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만성콩팥병 환자의 임상 경과에 혈청 hepcidin이 미치는 영향에 대한 연구

The effect of serum hepcidin levels on the clinical outcomes in patients with chronic kidney disease

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Abstract

The effect of serum hepcidin levels on the clinical outcomes in patients with chronic kidney disease

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Background

Anemia is common problem in patients with chronic kidney disease (CKD) and contributes to increased risk of poor clinical outcomes. In treating anemia in CKD patients, erythropoiesis stimulating agents (ESA) resistance is an important issue and hepcidin is suggested as a key peptide of ESA resistance. However, the clinical characteristics of hepcidin and its role on ESA resistance have not been validated in large-scaled multicenter cohort. Moreover, the relative contribution of hepcidin and iron indices on anemia severity in CKD has been studied little. Therefore investigator designed and performed this study to confirm the known association between hepcidin, kidney function, ESA resistance, and anemia, and to identify the effect of hepcidin on clinical outcomes in non-dialysis CKD patients.

Methods

Investigator reviewed data of 2238 patients from a large-scale multicenter prospective Korean study (2011–2016). Among 2238 patients whose mean age was 54.2 years, serum of 2113 patients were analyzed to measure serum hepcidin levels using competitive enzyme-linked immunosorbent assay. Iron indices were transferrin saturation (TSAT) and ferritin. Anemia was defined as hemoglobin (Hb) <13.0 g/dl in men and <12.0 g/dl in women. Mild, moderated, and severe anemia were defined as Hb <13.0 g/dl, <11.5 g/dl, and <10.0 g/dl, respectively. The studied clinical outcomes were renal events, defined as a >50% decrease in kidney function from the baseline values, doubling of serum creatinine, or dialysis initiation, which were detected and adjudicated annually.

Results

Markers of inflammation and iron status were positively associated with serum hepcidin levels, regardless of CKD stage. However, estimated glomerular filtration rate was inversely associated with serum hepcidin levels (beta -0.007, $P < 0.001$), particularly in patients with CKD stages 3b–5, but not in those with CKD stages 1–3a. Iron supplementation was associated with increased serum hepcidin levels (beta 0.306, $P = 0.001$), particularly in patients with CKD stages 1–3a, but not in those with CKD stages 3b–5. Use of ESA was associated with increased serum hepcidin levels (beta 0.802, $P <0.001$), particularly in patients with CKD stages 3b–5, but not in those with CKD stages 1–3a, and ESA dosage positively correlated with serum hepcidin levels. In subgroup analysis according to the causes of CKD, kidney function was negatively associated with serum hepcidin levels in patients with hypertensive nephropathy and glomerulonephritis. The positive association between ESA use and serum hepcidin levels was not

affected by causes of CKD. TSAT and serum hepcidin were significantly associated with anemia status, whereas serum ferritin was not, regardless of anemia severity. In patients with CKD1-3a, a 10% increase of TSAT was associated with severe anemia [odds ratio (OR) 0.628, 95% confidence interval (CI) 0.515-0.765; P <0.001] and moderate anemia (OR 0.672, 95% CI 0.476-0.950; P = 0.024), whereas a 10-ng/ml increase of serum hepcidin was associated with mild anemia (OR 1.360, 95% CI 1.115-1.659; P=0.002) and moderate anemia (OR 1.379, 95% CI 1.173-1.620; P <0.001) in patients with CKD 3b-5 on multivariate logistic analysis. During a mean of 2.4 years, 333 patients developed renal events (17.4%); 165 (8.6%) patients with functional deterioration and 275 (14.4%) patients with dialysis initiation. In penalized smoothing splines curve analysis, the hazard of renal events steadily increased with the increase of serum hepcidin levels. In multivariate Cox-proportional hazard regression analysis, the hazard ratio and its 95% CI in the third and the forth serum hepcidin quartile were 1.514 (1.025-2.237, P = 0.037) and 1.752 (1.183-2.596, P = 0.005), respectively, compared to the first serum hepcidin quartile. In subgroup analysis, increased serum hepcidin levels were associated with increased hazard of future renal events development, particularly in diabetic male patients with lower levels of Hb, TSAT, ferritin, inflammation, and kidney function.

Conclusions

Investigator observed that kidney function was an independent factor of serum hepcidin levels. Increased serum hepcidin levels with the increase of ESA dosage may suggest the key role of hepcidin in ESA resistance. Although ferritin was not associated with anemia in CKD patients, regardless of kidney function, TSAT was associated with less severe anemia in early CKD patients, whereas serum hepcidin was associated with more severe

anemia in advanced CKD patients. In this study, increased serum hepcidin levels independently predict the progression of CKD in non-dialysis CKD patients. Diabetic male patients with lower levels of Hb, TSAT, ferritin, inflammation, and kidney function may need to be treated more meticulously with special attention to the development of CKD progression.

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keywords: hepcidin, anemia, erythropoien resistance, transferrin saturation, ferritin, progression, chronic kidney disease

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Introduction

Anemia is more prevalent in chronic kidney disease (CKD) patients than in general population, and the severity of anemia is increased with the progression of CKD [1,2]. Since anemia contributes to an increased risk for end stage renal disease, cardiovascular events, and death in CKD patients [3,4], identification of anemia-associated factors in CKD patients is of utmost importance. Erythropoietin is a peptide which is produced by renal interstitial fibroblasts and stimulates red blood cells (RBC) production in bone marrow [5]. As CKD progresses, the production of erythropoietin in kidney is diminished, causing normocytic, normochromic, and hypo-proliferative anemia [5]. Based on these features in CKD patients, there have been several attempts to treat anemia by replacing erythropoietin [6-9], but ultimately failed to improve clinical outcomes [6,7]. Secondary analyses have revealed that resistance to erythropoiesis stimulating agents (ESA) played an important role on the poor outcomes [10,11].

Since Tomas Ganz and the colleges found a new cysteine-rich human peptide and named hepcidin after its origin in the liver (hep-) and antimicrobial properties (-cidin) in 2001 [12], many studies have found that it plays a key role on iron metabolism [13]. The biological receptor of hepcidin is ferroportin, an iron exporting transcellular channel located in cells that are sources of iron, including enterocytes, macrophages, and hepatocytes [14]. Binding of hepcidin to the ferroportin induces the internalization and degradation of ferroportin and disturbs iron efflux from cells to plasma, ultimately reducing serum iron levels and sequestering iron in iron storage sites [14]. This is why hepcidin is thought to be a fundamental peptide related to ESA resistance [15].

The primary site of hepcidin synthesis is hepatocytes. The biological stimuli of hepcidin production is iron overload and inflammation, and the hepcidin production is suppressed by anemia and erythropoietic activity [16]. Because hepcidin is very small peptide (2.7 kDa), it is easily cleared by kidney, and the decreased kidney function may cause accumulation of the peptide [17,18]. Nonetheless, the association between serum hepcidin and kidney function have been suggested only in small single-center studies and the results were not consistent [17-27]. Moreover, unlike hypothetical key role of hepcidin in ESA resistance, previous studies have failed to provide hepcidin-related ESA resistance in real world [17,28]. Therefore, the uncertainties in clinical characteristics of hepcidin and its role on ESA resistance are needed to be evaluated further.

Beyond the uncertainty of hepcidin and ESA resistance, the relative effect of conventional iron indices and hepcidin on anemia in CKD patients has also not been fully evaluated. Conventionally, transferrin saturation (TSAT) and serum ferritin have been used as serum iron indices [29,30]. TSAT and serum ferritin are markers of available serum iron and whole body iron storage, respectively [16,31]. Currently, guidelines for anemia in CKD patients have recommended iron replacement according to serum levels of TSAT and ferritin [9,32]. However, the effectiveness of these two iron indices with anemia in CKD is doubtful [33-36]. Unlike conventional iron indices, however, hepcidin has been significantly associated with anemia in CKD patients [18,19,22,27,37-39]. Therefore, comprehensive analysis of the relative contribution of conventional iron indices and hepcidin on anemia in CKD patients is warranted.

To dates, only few studies have explored the association between serum hepcidin level and adverse clinical outcomes. Niihata et al. reported that serum hepcidin levels predicted future anemia development in 335

non-dialysis CKD patients [37]. Wagner et al. analyzed 249 diabetic patients with CKD who were ESA-naïve, and reported that serum hepcidin levels were independently associated with CKD progression and mortality [40]. However, these previous studies relied on small population samples.

Therefore, investigator planned to perform the present study to confirm known association between hepcidin, kidney function, ESA resistance, and anemia, and to identify the effect of hepcidin on renal events in non-dialysis CKD patients using a large number of adults enrolled in the KoreaN cohort study for Outcome in patients With Chronic Kidney Disease (KNOW-CKD).

Material and methods

Participants

The KNOW-CKD is a multicenter prospective cohort study in Korea of 2238 patients with non-dialysis CKD stages 1–5 enrolled from February 2011 through January 2016. The detailed design and methods of the KNOW-CKD were previously published (NCT01630486 at http://www.clinicaltrials.gov) [41]. The protocol of the KNOW-CKD adhered to the principles of the Declaration of Helsinki and was approved by the Institutional Review Board at each participating hospital including Seoul National University Hospital, Yonsei University Severance Hospital, Kangbuk Samsung Medical Center, Seoul St. Mary's Hospital, Gil Hospital, Eulji Medical Center, Chonnam National University Hospital, and Pusan Paik Hospital. Written informed consent was obtained from all subjects. Estimated glomerular filtration rate (eGFR) was calculated by the equation of Modification of Diet in Renal Disease study formula [42]. CKD and its stages were defined using the Kidney Disease Improving Global Outcomes

2012 guidelines [43].

