡ ($n = 400$)			
Graft survival	1.46 (1.02–2.10)	4.26	$P = 0.039$
Survival	1.72 (1.14–2.60)	6.74	$P = 0.009$

*Adjustment for recipient age and gender.
†Adjustment for recipient age, gender, and model for end-stage liver disease (MELD) score.
‡Adjustment for recipient-related factors (age and gender), donor-related characteristics (age, gender, and bilirubin values), and transplant procedure-related factors (cold ischemia time, split liver, and living donor).

Table 4. Causes of graft failure according to donor haptoglobin (Hp) phenotype in the early and late follow-up period.

Cause of graft failure (relative percentage according to the Hp group, %)	Early (≤6 months post-transplantation)		Late (>6 months post-transplantation)	
	Hp 1-1/Hp 2-1 (n = 271)	Hp 2-2 (n = 179)	Hp 1-1/Hp 2-1 (n = 218)	Hp 2-2 (n = 116)
Liver failure (%)	–	2 (1.1)	4 (1.8)	3 (2.6)
Malignancies (%)	–	–	11 (5.1)	8 (0.7)
Sepsis/infection* (%)	7 (2.6)	9 (5.0)	6 (2.8)	7 (6.0)
Hepatitis B/C (%)	–	1 (0.6)	4 (1.8)	2 (1.7)
Cardiovascular death (%)	6 (2.2)	6 (3.4)	3 (1.4)	5 (4.3)
Alcohol abuse (%)	1 (0.4)	–	4 (1.8)	2 (1.7)
Rejection (%)	–	–	5 (2.3)	2 (1.7)
Biliary cirrhosis (%)	1 (0.4)	1 (0.6)	2 (0.9)	1 (0.9)
Biliary stricture (%)	–	–	1 (0.5)	1 (0.9)
Primary nonfunction (%)	2 (0.7)	1 (0.6)	–	–
Other† (%)	1 (0.4)	2 (1.1)	3 (1.4)	1 (0.9)
Multifactorial/unknown (%)	5 (1.8)	6 (3.4)	10 (4.6)	6 (5.1)
Total* (%)	23 (8.5)	28 (15.6)	53 (24.3)	38 (32.8)

Hp, haptoglobin.

*$P < 0.05$.

†The category 'Other' includes graft failure of various origin (trauma, kidney failure, gastric bleeding, and hemophagocytic syndrome).

serum ferritin concentration was significantly associated with survival ($P = 0.014$) and graft failure ($P = 0.003$).

Table 4 summarizes the causes of graft failure according to the donor Hp phenotype in the early (≤6 months post-transplantation) and late (>6 months post-transplantation) follow-up period. In both the early and late periods, recipients of an Hp 2-2 graft had a marked overrepresentation of graft failure because of infections and septicemia. Adjusted for age and gender, the Hp phenotype (Hp 1-1 or Hp 2-1 vs. Hp 2-2) was significantly ($P = 0.029$) associated with graft failure because of infectious complications.

Discussion

As Hp is almost exclusively synthesized by the liver, liver transplantation is a unique situation in the sense that the donor Hp phenotype determines the post-transplantation phenotype of the recipient [26]. We hypothesized that the donor Hp phenotype might have an important effect on post-transplant outcome by affecting the iron metabolism. This study investigated 450 transplant patients who underwent liver transplantation between 1985 and 2010 with a mean follow-up time of 37 months. Cox regression analysis adjusted for age and gender demonstrated a significant difference in graft survival ($P = 0.015$) between the Hp 2-2 group and the combined group of Hp 1-1 and Hp 2-1 donor phenotypes, which was associated with a clear difference in mortality ($P = 0.003$) between both groups. After additional adjustment for donor- and transplant-related factors, these results remained significant ($P = 0.039$ and $P = 0.009$ respectively) (Table 3). The group of Hp 1-1 and Hp 2-1 subjects had a marked advantage in survival, most prominent in the first year and a half after transplantation. The results differ from those observed following heart transplantation [33], in which the recipient Hp 2-1 phenotype was associated with the development of cardiac transplant vasculopathy. In the case of heart transplantation, the Hp phenotype remains unchanged following transplantation.

