Am J Transl Res 2022;14(10):7520-7527
www.ajtr.org /ISSN:1943-8141/AJTR0145026

Original Article

Circulating hepcidin and its associations with low-grade inflammation and iron indices among Arab adults with and without T2DM

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Received June 29, 2022; Accepted October 10, 2022; Epub October 15, 2022; Published October 30, 2022

Abstract: We investigated the correlations of serum and dietary intake of iron with low-grade inflammation as well as with circulating hepcidin in adult Arabs with or without type 2 diabetes mellitus (T2DM). Three hundred and twelve (N=312) Saudi adult males and females with a mean age of 56.3 ± 6.5 years were included and divided into two groups, the control group (n=151, 43 males, 108 females), and T2DM group (n=161, 58 males, 103 females). Data included demographic characteristics, medical history, and dietary intake using food frequency and a 24-hour dietary recall for 1 day. Anthropometric measurements were noted and fasting blood samples extracted for the analysis of glucose, lipids, iron indices, hepcidin, 25(OH)D and endotoxin using commercially available assays. Hepcidin levels among T2DM participants were significantly higher than the control group (P<0.001). In all participants, serum hepcidin was positively associated with WHR, HbA1c, TG and TSAT while inversely associated with LDL-C and ferritin. Using hepcidin as dependent variable and age, anthropometrics, blood pressure, glucose, lipids, 25(OH)D, serum iron, transferrin and ferritin as independent variables showed that only glucose and WHR significantly predicted hepcidin by as much as 33.5% of the variances perceived (P<0.001). Sub-analysis in female participants revealed that endotoxin, iron and 25(OH)D were significant predictors of hepcidin, predicting 26.8% of the variances perceived (P<0.001). To conclude, the present study suggests that hepcidin is significantly linked with major cardiometabolic parameters, while its influence in iron indices, including low grade inflammation, appears to be stronger in females.

Keywords: Hepcidin, metabolic disorders, lipopolysaccharides, T2DM

Introduction

Low-grade inflammation caused by moderate increases in plasma concentration of LPS is known as a triggering factor in obesity-induced insulin resistance (IR). It has been shown that moderate increases in plasma concentration of LPS are associated with metabolic disorders that occur in dietary high-fat-induced obesity [1]. LPS (endotoxin) are located in the external layer of gram-negative bacteria, which are large molecules composed of lipid and polysaccharide [2]. Gut-derived endotoxin is the likely primary cause of sub-clinical inflammation contributing to metabolic diseases, with accumulating evidence showing elevated levels of endotoxin in individuals various metabolic disorders including premature aging, fatty liver, obesity, type 2 diabetes mellitus (T2DM) and coronary artery disease [3-9].

Hepcidin is a hepatic peptide hormone produced in several other cell types that regulate iron homeostasis and it also acts as an antimicrobial peptide. It is secreted in response to iron load and inflammation. It regulates iron metabolism via ferroprotein, found on the macrophages and duodenal enterocytes, triggering its degradation and hence regulating absorption of iron [10]. Hepcidin has been shown to be associated with metabolic diseases such as polycystic ovarian syndrome (PCOS), obesity and

obesity-associated T2DM, and insulin resistance IR in humans [11-14]. The main objective of this study is to demonstrate the associations of serum and dietary intake of iron with low grade inflammation and hepcidin level in participants with (T2DM, MetS) and controls. Arguably, this investigation is the first to associate low grade inflammation with iron indices in patients with varying levels of insulin resistance.

Materials and methods

Study design and participants

In this cross-sectional study, 312 Saudi adults (101 males and 211 females) aged 56.3 ± 6.5 years were recruited from King Salman Social Center (KSSC), King Salman Hospital (KSH), King Khalid University Hospital (KKUH) and selected schools, before commencement, each participant provided informed consent. Informed consent was obtained prior to inclusion. They were equally divided into two groups (control group with apparently healthy individuals, and the patients' group with T2DM). Ethical approval (Ref. No.: KSU-SE-18-22) was obtained from the Institutional Review Board, College of Medicine, King Saud University (KSU), Riyadh, Saudi Arabia. A generalized questionnaire was requested to be filled out by all participants in order to obtain demographic and medical history information. Exclusion criteria included conditions that require immediate medical attention like diseases for infections and acute complications from other medical conditions. Personal history was obtained from all participants including age and medical history.