Serum hepcidin measurement

Serum hepcidin levels were measured at a central laboratory by competitive enzyme-linked immunosorbent assay (cELISA) using EIA5258 kit (DRG Diagnostics, Marburg, Germany), according to the manufacturer`s instructions. The intra- and inter-assay coefficients of variation ranged from 2.1–9.9% and from 11.5–14.6%, respectively. The detectable maximum level was 80 ng/ml, with higher levels recorded as 80 ng/ml.

Definitions

Clinical data, including detailed demographic information and baseline laboratory results, were extracted from the electronic data management system (PhactaX). Hypertension was defined as physician diagnosis; systolic blood pressure (BP) ≥ 140 mm Hg or diastolic BP ≥ 90 mmHg or treatment with anti-hypertensive drugs. Diabetes was defined as physician diagnosis; fasting glucose ≥ 126 mg/dl, or treatment with insulin or oral anti-diabetic drugs. High income was defined as monthly household income more than 4.5 million won (approximately 4000 US dollars). Ever smoking was defined as past or current smoking. Body mass index was calculated as weight (kg) per square of height (m^2). Anemia was defined as hemoglobin (Hb) <13.0 g/dl in men and <12.0 g/dl in women [32]. The severity of anemia were defined by the necessity of ESA: mild (Hb <13.0 g/dl, a level for which ESA should not be used to increase Hb levels intentionally), moderate (Hb <11.5 g/dl, a level for which ESA should be used to maintain Hb levels), and severe (Hb <10.0 g/dl, a level for which ESA

should be started), following KDIGO 2012 anemia guidelines [32]. TSAT (%) was calculated as serum iron × 100/ total iron binding capacity (TIBC). Dose of ESA was measured as weight-normalized epoetin-equivalent (IU/kg/week), with 1 μg of darbepoetin alfa converted to 331 units of epoetin [44]. Continuous erythropoietin receptor activator doses of 50 μg/month, 75 μg/month, 100 μg/month, and 150 μg/month were converted to epoetin equivalents of 3000 IU/week, 4000 IU/week, 6000 IU/week, and 8000 IU/week, respectively [2]. Patients were also sub-grouped by CKD stages into those with early (stage 1–3a) and advanced (stage 3b–5) CKD. The fourth quartile was defined as high serum hepcidin. Renal events are defined by a >50% decrease in eGFR from the baseline values, doubling of serum creatinine, or dialysis initiation, and detected and adjudicated annually [41].

Statistical analysis

The distributions of continuous variables were evaluated using histograms and Q-Q plots. Four variables, hepcidin, ferritin, high-sensitivity C-reactive protein (hsCRP), and urine protein-to-creatinine ratio (UPCR) were not normally distributed. Normally distributed continuous variables are expressed as mean ± standard deviation, non-normally distributed continuous variables as median (interquartile range), and categorical variables as percentages. *P*-trend was analyzed for normally distributed continuous variables by a linear-term of one-way analysis of variance (ANOVA), for non-normally distributed continuous variables by Jonckheere-Terpstra tests, and for categorical variables by a linear-by-linear association. Differences were analyzed by Bonferroni post-hoc analysis of one-way ANOVA for normally distributed continuous variables, Mann-Whitney U tests for non-normally distributed continuous variables, and chi-square tests for categorical variables.

The square roots of serum hepcidin levels and the logarithm of hsCRP, UPCR and ferritin were utilized in linear regression analysis. Odds ratio (OR) and 95% confidence interval (CI) were calculated by logistic regression analysis. A P value of < 0.05 was considered statistically significant. In multivariate analysis, variables with statistical significance on univariate analyses were chosen as covariates using enter method. For the estimated renal survival, the Kaplan-Meier method was employed, and the statistical significance was calculated using the log-rank test. Hazard ratio (HR) and its 95% confidence interval (CI) of serum hepcidin and its tertile group for renal events were assessed using Cox proportional hazard regression analysis. The assumption of proportional hazard was tested by log minus log plot for categorical variables and interaction analysis with time covariate using time-dependent Cox regression analysis for continuous variables. In multivariate Cox proportional hazard regression analysis, age, sex, serum ferritin, hsCRP, and variables with P <0.05 in univariate analysis were chosen as covariates. The relationship between serum hepcidin and renal events was plotted using the penalized smoothing spline method, using the "pspline" package in R Statistics (version 3.03). All analyses, unless otherwise specified, were performed using SPSS Version 22 (IBM Corp., Armonk, NY).

Results

1. Clinical characteristics of serum hepcidin and its role on ESA resistance

Of the 2238 cohort subjects, 148 were excluded, including 126 with missing serum hepcidin levels and 23 with missing Hb levels. This analysis therefore included 2090 patients. The mean age of the 2090 study patients was 53.6 years and 61.1% were men. Mean eGFR was 50.3 ml/min/1.73m^2 and the proportions of patients with CKD stages 1, 2, 3a, 3b, 4 and 5 were 11.9%, 18.3%, 18.0%, 21.7%, 23.5% and 6.6%, respectively. The causes of CKD were diabetic nephropathy (DMN) in 25.3% of patients, glomerulonephritis (GN) in 31.2%, hypertensive nephropathy (HN) in 20.1% and others (mostly autosomal dominant polycystic kidney disease, ADPKD) in 23.4%. ESA and iron supplements were administered to 7.6% and 14.7% of these patients, respectively.

Patients were divided into quartiles, with the first, second, third, and fourth quartiles defined as <6.6 ng/ml, 6.6–13.4 ng/ml, 13.4–25.1 ng/ml and ≥25.1 ng/ml, respectively. Exploration of baseline characteristics in patients assorted by serum hepcidin quartile (Table 1.1) showed that increased serum hepcidin quartile was associated with increased age and an increased percentage of men, as well as with high rates of hypertension and diabetes. Moreover, increased serum hepcidin quartile was associated with a significant reduction in eGFR and significant increases in white blood cells (WBC) counts and hsCRP. Higher serum hepcidin quartile was also associated with higher rates of anemia, treatment with ESA and supplemental iron, and higher serum levels of TSAT and ferritin.

Analysis of Hb levels and markers of iron metabolism and inflammation as a function of CKD stage (Table 1.2) showed that serum hepcidin levels increased with the progression of CKD stages. The median hepcidin levels in patients with CKD stages 1, 2, 3a, 3b, 4 and 5 were 7.7, 11.5, 11.6, 12.5, 20.5 and 31.6 ng/ml, respectively. Moreover, as CKD stage increased, Hb levels decreased with a statistically significant difference between stage

3a and stage 1. Serum ferritin levels were higher, while serum levels of iron and TIBC were lower, as CKD stage increased, with significant differences between stage 2 and stage 1. TSAT also showed decreasing trend with the progression of CKD stages. WBC count was higher in CKD stage 4 than in stage 1, whereas hsCRP level was higher in stage 2–5 than in stage 1.

Multivariate linear regression analysis of factors associated with serum hepcidin levels showed that lower Hb levels and eGFR and higher levels of inflammatory markers (hsCRP and WBC counts) and iron markers (TSAT and ferritin) were independently associated with higher serum hepcidin levels (Table 1.3). These findings were confirmed in multivariate logistic regression for high serum hepcidin (Table 1.4). CKD stage was independently associated with high serum hepcidin, particularly when comparing CKD stage 3b and higher with stage 1. Subgroup analysis by CKD stages (Table 1.5) showed that lower Hb level and higher CRP, ferritin, and TSAT were associated with higher serum hepcidin levels in early and advanced CKD. However, decreased eGFR was associated with higher hepcidin in advanced, but not in early, CKD.

In subgroup analysis according to the causes of CKD, eGFR was negatively associated with serum hepcidin levels in HN and GN. WBC counts were positively associated with serum hepcidin levels, only in DMN, whereas hsCRP levels were positively associated with serum hepcidin levels in GN and others. ESA use was positively associated with serum hepcidin levels, regardless of causes of CKD. Generally, levels of Hb and iron indices were associated with serum hepcidin levels, regardless of causes of CKD (Table 1.6).

We found that both ESA treatment and iron supplementation were associated with higher serum hepcidin levels (Table 1.3). Subgroup analysis

by CKD stages showed that serum hepcidin levels were associated with iron supplementation in patients with early CKD, and with ESA treatment in patients with advanced CKD (Table 1.5). Multivariate logistic regression analysis showed that ESA treatment, but not iron supplementation, was associated with high serum hepcidin levels (Table 1.4). Assessment of the relationships of ESA dose and iron supplement routs with serum hepcidin, showed that increased ESA dose was associated with a significant increase in the square root of serum hepcidin levels (Figure 1.1). Multivariate logistic analysis showed that patients taking 60–120 and ≥120 IU/kg/week ESA showed 1.911-fold (P = 0.041) and 2.462-fold (P = 0.049) higher odds for high serum hepcidin, respectively, than patients not taking ESA. Although the square root of serum hepcidin levels progressively and significantly increased from patients not treated with iron supplements to those taking oral iron to those taking intravenous iron, multivariate analysis showed that iron supplements, regardless of route, were not associated with high serum hepcidin (Figure 1.2).

