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Hepcidin expression and iron parameters change in Type 2 diabetic patients

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ABSTRACT

Aim: Iron may contribute to the pathogenesis of Type 2 diabetes mellitus (DM). The aim of this study was to determine iron regulator hepcidin and iron metabolic parameters in Type 2 DM patients, the relationships among them were evaluated in this specific sub-groups.

Materials and methods: The study included sixty-four people: 34 cases of diabetes and 30 age-matched controls. Serum hepcidin, IL-6, hsCRP, ferritin, sTfR, EPO as well as other clinical parameters were detected, and the associations between hepcidin levels and iron/inflammatory parameters were analyzed in diabetes and the controls.

Results: Serum ferritin and hepcidin levels in diabetic patients were significant higher than the controls ($p < 0.001$ respectively). A positive correlation between hepcidin and ferritin, as well as between ferritin and IL-6 levels was existed in diabetes and the control groups ($p < 0.001$ respectively).

Conclusion: All of these data demonstrated that the higher hepcidin levels in diabetic patients may be due to those higher ferritin and IL-6 levels, the elevated hepcidin might have adaptive value through down-regulated iron absorb and play an important role in pathogenesis of Type 2 DM.

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1. Introduction

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion or insulin action, or both. Diabetes and its complications have become a major public health problem in the world and its prevention has become a public health priority. Increasing evidence now suggests a potential role of iron in the pathogenesis of Type 2 DM [1]. Iron is a strong pro-oxidant that catalyses several cellular reactions leading to the formation of reactive oxygen species (ROS) and resulting in elevated oxidative stress, interfering with insulin secretion which is proposed to contribute to an increased risk of Type 2 DM [2]. In animal models, iron excess might result in β-cell oxidative stress and decrease insulin secretary capacity [3]. Apart from direct tissue damage, epidemiological studies have reported an association between iron overload and peripheral insulin resistance [4].

Ferritin is a widely used marker of iron status in epidemiological studies and accurately reflects body iron stores in healthy individuals [5]. Several cross-sectional and retrospective case–control studies have linked elevated ferritin levels with DM [6]. Furthermore, a few prospective studies have also reported that relatively high levels of ferritin are associated with an increased of risk of developing DM in apparently healthy individuals [7,8]. In diabetes, metabolic abnormalities may lead to increased ferritin levels through a variety of mechanisms, in certain insulin-sensitive cells such

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as adipocytes, receptors for transferrin, glucose and insulin-like growth factor II co-localize in the cell membrane, and the presence of insulin resulted in the simultaneous translocation of all three proteins. Therefore, it has been hypothesized that insulin mediated glucose transportation may lead to increased transferring receptors on the cell surface, resulted in increased uptake of extracellular iron [9]. Ferritin is also an acute-phase reactant, its synthesis is up-regulated by infection or inflammation. Studies have demonstrated that pro-inflammatory cytokines such as tumor necrosisfactor (TNF)-α, interleukin-1 (IL-1) and interleukin-6 (IL-6) induce the expression of ferritin in cultured hepatic cell lines [10]. Type 2 DM is closely correlated with chronic inflammation, increased circulating concentrations of IL-6 and TNF-α were found in Type 2 DM [11]. So, the increased ferritin levels in type 2 DM probably also induced by their elevated inflammatory cytokines.