Dietary data were collected from the participants during their visit using the food frequency and 24-hours questionnaire during the 3 days of the week with one day during the weekend. To facilitate quantification, standard measurements such as cups, spoons and plates were used, and we also used photos of some products for illustration. Analysis of food consumption to quantitatively determine macro and micronutrient intake was done using the Esha food processor software (version 11.7, Esha Research, Salem, OR, USA), as described previously [15].

Sample analysis

Fasting blood samples (>8 hours) were collected for each participant and immediately transferred to non-heparinized tubes. After that, centrifugation for all samples was done (5000 rpm for 10 minutes). All samples were at -80°C at the Chair for Biomarkers of Chronic Diseases, KSU, Riyadh.

Biochemical assessment of fasting lipids, glucose and calcium were done using routine autoanalyzer (Konelab, Espoo, Finland). Point-of-care (POC) devices (AccuCheck Active, Roche Diagnostics, Mannheim, Germany) were used to assess levels of HbA1c. Hepcidin and transferrin were measured using ELISA according to manufacturer instructions (R&D Systems, MN, USA) with 6.9% and 9% (intra- and inter-assay coefficients of variations respectively). Commercial assays (Biovendor, Karasek, Czech Republic) were used to assess circulating ferritin (intra- and inter-assay CVs were 6.9% and 9%, respectively). Serum LPS levels were quantified in female participants using Limulus Amebocyte Lysate (LAL) assay kit (QCL 1000, Lonza, MD, USA) and total serum 25(OH)D was measured using commercial electrochemiluminescence immunoassay as done in previous investigations [16, 17]. Intra- and inter-assay coefficients of variations were 4.6% and 5.3%, respectively (Roche Diagnostics, Penzberg, Germany).

Serum iron and total iron-binding capacity (TIBC) levels were determined using spectrophotometer (Spectra Max® M5, Molecular Devices LLC, US). Transferrin saturation (%) was calculated using the general formula: Transferrin saturation (%) = [Serum iron (μg/L)/TIBC (μg/L)] × 100. Transferrin was measured using ELISA.

Statistical analysis

Data were analyzed using SPSS (version 22 Chicago, IL, USA). Continuous normal data were presented as mean ± standard deviation (SD) while non-normal variables were presented as median (1^{st}-3^{rd} quartiles). Categorical variables were presented as n (%). Non-normal variables were log-transformed prior to analysis. Student T-test and Mann Whitney U test were used to compare differences in measured continuous variables. Pearson's and spearman correlation analysis were used to test associations. Stepwise linear regression analysis was done using hepcidin as dependent variable and all parameters of interest to determine significant predictors hepcidin in males and females. A *p*-value <0.05 was considered significant.