Table 1.1. Baseline characteristics of the hepcidin quartile group

	Serum hepcidin quartile group (n = 2090)				*P*-trend
	1Q (n = 515)	2Q (n = 529)	3Q (n = 521)	4Q (n = 525)	
Age (years)	51.2 ± 12.6	53.8 ± 12.5*	53.8 ± 11.7*	55.4 ±11.7*	<0.001
Male sex	49.3	62.0*	66.6*	66.1*	<0.001
High income	21.8	25.8	23.5	21.1	0.580
Ever smoking	38.8	45.5*	50.3*	51.6*	<0.001
Hypertension	96.3	97.2	97.9	99.0*	0.003
SBP (mm Hg)	125.6 ± 15.4	127.7 ± 15.2	128.5 ± 16.6*	129.3 ± 17.4*	<0.001
DBP (mm Hg)	76.7 ± 11.1	77.2 ± 10.3	77.3 ± 11.5	76.8 ± 11.7	0.880
Diabetes	28.2	36.9*	36.5*	43.8*	<0.001
Cause of CKD					
DMN	17.5	23.6*	26.5*	33.5*	< 0.001
GN	39.0	32.3*	29.4*	24.2*	< 0.001

HN	18.4	22.3	19.4	20.2	0.777
Others	25.0	21.7	24.8	22.1	0.485
BMI (kg/m^2)	24.1 ± 3.5	24.6 ± 3.3	25.0 ± 3.5*	24.4 ± 3.3	0.047
Glucose (mmol/l)	5.9 ± 1.9	6.2 ± 2.1	6.3 ± 2.6*	6.2 ± 2.1	0.018
BUN (mmol/l)	8.3 ± 4.8	8.6 ± 4.1	10.1 ± 5.1*	13.3 ± 6.6*	<0.001
Creatinine (μmol/l)	128.6 ± 77.6	137.0 ± 72.9	162.4 ± 103.3*	216.9 ± 123.3*	<0.001
eGFR ($ml/min/1.73m^2$)	60.8 ± 32.5	55.7 ± 29.6*	48.8 ± 28.5*	36.1 ± 23.9*	<0.001
Bilirubin (μmol/l)	11.5 ± 4.7	12.2 ± 5.6	11.6 ± 5.3	10.4 ± 5.0*	<0.001
Albumin (g/l)	41.7 ± 3.8	42.2 ± 4.0	41.8 ± 4.3	41.2 ± 4.9	0.013
Cholesterol (mmol/l)	4.6 ± 0.9	4.6 ± 1.0	4.6 ± 1.1	4.4 ± 1.0*	0.002
WBC ($\times 10^3/\mu L$)	6.4 ± 1.9	6.5 ± 1.8	6.7 ± 1.9	6.8 ± 2.1*	0.001
Hemoglobin (g/dl)	13.0 ± 1.9	13.3 ± 1.8	13.0 ± 2.1	11.9 ± 2.0*	<0.001
Anemia	35.9	33.3	42.4*	65.5*	<0.001
ESA use	2.9	3.8	5.4*	18.4*	<0.001

Iron use	7.4	8.5	13.7*	29.3*	<0.001
TSAT (%)	28.1 ± 12.4	31.4 ± 11.1*	33.4 ± 11.7*	33.6 ± 12.4*	<0.001
Ferritin (pmol/l)	95.4 (49.3−163.8)	180.7 (120.6−285.9)*	259.1 (170.5−394.3)*	446.7 (282.0−675.2)*	<0.001
Hepcidin (ng/ml)	3.9 (2.7−5.3)	9.4 (7.9−11.3)*	18.1 (15.5−21.1)*	38.1 (29.9−56.8)*	<0.001
hsCRP (nmol/l)	4.8 (1.9−12.4)	5.4 (1.9−14.3)	6.7 (2.9−17.1)*	7.6 (2.9−21.9)*	<0.001
UPCR (g/g)	0.4 (0.1−1.2)	0.4 (0.1−1.2)	0.5 (0.2−1.8)*	0.7 (0.2−2.1)*	<0.001

SBP, systolic blood pressure; DBP, diastolic blood pressure; CKD, chronic kidney disease; DMN, diabetic nephropathy; GN, glomerulonephritis; HN, hypertensive nephropathy; BMI, body mass index; BUN, blood urea nitrogen; eGFR, estimated glomerular filtration rate; WBC, white blood cells; ESA, erythropoiesis stimulating agents; TSAT, transferrin saturation; hsCRP, high sensitivity C-reactive protein; UPCR, urine protein-to-creatinine ratio. Values are expressed as mean ± standard deviation for normally distributed continuous variables, median (interquartile range) for non-normally distributed continuous variables, and percentage for categorical variables. * meant $P < 0.05$ when compared to 1Q of serum hepcidin by using Bonferroni post-hoc analysis of one-way ANOVA for normally distributed continuous variables, Mann-Whitney U test for non-normally distributed continuous variables, and chi-square test for categorical variables.

Table 1.2. Trends of hemoglobin, iron metabolism and inflammation by the stage of chronic kidney disease

	CKD Stage (n =2090)						*P*-trend
	1(n = 248)	2 (n = 383)	3a (n = 376)	3b (n = 454)	4 (n = 491)	5 (n = 138)	
eGFR (ml/min/1.73m^2)	110.9 ± 20.1	73.2 ± 8.6*	52.2 ± 4.3*	37.3 ± 4.3*	23.2 ± 4.4*	11.8 ± 2.4*	<0.001
UPCR (g/g)	0.2 (0.1-0.7)	0.2 (0.1-0.7)	0.4 (0.1-1.1)*	0.5 (0.2-1.6)*	1.0 (0.3-2.6)*	1.5 (0.7-3.9)*	<0.001
Hemoglobin (g/dl)	14.0 ± 1.5	14.1 ± 1.7	13.5 ± 1.9*	12.7 ± 1.8*	11.5 ± 1.5*	10.5 ± 1.2*	<0.001
TSAT (%)	34.3 ± 14.7	32.8 ± 12.5	32.4 ± 11.6	30.8 ± 10.8*	29.9 ± 11.5*	30.6 ± 12.0	<0.001
Serum iron (μmol/l)	19.2 ± 7.5	18.0 ± 6.6*	17.6 ± 6.3*	16.0 ± 5.6*	19.2 ± 7.5*	18.0 ± 6.6*	<0.001
Serum TIBC (μmol/l)	57.4 ± 8.7	55.9 ± 8.4*	54.8 ± 8.6*	52.9 ± 9.0*	57.4 ± 8.7*	55.9 ± 8.4*	<0.001
Ferritin (pmol/l)	170.3 (75.0-349.1)	223.5 (111.6-406.1)*	232.3 (129.2-380.8)*	204.7 (118.1-400.2)*	243.9 (136.0-404.4)*	278.6 (139.1-472.4)*	<0.001
Hepcidin (ng/ml)	7.7 (3.8-14)	11.5 (5.7-18.6)*	11.6 (6.4-20.3)*	12.5 (6.9-25.2)*	20.5 (9.9-35.3)*	31.6 (15.6-60.2)*	<0.001
WBC ($\times 10^3/\mu$l)	6.3 ± 1.8	6.5 ± 2.0	6.5 ± 1.9	6.7 ± 1.9	7.0 ± 2.0*	6.3 ± 1.8	0.001
hsCRP (nmol/l)	3.8 (1.0-10.1)	5.7 (1.9-15.2)*	5.7 (1.9-12.6)*	5.7 (2.9-17.9)*	7.6 (3.3-21.0)*	5.7 (2.4-16.2)*	<0.001

CKD, chronic kidney disease; eGFR, estimated glomerular filtration rate; UPCR, urine protein-to-creatinine ratio; TSAT, transferrin saturation; WBC, white blood cells; hsCRP, high sensitivity C-reactive protein. Values are expressed as mean ± standard deviation for normally distributed continuous variables and median (interquartile range) for non-normally distributed continuous variables. *P*-trend was analyzed by a linear-term of one-way ANOVA for normally distributed variables and Jonckheere-Terpstra test for non-normally distributed variables. * meant $P < 0.05$ when compared to CKD stage 1 by using Bonferroni post-hoc analysis of one-way ANOVA for normally distributed variables and Mann-Whitney U test for non-normally distributed variables.

Table 1.3. Linear regression analysis for the square root of serum hepcidin level.