The hereditary hemochromatosis (HH) is a disorder of abnormal iron absorption resulted in the progressive accumulation of iron in the liver, heart, pancreas, and other organs. The most penetrate cases of HH presented with a classic triad of symptoms: hepatomegaly (associated with iron overload and cirrhosis), diabetes, and hyperpigmentation. Type 2 DM occurs in 25–75% of patients with hemochromatosis. HH is also a disease caused by a deficiency of hepcidin which associated with several iron-related disorder [12,13]. Hepcidin, a 25-amino-acid antimicrobial peptide, is the central regulator of iron homeostasis. Under normal circumstances, hepcidin expression and subsequent release into plasma prevents further absorption of iron from the duodenal enterocytes by preventing the efflux of iron by ferroportin channels, hence reduced amounts of iron delivery via transferrin to hepatocytes [14]. In response to iron loading in animal studies, hepcidin expression increased to prevent the further uptake of iron. Conversely, during iron deficiency, hepcidin expression decreased. Significant decreased hepcidin expression was found to contribute to HH, and the juvenile hemochromatosis, the most severe iron-overload disease, which associated with mutations in HJV or HAMP, is in this severe form of iron overload that the greatest deficiency of hepcidin has been documented [15]. Recently, research reported that the serum concentration of prohepcidin (a precursor of the mature hepcidin) was significantly higher in men with impaired glucose tolerance or Type 2 DM than in those with normal glucose tolerance, and that the serum prohepcidin level was negatively correlated with insulin sensitivity evaluated by the glucose clamp technique. Thus, they concluded that prohepcidin was associated with insulin resistance or impaired glucose metabolism [16].

Previous data allow us to presume that there may be existed relationship between hepcidin and diabetes. However, at present, there is spare information about the expression of hepcidin in Type 2 DM, whether the increased iron load in Type 2 DM due to a deficiency of hepcidin, or on the contrary, iron overload would up-regulate hepcidin expression? The aim of this study was to determine serum hepcidin levels and iron metabolic/inflammatory parameters in patients with Type 2 DM, and try to elucidate the relationships among them in this specific sub-population.

2. Subjects

This study was approved by the Ethics Committee of Shantou University Medical College and conducted from April to October, 2010. The study included sixty-four people: 34 cases of Type 2 DM and 30 age-matched controls. Incident cases of diabetes were defined by the appearance of any one of the following during follow-up: (1) a fasting ($\geq$8 h) glucose level $\geq$7.0 mmol/l, (2) a non-fasting glucose level $\geq$11.1 mmol/l; (3) use of diabetes medication, or (4) a self-reported physician diagnosis. All patients and control peoples gave written informed consent to the project's aims and to collection, analysis and use of the data for publication. Patients with malignancies and hepatic disease were excluded from our study, since a radiotherapy and/or chemotherapy might had altered the metabolism of the organism. None of our patients were under any treatment with iron or immunosuppressive drugs and/or received blood transfusion prior to the start of our study.

3. Materials and methods

Blood samples were obtained for hematological and biochemical tests after overnight fasting, using two types of containers, one of which contained ethylenediamine tetraacetic acid (EDTA), while the other without any anticoagulant. Blood in EDTA-containing vacutainers were analyzed by an automatic cell counter (COULTER LH 750 Hematology Analyzer) for the determination of the complete blood count including hemoglobin (Hb), platelet (PLT) and white blood cell (WBC). Non-anticoagulant blood was kept at room temperature for 2 h to ensure serum separation. Serum simples were analyzed for liver transaminases (AST, ALT, GGT), lipids (triglycerides, total cholesterol, HDL and LDL cholesterol) by an Automatic biochemical analyzer (Beckman LX20 Biochemical Analyzer). Serum ferritin, IL-6, hsCRP, EPO, hepcidin and sTfR were measured by ELISAs method (CUSABIO BIOTECH, Newark, New Jersey) according to manufacturers' instructions.

3.1. *Statistical analysis*

All data were given as mean ± standard [mean ± SD]. Statistical differences between two groups were carried out by non-parametric Kruskal–Wallis test or student's *t*-test. The associations of serum hepcidin with ferritin, sTfR, IL-6, hsCRP and other parameters were tested with Spearman rank correlation. Linear regression models were used to evaluate the effective of hematological variables, iron and inflammatory indicators on serum hepcidin production in each group. *p*-Value < 0.05 was considered as statistically significant.