Table 1. Clinical characteristics of participants

Parameters	Control	T2DM	*P*-Value
N (M/F)	151 (43/108)	161 (58/103)	
Age (year)	55.8 ± 6.5	56.8 ± 6.5	0.19
BMI (kg/m^2)	31.2 ± 5.1	32.2 ± 5.2	0.08
Waist-Hip Ratio	0.88 ± 0.10	0.89 ± 0.10	0.26
Systolic BP (mmHg)	131.7 ± 12.2	131.9 ± 12.9	0.89
Diastolic BP (mmHg)	83.0 ± 6.7	82.1 ± 8.2	0.27
Glucose (mmol/l)	5.9 ± 0.7	13.2 ± 4.6	<0.001
HbA1c (%)	5.5 ± 0.7	6.7 ± 1.9	<0.001
Total Cholesterol (mmol/l)	5.2 ± 1.6	5.1 ± 1.4	0.62
HDL-Cholesterol (mmol/l)	1.08 ± 0.3	1.07 ± 0.3	0.95
LDL-Cholesterol (mmol/l)	3.4 ± 1.7	3.0 ± 1.5	0.02
Triglycerides (mmol/l)#	1.3 (0.9-1.9)	2.1 (1.6-2.8)	<0.001
25(OH) D (nmol/l)	34.1 (24.5-47.6)	35.3 (25.7-46.7)	0.71
Serum Iron (ug/l)	428.7 (345-588)	435.5 (322-599)	0.67
TIBC (ug/l)	1212 (831-1897)	986 (686-1580)	0.02
Transferrin (ug/ml)	278.5 (192-383)	310 (205.8-431)	0.27
TSAT (%)	35.5 (25.5-57.5)	46.2 (28.2-63.4)	0.08
Ferritin (ng/ml)	44.1 (29.3-63.7)	37.2 (20.9-76.1)	0.16
Hepcidin (ng/l)	3.0 (0.9-7.8)	5.6 (1.6-10.4)	<0.001
Endotoxin (IU/ml)*	2.0 ± 0.4	2.2 ± 0.2	0.046

Note: Data presented as mean ± SD for normal variables # while median (1st-3rd quartile) for non-normal variables; N = number, BMI = body mass index, BP = blood pressure, TIBC = total iron-binding capacity, and TSAT = Transferrin saturation; *measured only in female participants. Significant at P<0.05.

Results

Table 1 shows the clinical characteristics of participants according to T2DM status. Females outnumbered males by 2:1 [N=211 females (108 controls, 103 T2DM), N=101 males (43 controls, 58 T2DM)]. While there were no differences in age and anthropometric parameters between groups, the mean BMI of most participants fell within the obesity category. As expected, glycemic parameters were significantly higher in the T2DM than control group (*p*-values <0.001). With regards to lipids, LDL-C was significantly higher among controls than the T2DM group (P=0.02), while TG was significantly higher in the T2DM group than controls (P<0.001). Among the iron indices, TIBC was significantly higher among controls than T2DM participants (P=0.02), while hepcidin was significantly higher in the T2DM group than controls (P<0.001). Lastly, endotoxin was modestly but significantly higher among T2DM subjects than controls (P=0.046). The rest of the comparisons are shown in **Table 1**.

Table 2 shows no differences in the dietary intake of both groups in both macro- and macronutrients. Worthy of note is that the energy intake (kcal) was twice the normal and even higher among controls, although not significant. No significant differences were also seen when both groups were stratified according to gender.

Table 3 shows only the significant correlations between hepcidin and metabolic parameters assessed. In all subjects, circulating hepcidin was positively associated with WHR, HbA1c, TG and TSAT while inversely associated with LDL-C and ferritin. When stratified according to sex, hepcidin showed significant positive associations with glucose, HbA1c and TG and significant inverse association with SBP in males. In females, hepcidin was significantly associated with iron, TIBC and endotoxin. When stratified according to T2DM status, the control group showed significant positive associations between WHR and iron, and inverse associations with HbA1c and transferrin. Circulating hepcidin in the T2DM group showed significant positive associations only in WHR and HbA1c, and significant inverse associations in TC, LDL-C, TIBC and ferritin. When further stratified according to sex and T2DM status, no associations between hepcidin and all parameters were found in the T2DM males. In T2DM controls, hepcidin was inversely associated with age and SBP. In control females, hepcidin was positively associated with 25(OH)D, iron, TIBC and endotoxin. Consequently, hepcidin was inversely associated with transferrin also in control females. Lastly, in T2DM females, transferrin was significantly associated with hepcidin (**Table 3**).