	Univariate		Multivariate	
	Beta (95% CI)	*P*	Beta (95% CI)	*P*
Age (years)	0.019 (0.012−0.025)	<0.001	-0.003 (-0.008−0.002)	0.310
Sex (men vs. female)	0.348 (0.183−0.513)	<0.001	-0.125 (-0.300−0.050)	0.160
Income (high vs. non-high)	-0.114 (-0.308−0.081)	0.252	-	-
Ever smoking (yes vs. no)	0.267 (0.106−0.429)	0.001	0.060 (-0.094−0.213)	0.447
SBP (mm Hg)	0.011 (0.006−0.016)	<0.001	0.002 (-0.001−0.006)	0.243
DBP (mm Hg)	-0.002 (-0.010−0.005)	0.536	-	-
BMI (kg/m^2)	0.014 (-0.009−0.038)	0.236	-	-
Glucose (mmol/l)	0.038 (0.001−0.075)	0.042	-0.015 (-0.042−0.012)	0.284
eGFR ($ml/min/1.73m^2$)	-0.021 (-0.023− -0.018)	<0.001	-0.007 (-0.009−-0.004)	<0.001
Albumin (g/l)	-0.031 (-0.050− -0.012)	0.001	0.032 (0.015−0.049)	<0.001
Cholesterol (mmol/l)	-0.184 (-0.263− -0.105)	<0.001	-0.036 (-0.096−0.024)	0.239
WBC ($\times 10^3/\mu l$)	0.056 (0.014−0.098)	0.008	0.044 (0.012−0.077)	0.007
hsCRP (nmol/l)	0.229 (0.170−0.288)	<0.001	0.095 (0.051−0.139)	<0.001
UPCR (g/g)	0.160 (0.108−0.211)	<0.001	0.012 (-0.035−0.06)	0.607
Hemoglobin (g/dl)	-0.234 (-0.272− -0.195)	<0.001	-0.222 (-0.264−-0.181)	<0.001
Ferritin (pmol/l)	1.310 (1.243−1.377)	<0.001	1.226 (1.157−1.295)	<0.001
TSAT (%)	0.027 (0.020−0.034)	<0.001	0.011 (0.006−0.017)	<0.001
ESA use (yes vs. no)	1.951 (1.659−2.244)	<0.001	0.802 (0.563−1.041)	<0.001

Iron use (yes vs. no)	1.545 (1.327−1.763)	<0.001	0.306 (0.120−0.492)	0.001
Bilirubin (μmol/l)	-0.045 (-0.061− -0.030)	<0.001	0.012 (-0.002−0.026)	0.082

SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index; eGFR, estimated glomerular filtration rate; WBC, white blood cells; hsCRP, high sensitivity C-reactive protein; UPCR, urine protein-to-creatinine ratio; TSAT, transferrin saturation; ESA, erythropoiesis stimulating agents. Logarithmic transformations were done for hsCRP, UPCR and ferritin. In multivariate linear regression analysis, variables with $P < 0.05$ in univariate linear regression analysis were chosen as covariates.

Table 1.4. Logistic regression analysis for the high serum hepcidin

	Univariate		Multivariate	
	OR (95% CI)	*P*	OR (95% CI)	*P*
Age (year)	1.017 (1.009−1.026)	<0.001	1.004 (0.992−1.016)	0.541
Sex (men vs. women)	1.335 (1.085−1.641)	0.006	1.122 (0.742−1.697)	0.585
Income (high vs. non-high)	0.859 (0.674−1.095)	0.220	-	-
Ever smoking (yes vs. no)	1.310 (1.075−1.597)	0.008	1.119 (0.782−1.600)	0.540
Hypertension (yes vs. no)	3.079 (1.216−7.798)	0.018	1.205 (0.356−4.078)	0.764
Diabetes (yes vs. no)	1.521 (1.243−1.861)	<0.001	0.854 (0.622−1.174)	0.332
BMI (kg/m^2)	0.989 (0.960−1.019)	0.462	-	-
CKD stages (vs. stage 1)		<0.001		<0.001
Stage 2	1.759 (1.044−2.964)	0.034	1.621 (0.869−3.024)	0.129
Stage 3a	1.951 (1.163−3.273)	0.011	1.250 (0.650−2.404)	0.504
Stage 3b	3.444 (2.118−5.602)	<0.001	2.293 (1.208−4.354)	0.011
Stage 4	6.429 (4.002−10.327)	<0.001	3.639 (1.881−7.038)	<0.001
Stage 5	15.980 (9.170−27.847)	<0.001	6.958 (3.163−15.305)	<0.001
Hemoglobin (g/dl)	0.732 (0.694−0.773)	<0.001	0.743 (0.670−0.824)	<0.001
TSAT (%)	1.017 (1.009−1.025)	<0.001	1.013 (1.000−1.027)	0.044
Ferritin (pmol/l)	1.005 (1.004−1.006)	<0.001	1.005 (1.005−1.006)	<0.001
ESA use (yes vs. no)	5.375 (3.843−7.517)	<0.001	2.031 (1.248−3.305)	0.004
Iron use (yes vs. no)	3.786 (2.944−4.867)	<0.001	1.142 (0.767−1.701)	0.512

WBC (1000/μL)	1.061 (1.008-1.116)	0.022	1.088 (1.010-1.171)	0.026
hsCRP (nmol/l)	1.005 (1.003-1.007)	<0.001	1.003 (1.001-1.006)	0.014
UPCR (g/g)	1.111 (1.064-1.159)	<0.001	1.010 (0.929-1.098)	0.816
Albumin (g/l)	0.961 (0.940-0.982)	<0.001	1.041 (0.996-1.089)	0.074
Cholesterol (mmol/l)	0.797 (0.719-0.884)	<0.001	0.948 (0.820-1.096)	0.472
Bilirubin (μmol/l)	0.941 (0.921-0.963)	<0.001	1.034 (1.001-1.068)	0.043

OR, odds ratio; CI, confidence interval; BMI, body mass index; CKD, chronic kidney disease; TSAT, transferrin saturation; ESA, erythropoiesis stimulating agents; WBC, white blood cells; hsCRP, high sensitivity C-reactive protein; UPCR, urine protein-to-creatinine ratio. In multivariate logistic regression analysis, variables with $P < 0.05$ in univariate logistic regression were chosen as covariates.

Table 1.5. Subgroup analysis for square root of serum hepcidin level according to CKD stages in multivariate linear regression analysis

	CKD stage 1-3a		CKD stage 3b-5	
	Beta (95% CI)	*P*	Beta (95% CI)	*P*
Age (years)	0.002 (-0.004–0.009)	0.477	-0.003 (-0.011–0.004)	0.397
Sex (men vs. female)	0.105 (-0.124–0.335)	0.369	-0.260 (-0.519–0.000)	0.050
Ever smoking (yes vs. no)	0.075 (-0.113–0.263)	0.433	0.038 (-0.202–0.277)	0.758
SBP (mm Hg)	-0.002 (-0.007–0.003)	0.460	0.002 (-0.003–0.007)	0.381
Glucose (mmol/l)	-0.010 (-0.051–0.031)	0.641	-0.013 (-0.049–0.023)	0.466
eGFR (ml/min/1.73m^2)	-0.002 (-0.005–0.001)	0.279	-0.025 (-0.035–-0.015)	<0.001
Albumin (g/l)	0.049 (0.026–0.072)	<0.001	0.025 (0.001–0.050)	0.045
Cholesterol (mmol/l)	0.023 (-0.056–0.103)	0.569	-0.075 (-0.160–0.010)	0.085
WBC ($\times 10^3/\mu$l)	0.033 (-0.009–0.076)	0.125	0.049 (0.003–0.096)	0.037
hsCRP (nmol/l)	0.079 (0.021–0.136)	0.008	0.119 (0.055–0.182)	<0.001
UPCR (g/g)	-0.009 (-0.066–0.047)	0.745	0.019 (-0.059–0.097)	0.632
Hemoglobin (g/dl)	-0.165 (-0.221–-0.109)	<0.001	-0.187 (-0.250–-0.124)	<0.001
Ferritin (pmol/l)	0.939 (0.847–1.031)	<0.001	1.460 (1.359–1.562)	<0.001
TSAT (%)	0.010 (0.004–0.017)	0.002	0.010 (0.002–0.019)	0.015
ESA use (yes vs. no)	-1.366 (-2.944–0.213)	0.090	0.655 (0.386–0.924)	<0.001
Iron use (yes vs. no)	0.647 (0.317–0.978)	<0.001	0.125 (-0.104–0.354)	0.284
Bilirubin (μmol/l)	0.013 (-0.002–0.028)	0.093	0.017 (-0.008–0.043)	0.178

SBP, systolic blood pressure; eGFR, estimated glomerular filtration rate; WBC, white blood cells; hsCRP, high sensitivity C-reactive protein; UPCR, urine protein-to-creatinine ratio; TSAT, transferrin saturation; ESA, erythropoiesis stimulating agents. Logarithmic transformations were done for CRP, UPCR and ferritin. Variables with $P < 0.05$ in univariate linear regression analysis were chosen as covariates in multivariate linear regression analysis.

Table 1.6. Subgroup analysis for square root of serum hepcidin level according to causes of CKD in multivariate linear regression analysis

	DMN (n = 529)		HN (n = 420)		GN (n = 652)		Others (n = 488)	
	Adj. Beta (95% CI)	*P*	Adj. Beta (95% CI)	*P*	Adj. Beta (95% CI)	*P*	Adj. Beta (95% CI)	*P*
eGFR (ml/min/1.73m^2)	-0.007 (-0.014–0.001)	0.083	-0.016 (-0.023–-0.008)	<0.001	-0.006 (-0.010–-0.002)	0.005	-0.004 (-0.009–0.002)	0.178
UPCR (g/g)	0.096 (-0.038–0.230)	0.161	0.034 (-0.074–0.142)	0.534	0.046 (-0.055–0.146)	0.372	-0.062 (-0.180–0.057)	0.306
WBC (×10^3/μl)	0.105 (0.035–0.175)	0.003	0.050 (-0.033–0.133)	0.240	0.013 (-0.044–0.069)	0.657	0.052 (-0.027–0.131)	0.196
hsCRP (nmol/l)	0.049 (-0.055–0.153)	0.358	0.033 (-0.075–0.142)	0.548	0.193 (0.110–0.276)	<0.001	0.115 (0.024–0.205)	0.013
Hemoglobin (g/dl)	-0.290 (-0.388–-0.192)	<0.001	-0.120 (-0.216–-0.025)	0.014	-0.207 (-0.285–-0.129)	<0.001	-0.256 (-0.355–-0.157)	<0.001
Ferritin (pmol/l)	1.276 (1.112–1.440)	<0.001	1.339 (1.165–1.514)	<0.001	1.118 (0.992–1.244)	<0.001	1.191 (1.049–1.332)	<0.001
TSAT (%)	0.019 (0.004–0.034)	0.012	0.008 (-0.005–0.02)	0.247	0.010 (0.002–0.019)	0.021	0.009 (-0.002–0.02)	0.118
ESA use (Yes vs. no)	0.435 (0.003–0.867)	0.049	1.155 (0.466–1.844)	0.001	0.602 (0.141–1.062)	0.011	1.090 (0.488–1.692)	<0.001
Iron use (Yes vs. no)	-0.017 (-0.381–0.346)	0.926	0.528 (0.015–1.041)	0.044	0.547 (0.165–0.930)	0.005	0.497 (0.109–0.884)	0.012

CKD, chronic kidney disease; DMN, diabetic nephropathy; HN, hypertensive nephropathy; GN, glomerulonephritis; Adj., adjusted; eGFR, estimated glomerular filtration rate; UPCR, urine protein-to-creatinine ratio; WBC, white blood cells; hsCRP, high sensitivity C-reactive protein; TSAT, transferrin saturation; ESA, erythropoiesis stimulating agents. Logarithmic transformations were done for CRP, UPCR and ferritin. Variables with $P < 0.05$ in univariate linear regression analysis (all above with age, sex, smoking status, systolic blood pressure, fasting glucose, albumin, cholesterol, and bilirubin) were chosen as covariates in multivariate linear regression analysis.