4. Results

A summary of the results of the patients and control groups were presented in Table 1. The mean age of patients was 60.88 years. The levels of Glu and HbAc1 were significantly higher than the controls ($p < 0.001$ for both). The levels of

Table 1 – Clinical and laboratory data in healthy control subjects and patients with type 2diabetes.

	Diabetes	Control	*p*-Value
N (M/F)	34 (19/15)	30(16/14)	
Age	60.88 ± 10.99	60.19 ± 6.74	0.762
AST (IU/l)	23.26 ± 10.04	24.85 ± 8.97	0.523
ALT (IU/l)	25.53 ± 17.99	32.22 ± 20.59	0.188
GGT (IU/l)	49.12 ± 82.24	29.41 ± 20.01	0.229
CRE (IU/l)	192.26 ± 204.67	72.15 ± 17.95	0.002
BUN (IU/l)	8.85 ± 7.83	4.28 ± 1.18	0.002
GLU (mmol/l)	8.66 ± 4.56	5.41 ± 0.51	<0.001
HBAc1 (%)	7.05 ± 1.66	4.79 ± 0.51	<0.001
TC (IU/l)	5.35 ± 2.62	5.19 ± 0.86	0.732
TG (IU/l)	2.78 ± 2.68	1.36 ± 0.66	0.005
HDL (IU/l)	1.05 ± 0.29	1.08 ± 0.24	0.620
LDL (IU/l)	3.60 ± 2.52	3.52 ± 0.87	0.876
WBC ($\times 10^9$/L)	12.46 ± 13.59	6.98 ± 1.55	0.035
Hb (g/l)	107.76 ± 27.46	130.95 ± 28.84	0.002
PLT ($\times 10^9$/L)	239.21 ± 95.46	229.53 ± 63.45	0.652
BMI (kg/m^2)	25.72 ± 3.28	22.52 ± 2.44	<0.001
hsCRP (mg/l)	17.51 ± 9.37	12.67 ± 6.52	0.036
Il-6 (ng/l)	243.61 ± 85.41	60.98 ± 62.42	<0.001
EPO (IU/L)	26.16 ± 7.15	24.15 ± 10.98	0.392
Ferritin (μg/l)	423.84 ± 132.28	244.24 ± 173.56	<0.001
sTfR (μg/l)	1082.38 ± 48.56	1162.96 ± 32.12	<0.001
Hepcidin (μg/l)	778.91 ± 175.22	513.44 ± 281.73	<0.001

Data are presented as mean ± SD. M/F indicates males/females; ALT, alanine aminotransferase; AST, aspartate aminotransferase; sTfR, serum transferrin receptor; Hb, hemoglobin; Plt, platelet; GGT, galactosylhydroxylysyl glucosyltransferase; CRE, creatinine; BUN, urea nitrogen; TC, total cholesterin; TG, triglyceride; HDL, high density lipoprotein, LDL, low density lipoprotein; WBC, white blood cell; hsCRP, high-sensitivity c-reactive protein; EPO, erythropoietin.

BMI were elevated significantly in the diabetic patients when compared with the control ($p < 0.001$). Among these patients, there were 16 patients were diabetic nephropathy (DN) (3 cases in phase III: early nephropathy period, 8 cases in phase IV: clinical nephropathy period, and 5 cases in phase V: terminal renal failure stage). Thus, patients in our study had significantly higher levels of CRE and BUN than the control ($p = 0.002$ for both). Diabetic patients had significantly higher WBC counts, lower Hb when compared to the controls as shown in Table 1 ($p = 0.035$, $p = 0.002$ respectively). No significant differences in AST, ALT, GGT, TC, HDL, LDL and PLT values were found between patients and control groups ($p > 0.05$ for all).

Hepcidin levels were elevated significantly in the diabetic patients when compared with the control ($p < 0.001$). The indicators of iron metabolism, serum ferritin and sTfR were significantly elevated in diabetes than the controls ($p < 0.001$ respectively).

