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Published in:
European Journal of Clinical Investigation

DOI:
10.1111/eci.12682

Publication date:
2016

DOI: 10.1111/eci.12682

ORIGINAL ARTICLE

Association of hepcidin-25 with survival after kidney transplantation

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ABSTRACT

Background Hepcidin is considered the master regulator of iron homoeostasis. Novel hepcidin antagonists have recently been introduced as potential treatment for iron-restricted anaemia. Meanwhile, serum hepcidin has been shown to be positively associated with cardiovascular disease and inversely with acute kidney injury. These properties may lead to contrasting effects, especially in renal transplant recipients (RTR), which are prone to cardiovascular diseases and graft failure. To date, the role of serum hepcidin in RTR is unknown. We, therefore, prospectively determined the association of serum hepcidin with risk of graft failure, cardiovascular mortality and all-cause mortality in RTR.

Materials and methods Serum hepcidin was assessed in an extensively phenotyped RTR cohort by dual-monoclonal sandwich ELISA specific immunoassay. Statistical analyses were performed using univariate linear regression followed by stepwise backward linear regression. Cox proportional hazard regression models were performed to determine prospective associations.

Results We included 561 RTR (age 51 ± 12 years). Mean haemoglobin (Hb) was 8·6 ± 1·0 mM. Median [IQR] serum hepcidin was 7·2 [3·2–13·4] ng/mL. Mean estimated glomerular filtration rate was 47 ± 16 mL/min/1·73 m^2. In univariate Cox regression analyses, serum hepcidin was not associated with risk of graft failure, cardiovascular mortality or all-cause mortality. Notably, after adjustment for high sensitivity C-reactive protein and ferritin, serum hepcidin became negatively associated with all-cause mortality (hazard ratio 0·89; 95% confidence interval 0·80–0·99, $P = 0·03$).

Conclusions In this study, we did not find an association between serum hepcidin and outcomes, that is graft failure, cardiovascular mortality or all-cause mortality. Based on our results, it is questionable whether serum hepcidin may be used to predict a beneficial effect of hepcidin antagonists.

Keywords Determinants, graft failure, hepcidin, mortality, renal transplantation.

Eur J Clin Invest 2016; 46 (12): 994–1001

Introduction

In renal transplant recipients (RTR), post-transplant anaemia is associated with an increased risk for graft failure, cardiovascular mortality and all-cause mortality. Iron deficiency is one of the main contributors to post-transplant anaemia [1,2].

Hepcidin is known to be the master regulator of iron homoeostasis [3,4]. The biologically active 25-amino acid form of hepcidin (hepcidin-25) is mainly synthesized and secreted by the liver and regulates the amount of iron absorbed from the intestines and the iron release of the reticuloendothelial system [5,6]. Hepcidin-25 regulates iron homoeostasis by binding directly and causing internalization and degradation of ferroportin, the iron transporter on the duodenal enterocytes and macrophages [7,8].

In addition, serum hepcidin has been shown to be a potential important biomarker for cardiovascular disease, because in animals and humans, an association between serum hepcidin and atherosclerotic disease and clinical events was found, possibly mediated via iron sequestration [9]. In particular, in chronic haemodialysis (HD) patients, serum hepcidin-25 has been shown to be associated with fatal and nonfatal cardiovascular events [10].

Currently, hepcidin antagonists are being introduced as potential treatment to improve iron-restrictive anaemia, such as anaemia of inflammation, cancer or chronic kidney disease

(CKD) [11–13]. Hepcidin antagonists may act on several pathways, that is neutralize hepcidin-stimulating cytokines (e.g. IL-6), target the cytokine-signalling pathways, bind and neutralize the hepcidin peptide (e.g. antibodies), prevent hepcidin binding to ferroportin or interfere with ferroportin-internalizing pathways [14]. As a consequence, hepcidin antagonists may interfere with post-transplant anaemia and beneficially influence graft and patient survival.