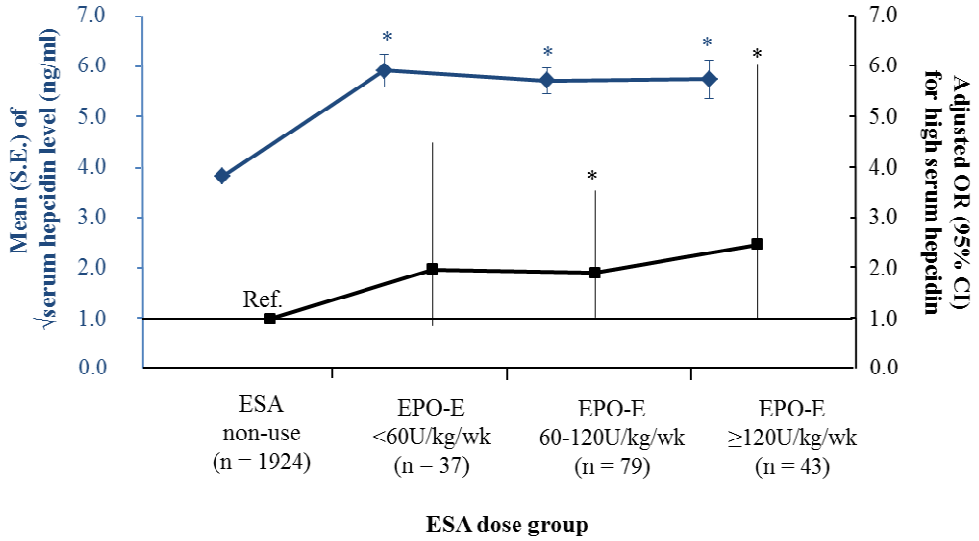


Figure 1.1. Dose relationship between erythropoietin stimulating agents (ESA) usage and serum hepcidin level. S.E., standard error; EPO-E, epoetin-equivalent. * meant $P < 0.05$ when compared to ESA non-use group.

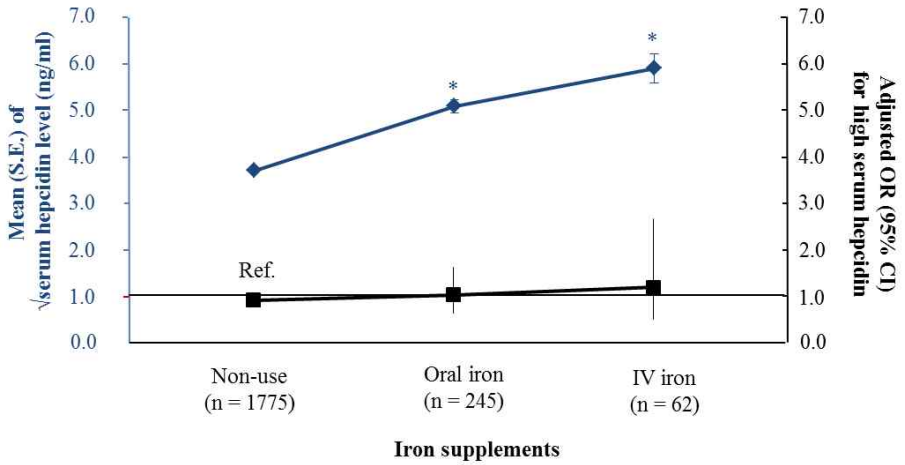


Figure 1.2. Dose relationship between route of iron supplements and serum hepcidin level. S.E., standard error; IV, intravenous. * meant $P < 0.05$ when compared to non-use group.

2. Relative contribution of conventional iron indices and hepcidin on anemia

Of the 2238 patients, 561 were excluded: these included 198 with missing data on serum hepcidin, Hb, TSAT, ferritin, and usage of ESA or iron, and 363 patients who have used ESA or iron. Therefore, 1677 patients were included in this analysis. The mean age of the 1677 patients was 53.5 years and 65.4% were men. The mean eGFR was 54.8 ml/min/1.73 m^2 with a median UPCR of 0.4g/g creatinine. The mean Hb level was 13.2g/dl with a mean TSAT of 31.9%. The median serum levels of hepcidin and ferritin were 11.8 ng/ml and 207.2 pmol/l, respectively. The prevalence of mild, moderate, and severe anemia was 45.1%, 18.8%, and 4.2%, respectively.

We explored the association between serum hepcidin and iron indices, and anemia severity (Table 2.1). Regardless of anemia severity, serum hepcidin and iron indices were significantly associated with anemia in univariate logistic regression analysis. In multivariate logistic regression analysis, however, TSAT and serum hepcidin maintained statistical significance whereas serum ferritin lost statistical significance.

We performed a subgroup analysis according to kidney function to evaluate the association between serum hepcidin and iron indices, and anemia severity (Table 2.2). In patients with early CKD, serum hepcidin

was not associated with anemia, regardless of anemia severity. Among iron indices, only TSAT was significantly associated with anemia, particularly in less severe anemia (Hb <13.0 g/dl and Hb <11.5 g/dl). However, in patients with advanced CKD, serum hepcidin was significantly associated with anemia, particularly in severe anemia (Hb <11.5 g/dl and Hb <10.0 g/dl), whereas the effect of TSAT and ferritin on anemia severity was minimal.

Table 2.1. Association of serum hepcidin and iron indices with anemia severity

Outcomes	Factors*	Univariate		Multivariate	
Hb		OR (95% CI)	*P*	OR (95% CI)	*P*
<13 g/dl	**TSAT (10 %)**	**0.660 (0.602-0.723)**	**<0.001**	**0.701 (0.610-0.807)**	**<0.001**
	Ferritin (10 pmol/l)	0.995 (0.991-0.999)	0.016	1.001 (0.993-1.008)	0.890
	Hepcidin (10 ng/ml)	**1.175 (1.100-1.255)**	**<0.001**	**1.200 (1.054-1.367)**	**0.006**
<11.5 g/dl	**TSAT (10 %)**	**0.671 (0.595-0.756)**	**<0.001**	**0.747 (0.620-0.901)**	**0.002**
	Ferritin (10 pmol/l)	1.001 (0.996-1.005)	0.785	0.992 (0.984-1.001)	0.072
	Hepcidin (10 ng/ml)	**1.316 (1.224-1.413)**	**<0.001**	**1.403 (1.221-1.613)**	**<0.001**
<10 g/dl	**TSAT (10 %)**	**0.765 (0.612-0.956)**	**0.019**	**0.691 (0.484-0.986)**	**0.041**
	Ferritin (10 pmol/l)	1.010 (1.003-1.017)	0.006	0.999 (0.988-1.009)	0.798
	Hepcidin (10 ng/ml)	**1.425 (1.283-1.583)**	**<0.001**	**1.422 (1.180-1.713)**	**<0.001**

Hb, hemoglobin; TSAT, transferrin saturation; OR, odds ratio; CI, confidence interval. OR and its CI were calculated using logistic regression analysis. In multivariate analysis, TSAT, ferritin and hepcidin were entered into together with age, sex, income status, smoking status, body mass index, fasting plasma glucose, systolic blood pressure, estimated glomerular filtration rate, bilirubin, albumin, and urine protein-to-creatinine ratio. * per 10 % increase in TSAT, per 10 pmol/l increase in ferritin, and per 10 ng/ml increase in hepcidin.