To examine whether elevated hepcidin may be partly attributable to the inflammatory signals, we measured WBC, hsCRP and IL-6 levels in each groups, significant higher of WBC ($p = 0.035$), hsCRP ($p = 0.036$) and IL-6 ($p < 0.001$) were found in the diabetic patients when compared with the controls.

Although diabetic patients had lower Hb, and serum EPO levels of diabetic patients was higher than the control, no significant difference of EPO concentration was found between two groups.

4.1. *What was correlated with hepcidin?*

Spearman rank correlation was used to evaluate any correlations between hepcidin and iron/inflammatory indicators in our study. Adjusted linear regression analyses for hepcidin levels in both groups are collected in Table 2. In diabetic group, a positive correlation between hepcidin and age was found ($R = 0.373$, $p = 0.015$) (Fig. c (1)), but not in the control group ($R = 0.276$, $p = 0.078$) according to the result of Spearmen analyses. A positive correlation was also found between ferritin and age in diabetic group ($R = 0.287$, $p = 0.050$). No correlation was found between BMI and ferritin in both diabetic ($R = 0.282$, $p = 0.059$) and control group ($R = -0.150$, $p = 0.223$). When investigating the correlations between hepcidin and hematological parameters, we found that no correlations were existed between serum hepcidin and hematological parameters including Hb, PLT and WBC counts in our study ($p > 0.05$ for all). A strong positive correlation between hepcidin and ferritin was existed in the control group ($R = 0.836$, $p = 0.000$) (Fig. a (1)) and in the diabetic group ($R = 0.704$, $p = 0.000$) (Fig. a (2)). No correlation was found between hepcidin and sTfR in diabetic group ($R = 0.277$, $p = 0.056$), but there was a positive correlation was found between hepcidin and sTfR in the control group ($R = 0.473$, $p = 0.006$). A positive correlation between hsCRP and WBC was existed in the diabetic group ($R = 0.369$, $p = 0.032$), whereas No correlation was found in the control group ($R = 0.266$, $p = 0.160$). No correlation was found between hepcidin and hsCRP in both diabetic ($R = -0.054$, $p = 0.384$) and control group ($R = -0.002$, $p = 0.495$). The same conditions were also found between hepcidin and IL-6 in the diabetic group ($R = 0.176$, $p = 0.160$).

5. Discussion

Type 2 DM is a metabolic disease in which the amount of insulin produced by the pancreas is inadequate to meet body needs. Type 2 DM is closely correlated with chronic inflammation, with increased levels of circulatory acute response proteins and cytokines in affected subjects. Immune responses and inflammation are suggested to play certain roles in the development and complications of Type 2 DM [17], and there is also increasing evidence that an ongoing cytokine-induced inflammatory response is related closely to the pathogenesis of Type 2 DM [18]. Among the elevated cytokines in Type 2 DM subjects, IL-6 is one of the type 2 T helper cell (Th2) cytokines that contribute to the exquisite regulation of balance between Th1 and Th2 cells. In our study, WBC, hsCRP and IL-6 levels increased significantly in all Type 2 DM patients when compared with the normal control, suggested that increased hsCRP, WBC together with IL-6 levels in patients with Type 2 DM might reflect their systemic inflammation. Under chronic inflammatory conditions, excessive cytokines produced by macrophages and T-lymphocytes, particularly IL-6 play a central role in the hepcidin production [19]. IL-6 acts directly on hepatocytes to stimulate hepcidin production. Binding of IL-6 to its receptor results in phosphorylation of the intracellular signaling

Table 2 – Linear regression analysis of relationships between the serum hepcidin or ferritin and characteristics of patients with diabetes.