Table 4 shows only the significant associations of circulating hepcidin with dietary intake. With the exception of riboflavin, hepcidin was inversely associated with all dietary parameters in the T2DM group (mostly *p*-values <0.01) and no

Table 2. Dietary intake of participants with and without T2DM

Parameters	Control	T2DM	*P*-Value
N (M/F)	151 (43/108)	161 (58/103)	
Water (ml)	3241 (1184-6554)	2642 (133-5565)	0.13
Fat (g)	295 (66.1-650.9)	212 (36-466)	0.14
Protein (g)	212.1 (62.2-388)	150 (17.3-319.6)	0.12
CHO (g)	575.8 (171.9-1183)	422.6 (41.6-936)	0.10
Fiber (g)	35.5 (9.8-89.9)	28.7 (3.2-68.5)	0.14
Ash (g)	31.5 (8.7-53.7)	19.8 (2.1-48.2)	0.14
Energy (Kcal)	4997 (1513-9610)	3753 (483-7615)	0.09
Retinol (μg)	1776 (485-3653)	1258 (157-3228)	0.19
B-Carotene (μg)	2881 (961-7447)	2565 (200-7093)	0.24
Thiamine (mg)	2.9 (1.3-5.7)	2.1 (0.9-4.5)	0.09
Riboflavin (mg)	8.6 (3.4-14.2)	6.5 (2.7-13.8)	0.12
Vitamin C (mg)	123.5 (32.7-360.4)	91 (8.3-281.6)	0.14
Sodium (mg)	5876 (1463-14705)	4394 (327-12163)	0.23
Potassium (mg)	8250 (2639-23658)	7496 (425-17942)	0.15
Calcium (mg)	447 (70.6-1545)	351 (11.5-1193)	0.14
Phosphorous (mg)	4231 (1563-8862)	3085 (228-7172)	0.15
Iron (mg)	58.1 (15.2-151.9)	40.6 (2.9-139.5)	0.20
Vitamin B12 (μg)	17.2 (4.7-37.3)	13.9 (2.7-33.6)	0.41
Vitamin D (μg)	7.4 (2.5-17.5)	5.6 (1.3-14.5)	0.13

Note: Data presented as median (1^{st}-3^{rd} quartile). Significant at P<0.05.

correlations were elicited in the controls. Interestingly, hepcidin was not associated with any dietary intake parameters in females regardless of T2DM status. In male controls, hepcidin was associated with dietary retinol, calcium and vitamin B12. Lastly, in T2DM males, serum hepcidin was inversely and significantly associated with dietary fat, vitamin C and iron (**Table 4**).

Furthermore, stepwise regression analysis for all subjects was performed using hepcidin as dependent variable and measured parameters as independent variables (age, anthropometrics, blood pressure, glucose, lipids, 25(OH)D, serum iron, transferrin and ferritin) and showed that only glucose and WHR significantly predicted hepcidin by as much as 33.5% of the variances perceived (P<0.001). Sub-analysis in female participants revealed that endotoxin, iron and 25(OH)D were significant predictors of hepcidin, predicting 26.8% of the variances perceived (P<0.001).

Discussion

The major findings of this cross-sectional study are that circulating hepcidin levels associated with both glycemia and visceral adiposity (WHR) independent of T2DM status, and that in females, the association with low grade inflammation via levels of endotoxin and vitamin D were significant predictors of hepcidin. The obtained data showed an inverse significant correlation between hepcidin among the controls and HbA1c (r=-0.19) and transferrin (r=-0.29, P<0.05), while data shows a significant positive relationship with serum iron (r=0.21, P<0.05). Thus, the elevated hepcidin levels in our participants suggest a physiologic response to excess liver iron which is consistent with several studies [12, 18-21] but not all [13, 22-24].

The liver has an important function in the control of iron metabolism through the hormone hepcidin. Under normal conditions, hepcidin regulates iron status, maintaining iron homeostasis [25]. HAMP gene regulates hepcidin synthesis by pathways such as BMP-SMAD (bone morphogenetic protein-sons of mothers against decapentaplegic) and STAT3. On the other hand, iron-transferrin complex (Fe2-Tf) regulates hepcidin hepatocyte transcription. Hepcidin synthesis is hindered under iron deficient conditions secondary to Fe2-Tf complex binding to transferrin receptor-1 (TfR1) as this does not activate the BMP-SMAD pathway. In contrast, transferrin bonds with iron at elevated iron levels, resulting in more Fe-bound transferrin (holo-transferrin). In this case, Fe2-Tf binds to transferrin-2 (TfR2) and forms a complex that activates BMP [26].