Hepcidin production is increased in response to high body iron stores and inflammatory processes and decreased by low circulatory iron and low iron body stores, via higher erythropoietin (EPO) activity and hypoxia [15]. In patients with CKD, it is well established that serum hepcidin levels are elevated as result of inflammation and possibly also due to diminished renal clearance [16,17]. Moreover, it has been shown recently that serum hepcidin is directly regulated by fasting insulin levels [18]. To date, it is unknown in RTR to which degree markers of iron availability (serum ferritin), inflammation [high sensitivity C-reactive protein (hs-CRP)] and insulin sensitivity (fasting insulin levels) determine the serum hepcidin level.

In the absence of prospective studies, we aimed to elucidate the main determinants of serum hepcidin in RTR and to assess the association of serum hepcidin with graft failure, cardiovascular mortality and all-cause mortality in RTR.

Materials and methods

Study design

All adult RTR who survived with a functioning allograft beyond the first year after transplantation (1-year post-transplantation was considered baseline) were eligible to participate in this study during their next visit to the outpatient clinic. Baseline data of the RTR were collected between August 2001 and July 2003 at a median 6·0 [interquartile range (IQR): 2–11] years after transplantation. Combined transplant recipients (i.e. kidney/pancreas or kidney/liver) were also invited to participate at the study. Patients with known or apparent systemic illnesses (i.e. malignancies, opportunistic infections) were excluded from participation. A total of 606 of 847 (72%) eligible RTR gave informed consent for the study. For the analyses, we excluded patients with missing data on hepcidin (n = 45), resulting in 561 RTR eligible for analyses. Informed consent was obtained in all participants, and approval for this study has been obtained by the institutional review board (METc 2001/039). Relevant donor, recipient and transplant characteristics were extracted from the Groningen Renal Transplant Database, which has been described in detail before [19].

Measurements

Blood was drawn in the morning after an 8- to 12-h overnight fasting period to determine serum creatinine and plasma glucose concentrations. Serum creatinine concentrations were determined using the Jaffé method (MEGA AU510; Merck Diagnostica, Darmstadt, Germany). Plasma glucose was measured by the glucose oxidase method (YSI 2300 Stat plus, Yellow Springs, OH, USA). Serum hepcidin was assessed by dual-monoclonal sandwich ELISA immunoassay, as described in detail previously [20]. Renal function was assessed by estimating glomerular filtration rate (eGFR) applying the Chronic Kidney Disease Epidemiology Collaboration equation [21]. Proteinuria was defined as $\geq$ 0·5 g protein/24 h urine. Blood pressure was measured as the average of three automated (Omron M4; Omron Europe B.V., Hoofddorp, the Netherlands) measurements with 1-min intervals after a 6-min rest in supine position. The primary end points of this study were death-censored transplant failure, defined as return to dialysis therapy or retransplantation, cardiovascular mortality and all-cause mortality. The continuous surveillance system of the outpatient programme ensures up-to-date information on patient status. Follow-up was performed for a median of nearly 7 years. There was no loss due to follow-up for the primary end points.

Statistical analyses

Data were analysed using IBM SPSS software, version 22.0 (SPSS Inc., Chicago, IL, USA). Data are expressed as mean $\pm$ SD normally distributed variables and as median (IQR) for variables with a skewed distribution, for example hepcidin-25. In cross-sectional analysis, one-way ANOVA was used for normally distributed data and Kruskal–Wallis test for skewed distributed data. Multinomial chi-square test was used for dichotomous or categorical data. Furthermore, univariate linear regression analysis was performed to assess the determinants of serum hepcidin, which was followed by multivariate linear regression analysis with a stepwise backward procedure. For inclusion and exclusion in the multivariate linear regression analysis, P-values were set at 0·2 and 0·1, respectively. The natural logarithm of hepcidin-25 was used as the dependent variable in all regression models due to the skewed distribution. Other skewed distributed variables were also ln-transformed for inclusion in regression analysis. To assess the possible interaction and modification of a specific determinant by another determinant, an interaction term was computed and added to the univariate model and to the multivariate model.