	Ferritin (ng/ml)		Hepcidin (ng/ml)		IL-6 (ng/l)		sTFR (ng/ml)	
	R	p	R	p	R	p	R	p
Age	0.287	0.050	0.373	0.015	0.229	0.096	−0.124	0.243
AST	0.075	0.337	0.167	0.172	0.315	0.035	−0.143	0.209
ALT	−0.085	0.317	0.062	0.363	0.122	0.246	0.146	0.206
GGT	0.165	0.175	0.006	0.487	0.166	0.174	0.005	0.488
CRE	−0.134	0.226	0.126	0.239	−0.350	0.021	0.086	0.314
BUN	−0.077	0.333	0.150	0.198	−0.383	0.013	0.107	0.273
GLU	0.274	0.059	0.282	0.053	0.052	0.386	0.046	0.398
HbAc1	0.275	0.058	0.141	0.213	0.420	0.007	−0.149	0.200
TC	−0.166	0.174	−0.048	0.395	−0.251	0.076	0.362	0.018
TG	−0.094	0.299	0.112	0.265	0.014	0.489	0.394	0.011
HDL	−0.150	0.199	−0.156	0.189	−0.210	0.116	0.338	0.025
LDL	0.167	0.172	−0.025	0.443	−0.238	0.088	0.419	0.007
WBC	0.252	0.075	0.064	0.359	0.121	0.248	0.024	0.446
Hb	0.070	0.075	−0.094	0.299	0.034	0.423	−0.116	0.257
PLT	0.046	0.347	0.231	0.094	−0.002	0.496	0.064	0.359
BMI	0.282	0.059	−0.050	0.394	0.160	0.191	0.036	0.423
hsCRP	−0.118	0.260	−0.054	0.384	−0.030	0.434	0.018	0.461
Il−6	0.371	0.015	0.176	0.160	1.000	–	−0.061	0.366
EPO	0.018	0.461	−0.223	0.110	−0.075	0.341	−0.436	0.006
Ferritin	1.000	–	0.704	0.000	0.371	0.015	0.102	0.284
sTfR	0.102	0.284	0.277	0.056	−0.061	0.366	1.000	–
Hepcidin	0.704	0.000	1.000	–	0.176	0.160	0.277	0.056

ALT, alanine aminotransferase; AST, aspartate aminotransferase; sTfR, serum transferrin receptor; Hb, hemoglobin; PIL, platelet; GGT, galactosylhydroxylysyl glucosyltransferase; CRE, creatinine; BUN, urea nitrogen; TC, total cholesterin; TG, triglyceride; HDL, high density lipoprotein; LDL, low density lipoprotein; WBC, white blood cell; hsCRP, high-sensitivity c-reactive protein; EPO, erythropoietin.

molecule STAT-3 (signal transducer and activator of transcription 3). Phospho-STAT-3 dimerizes and then is translocated to the nucleus, where it interacts with a characterized IL-6 response element in the hepcidin promoter [20]. Although we did not find any obvious association between hepcidin and the inflammatory mediators including hsCRP and IL-6, the possible reason may due to there are several cytokines co-expressed in type 2 DM, a setting that is much