Hepcidin binds to the ferroportin, resulting in degradation and suppression of enteric iron release as well as from macrophages and hepatocytes [27]. Ferroportin degradation requires binding of hepcidin to its ferroportin receptor [28]. The binding depends on cysteine in the extracellular ferroportin at position 326. In this position, as in the ferroportin variant, the lack of this amino acid makes hepcidin unable to bind, causing iron overload [29].

In addition, in male controls, an inverse significant correlation between hepcidin with age and systolic BP was observed. Suárez-Ortegón et al.

Table 3. Significant associations between hepcidin and metabolic variables

Parameters	All	Gender		Group		Males	Females	
		Males	Females	Ctrl	T2DM	Ctrl	Ctrl	T2DM
N	312	101	211	151	161	43	108	103
Age (year)						-0.30*		
WHR	0.32**			0.29**	0.34**			
SBP (mmHg)		-0.41**				-0.44**		
Glucose (mmol/l)		0.37**						
HbA1c (%)	0.21**	0.35**		-0.19*	0.42**			
T. Chol (mmol/l)					-0.26**			
LDL-C (mmol/l)	-0.13*				-0.27**			
Trigly (mmol/l)#	0.13*	0.30**						
25(OH) D (nmol/l)							0.27*	
Iron (ug/l)			0.25**	0.21*			0.33**	
TIBC (ug/l)			0.18*		-0.29**		0.32**	
Transferrin (ug/ml)				-0.29**			-0.28**	0.23*
TSAT (%)	0.18**							
Ferritin (ng/ml)	-0.16*				-0.23*			
Endotoxin (IU/ml)			0.27*				0.33*	

Note: Only significant coefficients (R) were presented, #represented log transform parameters; *denotes significance at 0.05 level; **denotes significance at 0.01 level. N = number, WHR = waist-to-hip ratio, SBP = Systolic Blood Pressure, T. Chol = Total cholesterol, Trigly = Triglycerides, TIBC = total iron-binding capacity, and TSAT = Transferrin saturation.

Table 4. Significant correlations of hepcidin (ng/l) with dietary intake

Parameters	All	Group type	Males	
		T2DM	Control	T2DM
N	312	161	43	58
Water (ml)	-0.14*	-0.27**		
Fat (g)		-0.20*		-0.32*
Protein (g)	-0.14*	-0.26**		
CHO (g)		-0.23**		
Fiber (g)	-0.13*	-0.21*		
Ash (g)	-0.13*	-0.25**		
Energy (Kcal)	-0.14*	-0.26**		
Retinol (μg)	-0.16*	-0.28**	0.44*	
B-Carotene (μg)	-0.13*	-0.24**		
Thiamine (mg)	-0.14*	-0.20*		
Riboflavin (mg)	-0.15*			
Vitamin C (mg)	-0.21**	-0.31**		-0.32*
Sodium (mg)	-0.15*	-0.26**		
Potassium (mg)	-0.16*	-0.29**		
Calcium (mg)	-0.16*	-0.23*	0.45*	
Phosphorous (mg)	-0.15*	-0.27**		
Iron (mg)	-0.23**	-0.37**		-0.29*
B12 (μg)		-0.22*	0.36*	
Vitamin D (μg)	-0.19**	-0.33**		

Note: Data presented as coefficient (R), *denotes significance at 0.05 level; **denotes significance at 0.01 level. CHO = carbohydrates.

showed the same correlation between SBP and hepcidin in men and that more future research is needed to conclude a causal relationship; however, this observation is not consistent with these results [22].