To study whether serum hepcidin was associated with risk of graft failure, cardiovascular mortality and all-cause mortality, Cox proportional hazards regression analyses were performed. We performed analyses in which we adjusted for age and sex (model 1), additionally for eGFR (model 2) and additionally for hs-CRP and ferritin (model 3). Serum hepcidin was used as categorical variable (tertiles) and as continuous variable to

obtain the best fitting model; a 2 base of log-transformed values was used to allow for expression of the hazard ratios (HR) per doubling of serum hepcidin. Splines were fit by a Cox proportional hazards regression model based on restricted cubic splines and adjustments as in model 3. In all analyses, a two-sided *P*-value < 0·05 was considered significant.

Results

Baseline characteristics

We included 561 stable RTR. Mean age was 51 ± 12 years; 55% of participants were male, and their body mass index (BMI) averaged 26·0 ± 4·3 kg/m^2. Patients were included at 6.0

Table 1 Baseline characteristics of renal transplant recipients over tertiles of serum hepcidin

Variables	Tertiles of serum hepcidin			*P*-value
	1st tertile	2nd tertile	3rd tertile	
Age (years)	51 ± 12	50 ± 13	53 ± 11	0·03
Male sex (%)	51	57	56	0·52
Body mass index (kg/m^2)	25·9 ± 4·5	25·9 ± 4·1	26·4 ± 4·4	0·39
Body surface area (m^2)	1·86 ± 0·19	1·87 ± 0·19	1·88 ± 0·19	0·55
Never smoker (%)	36	37	34	
Former smoker (%)	44	41	42	0·45
Current smoker (%)	19	22	23	
Time since transplantation (years)	6.1 (2.8–10.9)	6.0 (3.3–11.8)	5.9 (2.3–11.9)	0·80
Alcohol use (%)	48	56	54	0·31
Diabetes (%)	18	18	16	0·73
Systolic blood pressure (mmHg)	156 ± 23	151 ± 22	152 ± 23	0·11
Diastolic blood pressure (mmHg)	91 ± 10	90 ± 9	89 ± 10	0·05
Laboratory measurements				
Hepcidin (ng/mL)	2·1 (0·9–3·3)	7·3 (5·8–8·9)	16·5 (13·4–25·3)	–
hs-CRP (mg/L)	1·5 (0·7–3·9)	1·8 (0·6–4·2)	2·8 (1·2–7·4)	< 0·001
Albumin (g/L)	40·4 ± 3·0	41·2 ± 3·0	40·4 ± 3·3	0·02
Total protein (g/L)	67·0 ± 4·4	67·6 ± 4·5	67·1 ± 5·1	0·43
Total cholesterol (mM)	5·5 ± 1·0	5·6 ± 0·9	5·7 ± 1·3	0·20
Creatinine (μM)	129 (109–157)	133 (114–155)	139 (115–192)	0·01
eGFR (mL/min/1·73 m^2)	48·5 ± 15·8	49·2 ± 14·6	43·3 ± 16·3	< 0·001
HbA1c (mmol/mol)	46·8 ± 12·0	47·0 ± 11·5	48·8 ± 11·1	0·19
Insulin (μU/mL)	12·0 (8·5–17·5)	12·2 (8·0–14·7)	10·8 (7·6–16·8)	0·06
Haemoglobin (mM)	8·7 ± 1·0	8·7 ± 0·9	8·4 ± 1·0	< 0·001
MCV (fL)	90·2 ± 7·0	92·3 ± 5·6	91·0 ± 6·9	0·007
Ferritin (μg/L)	70 (38–112)	171 (108–258)	291 (177–452)	< 0·001
NT-pro-BNP (pg/mL)	291 (144–683)	268 (104–544)	354 (139–706)	0·06
EPO (IU/L)	20 (13–28)	17 (12–23)	16 (11–23)	0·01

hs-CRP, high sensitivity C-reactive protein; eGFR, estimated glomerular filtration rate; EPO, erythropoietin; HbA1c, hemoglobin A1c; MCV, mean corpuscular volume; NT-pro-BNP, N-terminal prohormone of brain natriuretic peptide.
A *P*-value across tertiles of serum hepcidin was calculated with an one-way ANOVA for normally distributed data, and with a Kruskal–Wallis test for skewed distributed data. Chi-square test was used for dichotomous or categorical data. Alcohol use was defined as alcohol consumers vs. abstainers.