Table 2.2 Subgroup analysis according to the kidney function for the association of serum hepcidin and iron indices with anemia severity

Outcomes	Factors*	Estimate glomerular filtration rate			
Hb		≥45 ml/min/1.73m^2(n=919)		<45 ml/min/1.73m^2(n=758)	
		Adj. OR (95% CI)	*P*	Adj.OR (95% CI)	*P*
<13.0 g/dl	**TSAT (10 %)**	**0.628 (0.515-0.765)**	**<0.001**	0.805 (0.648-0.998)	0.048
	Ferritin (10 pmol/l)	0.997 (0.984-1.011)	0.677	1.008 (0.997-1.019)	0.179
	Hepcidin (10 ng/ml)	1.112 (0.887-1.394)	0.357	1.138 (0.946-1.369)	0.172
<11.5 g/dl	**TSAT (10 %)**	**0.672 (0.476-0.950)**	**0.024**	0.791 (0.626-1.001)	0.051
	Ferritin (10 pmol/l)	1.006 (0.983-1.030)	0.592	0.993 (0.984-1.003)	0.166
	Hepcidin (10 ng/ml)	1.060 (0.684-1.642)	0.794	**1.379 (1.173-1.620)**	**<0.001**
<10.0 g/dl	TSAT (10 %)	0.350 (0.116-1.058)	0.063	0.717 (0.477-1.078)	0.110
	Ferritin (10 pmol/l)	1.022 (0.970-1.077)	0.417	1.002 (0.991-1.014)	0.726
	Hepcidin (10 ng/ml)	1.293 (0.377-4.436)	0.683	**1.360 (1.115-1.659)**	**0.002**

Hb, hemoglobin; TSAT, transferrin saturation; Adj. OR, adjusted odds ratio; CI, confidence interval. Adj. OR and its CI were calculated using multivariate logistic regression analysis. TSAT, ferritin and hepcidin were entered into together with age, sex, income status, smoking status, body mass index, fasting plasma glucose, systolic blood pressure, estimated glomerular filtration rate, bilirubin, albumin, and urine protein-to-creatinine ratio. * per 10 % increase in TSAT, per 10 pmol/l increase in ferritin, and per 10 ng/ml increase in hepcidin.

3. Serum hepcidin and progression of CKD

Of the 2238 patients, 328 were excluded: these included 198 with missing data on serum hepcidin, Hb, transferrin saturation, ferritin, and usage of ESA or iron, and 130 patients who missed the follow-up of renal events. Therefore, 1910 patients were included in the final analysis. The mean age of the 1910 patients was 54.2 years and 61.0% were men. The causes of CKD were DMN in 25.6% of patients, HN in 20.1%, GN in 31.1%, and other causes in 23.2%. At enrollment, the median serum hepcidin levels were 13.4 ng/ml. During a mean of 2.4 years, 333 patients developed renal events (17.4%); 165 (8.6%) patients with functional deterioration and 275 (14.4%) patients with dialysis initiation.

We analyzed the association between serum hepcidin and renal events development. As serum hepcidin levels increased, the hazard of renal events development increased in penalized smoothing splines (Figure 3.1). The positive association between serum hepcidin and renal events development was also identified in Kaplan-Meier`s survival curve analysis (Figure 3.2). Compared to the first quartile of serum hepcidin (4.59 years, 95% CI 4.47-4.70), the estimated mean renal survival was similar in the second quartile of serum hepcidin (vs. 4.43 years, 95% CI 4.29-4.57; P = 0.168), but shorter in the third (vs. 4.18 years, 95% CI 4.02-4.35; P <0.001) and the forth quartile (vs. 3.49 years, 95% CI 3.31-3.67; P <0.001) of serum hepcidin. The increased hazard of renal events development with the

increase of serum hepcidin was also maintained in multivariate Cox hazard regression analysis after adjusting for confounders including kidney function, Hb, conventional iron indices and status of ESA or iron usage, and other chronic diseases (Table 3.1).

We performed a subgroup analysis according to the risk factors of renal events development using multivariate Cox proportional hazard regression analysis (Table 3.2). The hazard of increased serum hepcidin levels was evident in diabetic male patients. In addition, the hazard of increased serum hepcidin to develop renal events was obvious only in patients with decreased kidney function. The hazard of increased serum hepcidin levels was also prominent in patients with decreased levels of Hb, TSAT, and ferritin. Serum hepcidin levels were independently associated with the renal events development only in patients with lower hsCRP levels.

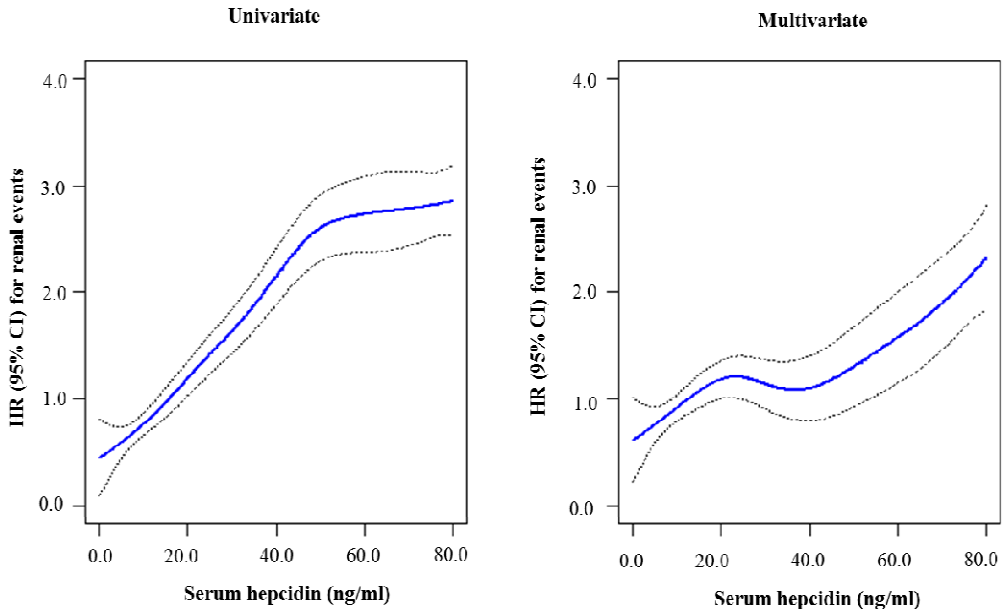
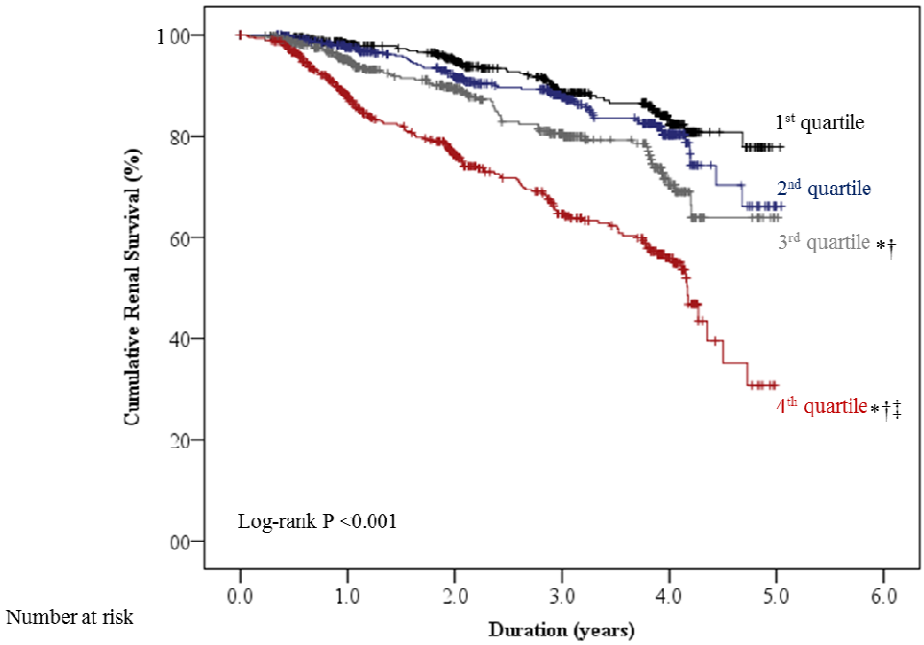


Figure 3.1. Penalized smoothing splines showing the relationship between serum hepcidin and renal events development. HR, odds ratio; CI, confidence interval. The blue line indicated the HR and the black dotted line indicated the 95% CI for which serum hepcidin influences the renal events development. In multivariate analysis, age, sex, serum ferritin, high sensitivity C-reactive protein, transferrin saturation, hemoglobin, usage of erythropoiesis stimulating agents or supplemental iron, causes of chronic kidney disease, systolic and diastolic blood pressure, fasting plasma glucose, smoking status, estimated glomerular filtration rate, bilirubin, albumin, white blood cells, and urine protein-to-creatinine ratio were chosen as covariates.



Number at risk

1st quartile	437	358	268	155	49	1
2nd quartile	442	352	252	150	47	1
3rd quartile	442	294	199	108	30	1
4th quartile	440	302	207	118	38	0

Figure 3.2. Kaplan-Meier survival curve of serum hepcidin quartile for renal events development. *, †, and ‡ meant $P < 0.05$ when compared to the first, second and third quartile of serum hepcidin group, respectively, using log-rank test.

Table 3.1. Cox-proportional hazard regression analysis of serum hepcidin for renal events

	Univariate		Multivariate	
	HR (95% CI)	*P*	HR (95% CI)	*P*
Serum hepcidin quartile				
2Q vs. 1Q	1.299 (0.890–1.894)	0.175	1.290 (0.861–1.932)	0.217
3Q vs. 1Q	1.967 (1.369–2.826)	<0.001	1.514 (1.025–2.237)	0.037
4Q vs. 1Q	3.954 (2.863–5.461)	<0.001	1.752 (1.183–2.596)	0.005

HR, hazard ratio; CI, confidence interval; Q, quartile. HR and its CI were analyzed using Cox proportional hazard regression analysis. In multivariate analysis, age, sex, serum ferritin, high sensitivity C-reactive protein, and variables with P <0.05 in univariate analysis (transferrin saturation, hemoglobin, usage of erythropoiesis stimulating agents or supplemental iron, causes of chronic kidney disease, systolic and diastolic blood pressure, fasting plasma glucose, smoking status, estimated glomerular filtration rate, bilirubin, albumin, white blood cells, and urine protein-to-creatinine ratio) were chosen as covariates.