The inverse significant correlation between hepcidin in female controls and transferrin is consistent with Tussing-Humphreys et al. (2010) [30], and inconsistent with results of Young et al. (2009) [31], who reported no correlations between hepcidin and transferrin, and the small sample size may be the reason for this result. While a positive significant relationship with 25(OH)D, iron, TIBC and endotoxin are consistent with previous works [30, 32]. The positive correlation between hepcidin and endotoxin can also be supported with study of Kemna et al. (2005) [33] who elucidated that the rapidity of the response of hepcidin could be attributed to its proposed role as a hypoferremia inducer that would limit the flow of critical iron to infecting microbes and delay their multiplication in tissue. Growth of

many microbes and pathogenicity require the presence of iron [34]. Protein-bound iron is not readily accessible for microbial absorption, as is the case for ferritin, transferrin, lactoferrin or ovotransferrin. Hepcidin reduces the level of blood iron by ferroportin degradation to protect against infection. An increase in hepcidin lowers blood iron levels, which can be beneficial in protecting against iron-dependent microbes [35].

Another stimulator of hepcidin synthesis is known to be inflammation through activin-B (Act-B) and interleukin-6, through BMP and Janus kinase2-signal activator and trans-ducer of transcription-3 (JAK2-STAT3) [36]. The synthesis of hepcidin is enhanced during infection or inflammatory cycles. In such states, the liver production of hepcidin is regulated via the signaling pathway of STAT-3 [35]. Hepcidin expression is increased by LPS via the IL-6/STAT3 axis, in study of You et al. (2017) treatment with LPS led to a major activation in these brain tissues of hepcidin mRNA expression [37].

There was no correlation between hepcidin and iron among female controls, which supports the work of Young et al. (2009) who proved an inverse correlation between hepcidin and iron absorption from food or supplements in healthy women. Total iron reserves are almost 4 g with 1-2 mg of daily iron loss to be replenished by dietary iron absorption [31]. Erythropoiesis needs 20-25 mg of daily iron, mostly coming from erythrophagocytosis. Due to iron loss in menstruation, daily dietary iron requirements vary according to age and sex, especially in young women. Iron absorption and plasma availability are controlled by the regulation of hepcidin [35].

The inverse correlation between retinol and hepcidin is consistent with results of recent work done by la Cruz-Góngora et al. who observed that retinol levels were correlated with hepcidin levels [38]. According to that study, the ability of vitamins A and D to regulate hepcidin expression is unknown, and vitamin D was proposed as a potent modulator of hepcidin production [38]. The inverse association between hepcidin and 25(OH)D has been shown in other studies where hepcidin levels decreased after taking supplements [39]. In hepatocytes, vitamin D has been observed to inhibit HAMP gene expression. High-dose vitamin D supplementation decreases serum levels of hepcidin in healthy adults [40]. Hepcidin production is enhanced due to extra plasma iron, ultimately preventing dietary iron uptake, thereby avoiding further iron absorption [41].

The authors acknowledge some caveats including the cross-sectional design and small sample size. Endotoxin was only measured in females and as such the low-grade inflammation findings cannot be applied to men and needs to be investigated in a separate study. Other important parameters including creatinine, albumin, erythropoietin and interleukin 6 were not assessed, and these are known mediators of hepcidin and iron metabolism that are altered in hepatic damage [42], which in the case of T2DM in the present study, are endotoxin-mediated resulting in low-grade inflammation.

Conclusions

In summary, hepcidin levels were observed to be higher in the T2DM group compared to age- and BMI-matched controls. While both glucose and WHR significantly predicts circulating levels of hepcidin independent of T2DM status, the association with low grade inflammation together with iron indices appears to be stronger in females. Further prospective investigations are warranted as to whether lifestyle modifications altering obesity and insulin resistance can favorably affect hepcidin levels and iron indices.

Acknowledgements

The authors extend their appreciation to the Deputyship for Research and Innovation, Ministry of Education in Saudi Arabia for funding this research work through project number IFKSURG-2-27.

Disclosure of conflict of interest

None.

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