(2.6–11.5) years after transplantation. Serum hepcidin-25 concentrations were 7·2 (3·2–13·4) ng/mL, haemoglobin concentrations were 8·6 ± 1·0 mM, hs-CRP concentrations were 2·0 (0·8–4·8) mg/L, and ferritin concentrations were 156·0 (78·0–283·0) μg/L. Across tertiles of serum hepcidin, significant differences were noted in hs-CRP, serum albumin, serum creatinine, eGFR, haemoglobin, mean corpuscular volume (MCV), ferritin, and EPO concentrations (Table 1).

Determinants of serum hepcidin

In univariate regression analysis, ferritin (β = 0·69, $P < 0·001$, Fig. 1), hs-CRP (β = 0·24, $P < 0·001$), eGFR (β = −0·14, $P = 0·001$), Hb (β = −0·12, $P = 0·006$), EPO (β = −0·12, $P = 0·006$), fasting insulin (β = −0·09, $P = 0·03$) and age (β = 0·09, $P = 0·03$) were associated with serum hepcidin.

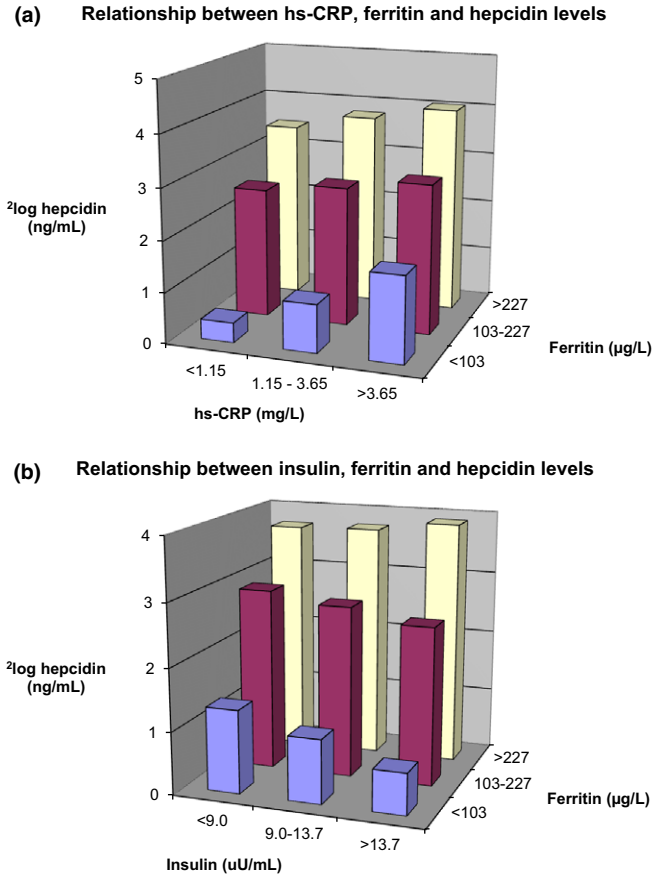

Figure 1 Determinants of serum hepcidin. (a) The interaction between high sensitivity C-reactive protein (hs-CRP) and serum ferritin on hepcidin is shown. hs-CRP and ferritin levels were divided in tertiles. (b) The interaction between serum insulin levels and serum ferritin on serum hepcidin is shown. Insulin and ferritin levels were divided in tertiles.

In multivariate regression analysis, ferritin (β = 0·66, $P < 0·001$), hs-CRP (β = 0·19, $P < 0·001$), EPO (β = −0·13, $P < 0·001$), fasting insulin (β = −0·08, $P = 0·01$) and Hb (β = −0·06, $P = 0·06$; total model $R^2 = 0·53$) were identified as independent determinants of serum hepcidin, while the univariate association of eGFR was lost (Table 2). To assess specifically which additional determinant caused that eGFR lost the association with serum hepcidin, we analysed adding all separate determinants of the multivariate model in combination with eGFR. The association of eGFR with serum hepcidin disappeared after inclusion of serum ferritin in the model (β = −0·05, $P = 0·11$).