Table 3.2. Subgroup analysis of the association between serum hepcidin and renal events development

Subgroup	No. of patients	4Q vs. 1Q of serum hepcidin	*P*
		Adjusted HR (95% CI)	
Age	<55 years (n =939)	2.025 (1.089–3.763)	0.026
	≥55 years (n =971)	1.868 (1.059–3.297)	0.031
Sex	Women (n = 745)	1.753 (0.932–3.295)	0.082
	Men (n = 1165)	1.865 (1.087–3.200)	0.024
Diabetes	No (n = 1390)	1.592 (0.969–2.615)	0.067
	Yes (n = 513)	2.102 (1.009–4.378)	0.047
CKD stage	Advanced (n = 1001)	1.718 (1.138–2.592)	0.010
	Early (n = 909)	4.211 (0.738–24.011)	0.106
Hemoglobin	< 12.8 g/dl (n = 952)	1.920 (1.232–2.992)	0.004
	≥12.8 g/dl (n = 958)	0.816 (0.249–2.670)	0.736
ESA/iron use	No (n = 1567)	1.830 (1.090–3.070)	0.022
	Yes (n = 343)	2.068 (1.033–4.141)	0.040
TSAT	<30.3% (n = 948)	2.303 (1.309–4.052)	0.004
	≥30.3% (n = 962)	1.314 (0.704–2.451)	0.391
Ferritin	<223.0 pmol/l(n = 955)	2.451 (1.264–4.750)	0.008
	≥223.0 pmol/l(n = 955)	2.863 (0.968–8.467)	0.057
hsCRP	<5.71 nmol/l(n = 850)	1.992 (1.086–3.652)	0.026

	≥5.71 nmol/l(n = 964)	1.643 (0.948–2.848)	0.077

HR, hazard ratio; CI, confidence interval; Q, quartile; CKD, chronic kidney disease; TSAT, transferrin saturation; ESA, erythropoiesis stimulating agents; hsCRP, high sensitivity C-reactive protein. HR and its CI were analyzed using Cox proportional hazard regression analysis. In multivariate analysis, age, sex, serum ferritin, hsCRP, and variables with P <0.05 in univariate analysis (TSAT, hemoglobin, usage of ESA or iron, causes of CKD, systolic and diastolic blood pressure, fasting plasma glucose, smoking status, estimated glomerular filtration rate, bilirubin, albumin, white blood cells, and urine protein-to-creatinine ratio) were chosen as covariates.

Discussion

Although several studies have evaluated the clinical significance of serum hepcidin in non-dialysis CKD patients, the relationship between kidney function and serum hepcidin levels is still inconclusive [17-27]. In this analysis, investigator found that serum hepcidin levels inversely correlated with kidney function. In multivariate logistic analysis, the association between serum hepcidin and kidney function seemed to be non-linear because the odds for high serum hepcidin was statistically evident from CKD stage 3b, compared to CKD stage 1. Subgroup analysis by CKD stage showed that factors associated with anemia (Hb levels), iron metabolism (TSAT and ferritin levels) and inflammation (hsCRP levels) were associated with serum hepcidin levels, regardless of CKD stages. In contrast, eGFR was associated with serum hepcidin levels only in patients with advanced (stages 3b–5), but not in early (stage 1–3a) CKD, suggesting that the pathogenesis of elevated serum hepcidin may differ in advanced and early CKD, and that decreased renal clearance may significantly contribute to elevated serum hepcidin levels in advanced CKD. However, the meaning of the null-association between kidney function and serum hepcidin levels in patients with DMN and others (mostly ADPKD), is not clarified with this study, and needs to be studied more in the future.

Investigator found that studies reporting kidney function as an independent predictor of serum hepcidin levels have used cELISA [17, 18, 23-25],

whereas studies suggesting kidney function as a confounding factor for serum hepcidin levels have used mass spectrometry (MS) [19-22], when measuring serum hepcidin levels. Therefore, the lack of agreement between studies assessing kidney function as a predictor of serum hepcidin levels may be derived from different assay methods. The bioactive isoform of serum hepcidin is hepcidin-25. However, hepcidin can exist in other isoforms, such as hepcidin-20, 22 and 24 [45]. Although MS can differentiate among hepcidin isoforms, cELISA is poor at differentiating hepcidin-25 from other isoforms [46, 47]. If the association between hepcidin and kidney function is affected by hepcidin isoforms [21], then hepcidin measured by cELISA and by MS may possess different clinical meanings.

Hepcidin and ESA resistance

This study also found that treatment with ESA was significantly associated with increased serum hepcidin levels, particularly in patients with advanced CKD. Moreover, investigator observed a positive relationship between serum hepcidin and ESA dose. The positive association between ESA use and serum hepcidin levels was not affected by causes of CKD. These results differ from those of previous studies. Ashby et al. measured serum hepcidin levels in seven CKD patients as they were starting ESA (20

mg darbepoietin alfa weekly) for the first time. Plasma hepcidin fell from 70.0±4.7 to 60.7±6.0 ng/ml during the first few days (P = 0.045) and remained at similar levels when patients were retested after 2–4 weeks of continued therapy [17]. The findings are confirmed by van der Putten et al. who analyzed EPOCARES trial. In this study, the hepcidin level decreased after 2 weeks of ESA treatment [28].

These previous studies, however, found that ESA affected serum hepcidin level, not vice versa. Unlike these studies, investigator identified the positive association between ESA dose and hepcidin levels. In this study, ESA users showed lower Hb levels despite of higher iron supplement. Also they revealed more iron sequestration (higher serum ferritin despite of similar TSAT) than ESA non-users. Taken together, we assumed that higher hepcidin level has caused the necessity of ESA prescription. To identify the effect of hepcidin on ESA responses, therefore, future prospective studies should compare the ESA response, such as change of Hb and iron indexes, in both high and low serum hepcidin group.

The current study also found that iron supplementation was associated with increased serum hepcidin level, in agreement with previous studies [20, 22]. In addition, investigator observed a positive association between iron supplementation and serum hepcidin levels in patients with early, but not advanced, CKD. In this cohort patients, serum hepcidin levels are about two-fold higher in advanced than in early CKD. This may be the reason why iron supplements was not associated with high (≥25.1 ng/ml) serum

hepcidin.

Hepcidin, iron indices, and anemia severity

Serum ferritin levels were not related to anemia and its severity. Although the serum ferritin levels in CKD patients were still related to hepatosplenic iron stores [48], those in CKD patients did not correlate with bone marrow iron stores [33, 48, 49]. This iron sequestration in CKD patients may nullify the association between serum ferritin and anemia status. The null-association between serum ferritin and anemia status was evident even in patients with early CKD. This implied that the iron sequestration in CKD patients may occur in the early stage of CKD. Hepcidin induces iron sequestration. Serum hepcidin levels in CKD patients are higher than those of healthy volunteer [24, 25]. Taken together, investigator can hypothesize that a small elevation of serum hepcidin even in early CKD may nullify the effect of serum ferritin on Hb levels.

In the current study, unlike ferritin, TSAT was independently related to anemia and its severity, which was in accordance with the fact that TSAT is more predictive of bone marrow iron contents [33] and Hb response after intravenous iron replacement [50] than ferritin in CKD patients. Serum hepcidin was also significantly associated with anemia of several severities in non-dialysis CKD patients. In subgroup analysis according to the CKD stages, TSAT was associated well with less severe anemia in patients with

early CKD, whereas serum hepcidin was significantly related to more severe anemia in patients with advanced CKD. These suggest that TSAT is a major determinant of anemia in early CKD patients, whereas serum hepcidin is a key determinant of anemia in advanced CKD patients.

Hepcidin and CKD progression

As serum hepcidin levels increased, the hazard of renal events development increased steadily as demonstrated in penalized smoothing splines analysis. The hazards to develop renal events of the third and the forth quartiles of serum hepcidin were 1.51-times and 1.75-times higher than that of the first quartile of serum hepcidin, respectively. To our knowledge, this was the first study which showed the independent hazard of serum hepcidin on future renal events development. Although the exact mechanism has not been fully elucidated, investigator postulated that the intimate association between serum hepcidin levels, inflammation, cardiovascular risk factors and disturbed homeostasis in anemia and iron metabolism [13, 16] may explain the harmful impact of increased serum hepcidin on the progression of CKD partly. However, the dangerous effect of serum hepcidin on the progression of CKD was independent from those confounding factors. We assumed that increased oxidative stress, mitochondrial dysfunction, and increased arterial stiffness shown in subjects with increased serum hepcidin

levels could be a good explanation for the possible underlying mechanisms [51, 52].

In subgroup analysis, diabetic patients are at increased risk of CKD progression by increased serum hepcidin levels than non-diabetic patients. Men are affected more by increased serum hepcidin levels than women. Investigator also identified that patients with disturbed homeostasis in anemia and iron metabolisms (lower levels of Hb, TSAT, and ferritin) were influenced more by increased serum hepcidin levels. The harmful effect of increased serum hepcidin was evident in patients with lower levels of eGFR and hsCRP.