The mechanisms through which the donor Hp phenotype may determine patient outcome following liver transplantation are complex. The Hp phenotypes have been associated with a number of infections, malignancies, metabolic disorders, and autoimmune diseases [19–22]. In the first months following liver transplantation, graft failure is determined by a broad variety of factors related to the recipient, the transplant procedure, and the immunosuppressive therapy. Infections remain the most common cause of death after liver transplantation [34]. Most infections are bacterial, followed by fungal and viral. Infections in the first month are mostly caused by pathogens related to surgery and hospital environment. From 2 to 6 months, the cumulative immunosuppressive therapy makes transplant patients more vulnerable to opportunistic infections. From 6 to 12 months and beyond, the pathogens and the incidence of infections become similar to the standard community statistics [35].

In our study, Kaplan–Meier analysis showed a significant overrepresentation of mortality because of infection or septicaemia ($P = 0.029$), which is most pronounced in male patients ($P = 0.005$). The similar rate of graft

rejection between the Hp phenotypes suggests the involvement of another causative factor. Mounting evidence associates elevated iron load to increased microbacterial virulence and impaired host immunity. Although not an ideal marker of total body iron, the ferritin test is a noninvasive, widely clinically accepted, and convenient method to estimate the iron storage in a large cohort of patients [36]. Following liver transplantation, the combined Hp 1-1 and Hp 2-1 group had a lower serum ferritin concentration in comparison with Hp 2-2 in male patients. The female group showed only modest differences in iron status between donor Hp phenotypes. Similar results have been obtained earlier in a reference population and in HIV patients [12,14]. However, this is the first study demonstrating the association between the serum ferritin concentration and the Hp phenotype of the donor after liver transplantation. These findings may have clinical consequences, as the Hp polymorphism has been associated with the prevalence and the clinical outcome of many diseases characterized by an altered iron metabolism [37]. Iron is a key molecule for survival and growth of many life-threatening bacterial and viral pathogens. Recently, higher serum ferritin levels have been associated with an increased frequency of infectious complications and mortality after liver transplantation [38].

Iron overload leads to diminished immune responses by impairing the antimicrobial activity of the neutrophils and macrophages because of a reduced interferon-gamma (IFN-γ) responsiveness and a decreased production of tumor necrosis factor-alpha (TNF-α) and nitric oxide [39]. *In vitro* and *in vivo* mammalian experiments have suggested immunomodulatory effects of iron by downregulation of $CD4^+$, an increase of $CD8^+$ lymphocytes, impairment of the generation of cytotoxic T cells and an altered immunoglobulin secretion. These conditions would lead to an increased susceptibility to infections [40,41]. In addition, a positive association between the plasma Hp concentration and the absolute numbers of leukocytes, neutrophils, $CD4^+$ cells, and the $CD4^+/CD8^+$ ratio was found [42]. Hp has a negative effect on the lectin-induced lymphocyte transformations [10]. Furthermore, it binds to CD22 on the cell surface of B-lymphocytes. CD22 is involved in the antigen-induced B-cell activation and the signal transduction [42]. Acting as an adhesion receptor, Hp mediates the interactions of B-cells with erythrocytes, T-lymphocytes, monocytes, neutrophils, and endothelial cells [43]. These conditions may worsen the prognosis of infections in Hp 2-2 individuals, especially in reconvalescent patients with a recent transplantation [44].

In recent years, a number of genetic polymorphisms have been associated with the outcome following liver transplantation [45–48]. Our findings further illustrate the importance of genetic factors influencing the outcome of liver transplantation. As the Hp phenotype of the recipient changes according to the phenotype of the graft, liver transplantation is an excellent model to investigate the functional differences of the Hp phenotypes. Our study demonstrates a clear survival benefit of the Hp 1-1 and Hp 2-1 grafts in comparison with Hp 2-2 donor livers, which is more pronounced in male patients and is partly explained by an increased frequency of infectious complications. As a result of the immunosuppressed state, infections are more difficult to recognize in transplant patients. A closer follow-up of liver transplant patients with an Hp 2-2 graft might aid early detection of infectious signs. Increasing evidence documents the key role of iron in the pathophysiology of infections and outcome of immunodepressed patients. The observed concordance between the Hp 2-2 phenotype and an elevated serum ferritin concentration and the significant association of the ferritin values in serum with graft failure confirm our research hypothesis. Differences in iron metabolism induced by the Hp phenotype of the graft may determine the clinical outcome of liver transplant patients, especially in male patients. More studies are needed to investigate the functional effects of the Hp polymorphism.

Authorship

RS, HvV, RT, MS, MDB and JD: wrote the paper. RS, LC and JD: performed the research/study. RS, DDB, MDB and JD: analyzed the data. HvV, RT, MS and BdH: collected the data. JD: designed the research study.

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