An interaction term between hs-CRP and serum ferritin was noted to be significant on serum hepcidin concentrations

Table 2 Determinants of serum hepcidin values in renal transplant recipients

	Univariate analysis		Multivariate analysis	
Parameter	**Std. β**	***P*-value**	**Std. β**	***P*-value**
Age (years)	0·09	0·03		
Male sex	0·01	0·77		
BMI (kg/m^2)	0·07	0·10		
Time since transplantation (years)	−0·02	0·72		
Albumin (g/L)	−0·02	0·65		
HbA1c (mmol/mol)	0·07	0·10		
Insulin (μU/mL)	−0·09	0·03	−0·08	0·01
Glucose (mM)	0·07	0·10		
Smoking (yes vs no)	0·03	0·49		
Alcohol use (yes vs no)	0·01	0·78		
hs-CRP (mg/L)	0·24	< 0·001	0·19	< 0·001
Creatinine (μM)	0·13	0·003		
eGFR (mL/min/1·73 m^2)	−0·14	0·001		
Ferritin (μg/L)	0·69	< 0·001	0·66	< 0·001
Haemoglobin (mM)	−0·12	0·006	−0·06	0·06
EPO (IU/L)	−0·12	0·006	−0·13	< 0·001
NT-pro-BNP (pg/mL)	0·05	0·27		
Total cholesterol (mM)	0·11	0·007		
Total protein (g/L)	0·009	0·83		

BMI, body mass index; hs-CRP, high sensitivity C-reactive protein; eGFR, estimated glomerular filtration rate; EPO, erythropoietin; NT-pro-BNP, N-terminal prohormone of brain natriuretic peptide.
Univariate and multivariate linear regression analyses of potential determinants of serum hepcidin concentrations. Smoking was defined as current use of cigarettes.

(P = 0·01). When adding the interaction to the multivariate model, still a significant interaction was present (P = 0.02). The relation between serum ferritin and serum hepcidin was present irrespective of inflammation reflected by hs-CRP concentration (Fig. 1a). Moreover, the higher the hs-CRP concentration at lower levels of serum ferritin, the higher the serum hepcidin levels. Also, an interaction between insulin concentrations and serum ferritin concentrations was noted (P = 0·005). When adding the interaction term to the multivariate model, the interaction term remained a determinant of serum hepcidin (Fig. 1b; P = 0·02).

Prospective analyses

During a median follow-up of 6·9 (IQR: 6·2–7·2) years, 50 RTR experienced graft failure, 61 died due to a cardiovascular event, and in total, 119 RTR died. Next to the 61 cardiovascular deaths (51%), other causes of death were 19 infection (16%), 26 malignancy (22%) and 12 miscellaneous and other causes (10%).

In Cox regression analysis, serum hepcidin as continuous variable was not significantly associated with graft failure, cardiovascular mortality and all-cause mortality in age- and sex-adjusted analysis (Table 3). However, after adjustment for ferritin and hs-CRP, the association of serum hepcidin as continuous variable with all-cause mortality became inversely significant (HR 0·84; 95% confidence interval (CI) 0·72–0·98, P = 0·03), whereas the association of serum hepcidin with cardiovascular mortality and graft failure remained nonsignificant (Fig. 2).

When divided in tertiles, serum hepcidin was not significantly associated with graft failure, cardiovascular mortality and all-cause mortality in age- and sex-adjusted analysis. However, after adjustment for ferritin and hs-CRP, the upper tertile of serum hepcidin was strongly associated with a decreased risk of cardiovascular mortality (HR 0·36; 95% CI 0·18–0·73, P = 0·005) and all-cause mortality (HR 0·48; 95% CI 0·29–0·79, P = 0·004; Table 3).

Discussion

This study, in stable RTR patients with a large variation in kidney function, did not show an association of serum hepcidin with graft failure, cardiovascular mortality and all-cause mortality in age- and sex-adjusted analysis. This is in contrast with results from other populations where serum hepcidin has been shown to be associated with increased risk for cardiovascular events as well as all-cause mortality, and with a protective effect on kidney function [9,22–25].