Limitations

The study had several limitations. First, as the study was observational, a causal relationship cannot be accurately determined and the results need to be interpreted carefully. Second, investigator measured serum hepcidin by cELISA only, which cannot distinguish among hepcidin isoforms. Since there have been no known methods which provide best clinical implication yet, additional studies are required to determine more accurate methods of measuring hepcidin level and to determine the significance of hepcidin isoforms other than hepcidin-25 [53]. Third, investigator did not have data

on the causes of anemia because KNOW-CKD was not designed specifically for anemia in CKD. Fourth, there were no data from healthy volunteers and patients with ESRD on dialysis. Fifth, a mean of 2.4 years was relatively short. Therefore, long-term effect of increased serum hepcidin needs to be analyzed in the next studies. Finally, although our study included a large number of patients, they were from a single nation with a single ethnic group, limiting the generalizability of our results.

Conclusions

Decreased kidney function was associated with increased serum hepcidin levels, especially in patients with advanced CKD. Decreased Hb levels and higher levels of iron markers were also associated with higher serum hepcidin levels. Iron supplementation was positively correlated with serum hepcidin levels, especially in patients with early CKD. The higher hepcidin level in ESA users, particularly in those with advanced CKD, suggests that hepcidin is a key peptide in ESA resistance. Ferritin was not associated with anemia, regardless of the severity of anemia and the level of kidney function. TSAT was associated with less severe anemia in patients with early CKD, while serum hepcidin was associated with more severe anemia in patients with advanced CKD. Increased serum hepcidin levels independently predict the progression of CKD in non-dialysis CKD patients.

Diabetic male patients with anemia and iron deficiency tended to be affected more by the increased serum hepcidin levels. The results of this study may prompt future longitudinal studies on the clinical significance of serum hepcidin, measured by cELISA, in non-dialysis CKD patients.

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초록

만성콩팥병 환자의 임상 경과에 혈청 **hepcidin**이 미치는 영향에 대한 연구

배경

빈혈은 만성콩팥병 환자들에게 흔하며 심혈관 질환, 입원 및 사망의 위험도를 높이므로 적절한 지표를 통해 만성콩팥병 환자의 빈혈을 잘 감시하고 치료하는 것은 매우 중요하다.

혈청 hepcidin은 체내 철 이용을 억제하는 물질로 주로 콩팥에서 배설되므로 콩팥기능이 감소하면서 증가한다. 만성콩팥병 환자에서 hepcidin 이 증가하면 저장철이 충분함에도 불구하고 철분 사용이 원활하지 않아 빈혈이 발생하며, 이런 경우 기존에 사용하던 페리틴은 빈혈에 대한 좋은 지표가 될 수 없다. 또한 만성콩팥병 환자에서는 콩팥에서 생성되는 조혈호르몬이 감소되어 빈혈이 생기므로 치료를 위해 조혈호르몬을 투여하는데, 조혈호르몬 저항성이 있는 경우에는 빈혈이 잘 교정되지 않아 문

제가 된다. 특히, 당뇨병과 같은 경우 이런 조혈호르몬 저항성이 더욱 있을 것으로 생각되며, 원인질환에 따라 신장기능 감소에 따른 hepcidin 증가정도가 다르고, 이로 인한 조혈호르몬 저항성에도 차이가 있을 것으로 생각된다. 소규모 단일 기관 연구들에서 hepcidin이 페리틴 보다 철분의 지표로 우월하며 hepcidn 이 조혈호르몬 저항성과 관련이 있다는 보고를 했으나 아직까지 대규모 다기관 코호트에서 확인된 바는 없다. 또한, 만성콩팥병 환자에서 hepcidin 이 콩팥병의 진행에 미치는 영향에 대해서는 아직까지 연구된 바가 없다.

이에 본 연구자는 대규모 다기관 코호트 자료를 이용하여 기존의 hepcidin의 임상적 특징 및 조혈호르몬 저항성과의 관련성을 확인하고, 혈청 hepcidin이 만성 콩팥병의 진행에 미치는 영향에 대해 알아보고자 본 연구를 진행하게 되었다.

방법

2011년부터 2016년까지 성인 만성콩팥병 1기-투석 전 5기 까지의 환자들 2238 명의 환자들이 등록된 다기관 전향적 코호트의 기본 자료를 분석했다. 대상자의 평균나이는 54.2세였다. 2238명중 혈청 hepcidin 값을 측정한 2113명을 대상으로 연구를 진행했다. 트랜스페린 포화도와 페리틴을 철 지표로 사용했다. 빈혈은 남성은 혈색소 13.0 g/dl 이하, 여성은 12.0 g/dl 이하로 정의했고, 경도, 중등도, 중증의 빈혈은 각각 혈색소 13.0 g/dl 이하, 11.5 g/dl 이하, 10.0 g/dl 이하로 정의하였다. 코호트 환자

들은 1년마다 중요 임상지표들을 추적관찰 했으며, 만성 콩팥병의 진행은 신기능의 50% 이상의 감소, 크레아티닌의 2배 상승 혹은 투석의 시작으로 정의하였다.

결과

혈청 hepcidin 과의 관계에 있어서 혈액 내 염증수치와 철 지표들은 만성콩팥병의 병기와 무관하게 양의 상관관계를 보였다. 그러나 사구체여과율은 콩팥 기능이 저하된 환자들에서만 (만성 콩팥병 3b-5기) 통계적으로 유의한 음의 상관관계를 보였다. (beta -0.007, P < 0.001) 철분 치료 여부는 콩팥 기능이 유지된 환자들에서만 (만성 콩팥병 1-3a기) 혈청 hepcidin 값과 양의 상관관계를 보인 반면 (beta 0.306, P = 0.001), 조혈호르몬 사용은 콩팥 기능이 저하된 환자들에서만 (만성 콩팥병 3b-5기) 독립적인 양의 상관관계를 보였다 (beta 0.802, P < 0.001). 원인 질환별 분석에서, 콩팥 기능은 고혈압성 신병증 및 사구체 신염 환자에서 통계적으로 유의한 음의 상관관계를 보였다. 조혈 호르몬의 사용은 원인 질환과 무관히 혈청 hepcidin값과 양의 상관관계를 보였다.

트랜스페린 포화도와 혈청 hepcidin은 빈혈의 중등도와 상관없이 빈혈과 관련성이 있었지만, 혈청 페리틴은 빈혈과 관련성이 없었다. 만성 콩팥병 1-3a기의 환자에서 트랜스페린 포화도의 10%의 상승은 중증 빈혈 (오즈비 0.628, 95% 신뢰구간 0.515-0.765; P < 0.001)과 중등도 빈혈 (오즈비 0.672, 95% 신뢰구간 0.476-0.950; P = 0.024)와 관련성이 있었다.

그러나, 만성 콩팥병 3b-5기의 환자에서는 10 ng/ml의 혈청 hepcidin의 상승이 경도 빈혈 (오즈비 1.360, 95% 신뢰구간 1.115-1.659; P=0.002)와 중등도 빈혈 (오즈비 1.379, 95% 신뢰구간 1.173-1.620; P <0.001)과 관련성을 보였다.

코호트 환자들의 평균 관찰 기간은 2.4 년이었고, 관찰에서 탈락하지 않은 1910명 중 333명의 환자에서 만성 콩팥병의 진행이 발생했다 (17.4%). 신기능의 50% 감소 및 크레아티닌 2배 상승에 해당되는 환자는 165명 (8.6%) 이었고, 투석을 시작한 환자는 275명 (14.4%) 이었다. 혈청 hepcidin 값이 증가함에 따라 만성 콩팥병 진행의 위험도는 지속적으로 증가했고 다변량 콕스 비례위험모형에서 hepcidin 1 사분위수에 비해 3 사분위수와 4 사분위수의 위험비 (95% 신뢰구간)는 각각 1.514 (1.025-2.237, P = 0.037)과 1.752 (1.183-2.596, P = 0.005)이었다. 세부 분석에서 당뇨가 있는 남성이면서 혈색소 값, 트랜스페린 포화도, 페리틴이 낮고, 콩팥 기능이 저하되어 있고, 염증수치가 낮은 환자들의 경우 혈청 hepcidin이 높았을 때 만성 콩팥병이 진행할 위험이 더 높았다.

결론

본 연구에서 신기능과 혈청 hepcidn 값이 독립적인 음의 상관관계가 있음을 대규모 코호트를 통해 확인했다. 혈청 hepcidin 값은 조혈 호르몬을 높은 용량으로 사용한 환자에서 높았는데, 이는 hepcidin이 조혈 호르몬 저항성에 기여하고 있음을 시사한다. 본 연구에서 페리틴은 만성 콩팥병

환자에서 빈혈과 관련성이 없었지만, 트랜스페린 포화도는 초기 콩팥병 환자들에 있어서 비교적 경한 빈혈과 관련성을 보였고 혈청 hepcidin은 진행성 콩팥병 환자들에서 중증의 빈혈과 관련성을 보였다. 혈청 hepcidin 과 콩팥병 진행에 대한 연구에서는 초기 혈청 hepcidin 값이 높을수록 향후 만성 콩팥병 진행의 위험이 증가함을 확인했고, 이런 hepcidin의 위험도는 당뇨가 있는 남성에서 콩팥기능이 저하되어 있고, 철 결핍과 빈혈이 있으며 염증수치가 낮을 때 분명했다. 헵시딘이 콩팥병 진행 및 사망률 등에 미치는 영향에 대해서는 추가적인 추적자료가 필요할 것으로 생각된다.

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주요어: hepcidin, 빈혈, 조혈호르몬 저항성, 트랜스페린 포화도, 페리틴, 진행, 만성 콩팥병

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