As expected, serum hepcidin-25 levels were mainly determined by iron stores (as reflected by serum ferritin),

Table 3 Prospective analysis serum hepcidin on graft failure, cardiovascular mortality, and all-cause mortality in renal transplant recipients

	Tertiles of hepcidin, ng/mL			Serum hepcidin as continuous variable (2log), ng/mL	
	Reference < 4·44	HR (95% CI) 4·44–10·76	HR (95% CI) > 10·76	HR (95% CI)	*P* value
Graft failure					
Model 1	1·00	0·68 (0·31–1·48)	1·77 (0·92–3·39)	1·20 (1·00–1·44)	0·05
Model 2	1·00	0·80 (0·37–1·77)	0·91 (0·46–1·79)	1·01 (0·86–1·18)	0·96
Model 3	1·00	0·79 (0·35–1·76)	0·81 (0·40–1·63)	0·99 (0·84–1·17)	0·88
Cardiovascular mortality					
Model 1	1·00	0·75 (0·40–1·39)	0·72 (0·39–1·33)	1·04 (0·90–1·19)	0·63
Model 2	1·00	0·77 (0·41–1·42)	0·62 (0·34–1·16)	1·00 (0·87–1·15)	0·99
Model 3	1·00	0·59 (0·31–1·14)	0·36 (0·18–0·74)	0·89 (0·77–1·03)	0·11
All-cause mortality					
Model 1	1·00	0·67 (0·43–1·05)	0·75 (0·49–1·15)	1·00 (0·91–1·10)	0·94
Model 2	1·00	0·69 (0·44–1·08)	0·64 (0·41–0·98)	0·95 (0·87–1·05)	0·33
Model 3	1·00	0·63 (0·39–1·01)	0·48 (0·29–0·79)	0·89 (0·80–0·99)	0·03

Model 1: Adjustment for age and sex.
Model 2: Model 1+ additional adjustment for estimated glomerular filtration rate.
Model 3: Model 2+ additional adjustment for hs-C-reactive protein and ferritin.

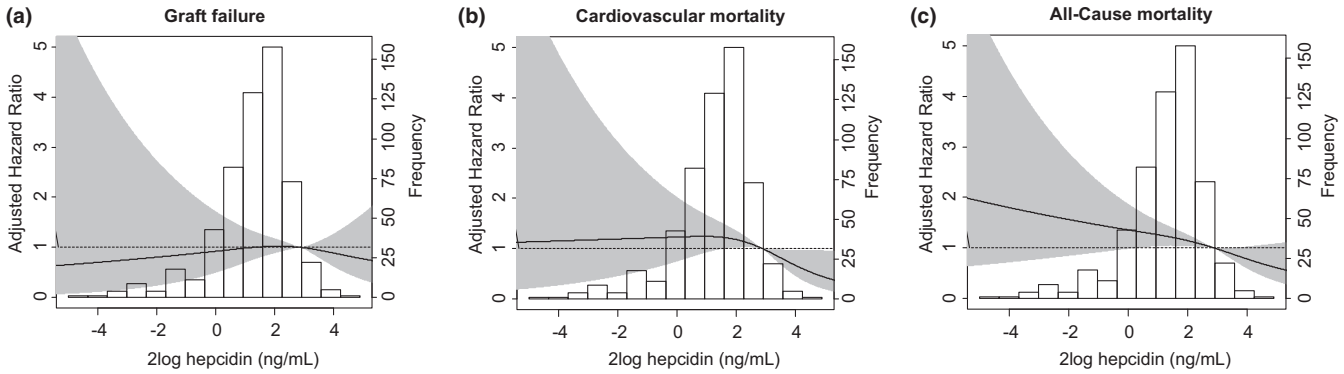

Figure 2 Association between serum hepcidin and risk of graft failure (a) of cardiovascular (b) and all-cause mortality (c) according to model 3, Table 3. The line in the graph represents the hazard ratio. The grey area represents the 95% confidence interval of the hazard ratio.

inflammation (as reflected by hs-CRP levels), tissue hypoxia (which is the primary stimulus for and reflected by increased EPO levels), insulin sensitivity (as reflected by fasting insulin levels) and serum haemoglobin. These factors accounted for 53% of the variance in the level of serum hepcidin in the present study.

As hepcidin antagonists are currently introduced as potential treatment for iron-restricted anaemia [13], we deemed it clinically relevant to assess whether increased serum hepcidin levels were associated with renal graft failure, cardiovascular mortality and all-cause mortality. Hepcidin-25 has been shown to predict cardiovascular events in chronic HD patients [10]. It has been postulated that increased hepcidin concentrations are associated with arteriosclerotic disease by retaining iron in macrophages in the vascular wall. This intracellular iron sequestration may result in pro-atherogenic environment mediated by oxidative stress, inflammatory responses and macrophage apoptosis [9,15]. Moreover, it has been shown that hepcidin-25 in diabetic patients with CKD is associated with mortality [22]. We found no such relationship in RTR. However, after adjustment for serum ferritin and hs-CRP, serum hepcidin was *inversely* associated with cardiovascular mortality and all-cause mortality. As a consequence, it may be speculated that under circumstances where hepcidin reflects mainly lack of hypoxia or lack of anaemia [as it is corrected for iron load (serum ferritin) and inflammation (hs-CRP)], it may convey a protective effect [18,23,26].

Next to the association with cardiovascular mortality and all-cause mortality, we assessed the association of serum hepcidin with renal risk. Wagner *et al*. [22] found elevated hepcidin-25 levels to be predictive of progression of CKD, whereas van Swelm *et al*. [23] recently suggested that hepcidin protects against haemoglobin-induced acute kidney injury. In our study, we did not find an association of serum hepcidin with risk of graft failure.

With respect to the determinants of serum hepcidin in our cohort of RTR, as expected, serum ferritin was the strongest determinant of serum hepcidin. Serum ferritin has already been acknowledged to be a major determinant of serum hepcidin in other populations, for example healthy controls, patients with CKD and HD patients [16,27–29]. In RTR, serum ferritin has been shown to be an important determinant of serum hepcidin in smaller cohorts [30]. As serum ferritin is also an acute phase reactant, inflammation can elevate serum ferritin, which could be a cause of high correlation with serum hepcidin in RTR. However, after inclusion of hs-CRP in the model, the association with serum ferritin remained. Taken the results of the models in Table 2 together, it seems that serum hepcidin levels in RTR are mainly determined by inflammation and iron status. Additionally, we found that serum hepcidin concentrations are inversely associated with fasting insulin levels because in the interaction at low serum ferritin, higher serum insulin levels are associated with lower serum hepcidin levels [18]. These findings underline the close relationship of markers of iron metabolism and insulin resistance which has been recently been evaluated by Krisai *et al*.[31].

The present study has several limitations. We used serum ferritin as a marker of iron availability. Although ferritin is the most frequently used marker of iron status both experimentally and clinically, it is also upregulated by inflammation [32]. Possibly, a combination with transferrin saturation or percentage hypochromic red blood cells would reflect better iron status than serum ferritin alone [33]. However, these data are not available. Another limitation is that our study is a single-centre experience.

The most important strength of our study is that as far as we know, we are to date the first who assessed the prospective association of serum hepcidin in RTR with outcomes, that is

graft failure, cardiovascular mortality, and all-cause mortality. Moreover, our study comprises the largest cohort of RTR where serum hepcidin levels have been determined so far [34,35]. Another strength of this study is the dual-monoclonal sandwich ELISA that has been used to determine serum hepcidin, which has been shown to be highly specific and to correlate robustly well with the gold standard, liquid chromatography–mass spectrometry assay for serum hepcidin-25 [20].

In conclusion, we did not find an association between serum hepcidin and prospective outcomes, that is graft failure, cardiovascular mortality or all-cause mortality with serum hepcidin concentrations in RTR being mainly determined by iron status and inflammation. Taken these findings together, it is questionable that serum hepcidin levels may be used to predict the potential beneficial effect of hepcidin antagonists in RTR. Further studies are needed to validate these results and to evaluate whether there is a potential role for hepcidin antagonists to improve iron deficiency, anaemia and outcome in RTR.

Acknowledgements

Parts of this study were presented in abstract form at the American Society of Nephrology Kidney Week 2015, San Diego, CA, 5–8 November 2015. This research did not receive any specific grant from funding agencies in the public, commercial or not-for-profit sectors.

Disclosure

None.

Address

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Received 26 May 2016; accepted 29 September 2016

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