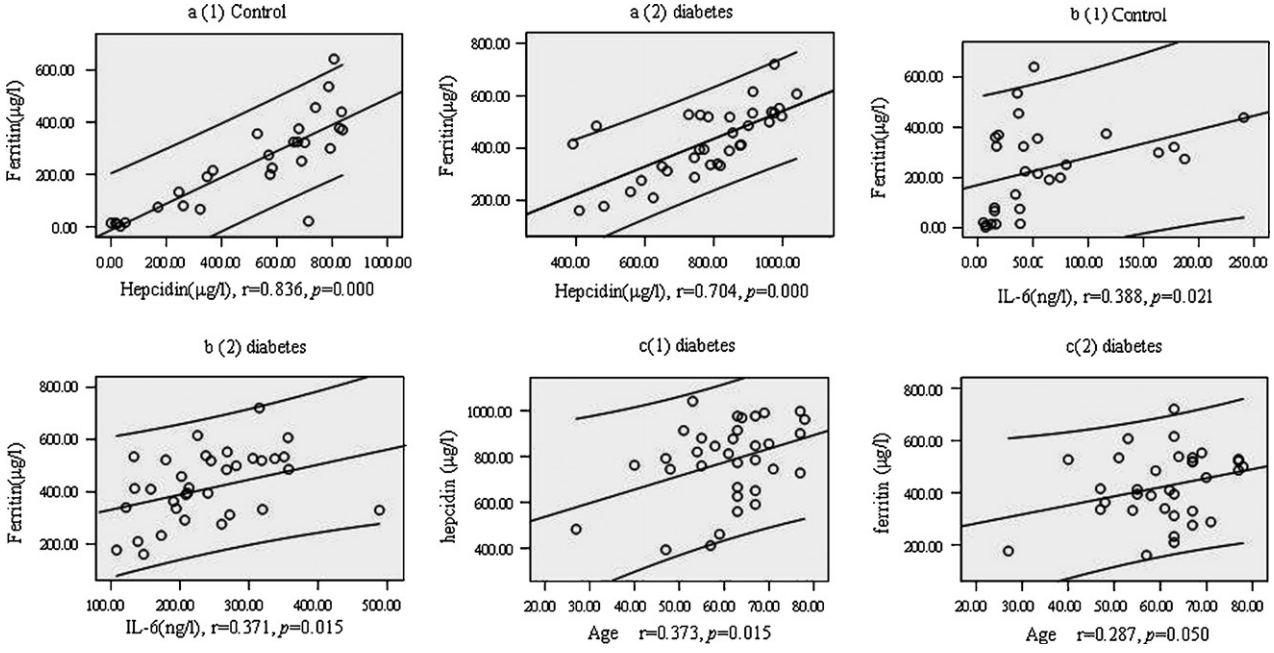

Fig. 1 – Correlations between serum hepcidin, ferritin and IL-6 levels in diabetes and control groups. (a) Correlations between serum ferritin and hepcidin levels in type 2 diabetic and control groups. (b) Correlations between serum ferritin and IL-6 levels in type 2 diabetic and control groups. (c) Correlations between serum hepcidin/ferritin levels with age in type 2 diabetic groups

different from the controlled application of single cytokine or LPS during an experiment, which reflect the net effects of several agonistic and antagonistic cytokines toward a specific target, and thus the potential effect of a single immune effecter molecule found in experiments may be masked in this in vivo setting [21]. This finding could possible explained our data that hepcidin levels did not directly correlated with hsCRP and IL-6 levels in Type 2 DM patients.

In anemia, increases in erythropoiesis require a considerable increase in the flow of iron from diet or storage pools, it is believed that excess hepcidin in inflammatory states reduce the iron recycling that is necessary for erythropoiesis, leading to the anemia of chronic disease (ACD), which is characterized by reduced erythropoiesis and reduced serum iron despite apparently normal stores. At present, anemia is common in chronic disease and elevated hepcidin expression has been proposed to be the underlying cause of ACD. In this study, Hb was lower in DN as well as non-DN group when compared to the controls, may be partly attributable to their higher hepcidin levels. Hepcidin could also have a direct effect on erythroid precursor proliferation and survival as erythroid colony formation in vitro was inhibited by the simultaneous addition of hepcidin and erythropoietin, at least at moderate EPO concentrations [22]. Although EPO levels in diabetic group were higher than the control in our study, we found no correlation existed between serum hepcidin and EPO.

The synthesis of hepcidin is also enhanced by an increased body iron stores [23]. Ferritin levels were increased significantly in all diabetic patients compared with normal control in our research, and a significant positive correlation between hepcidin and ferritin which was also marked in both control and diabetic group, indicated the associations between hepcidin and iron stores under normal conditions as well as in Type 2 DM. The regulation of hepcidin by body iron acts as a feedback mechanism to allow sufficient iron to enter the plasma when the demand for iron is high, but to limit iron release into the plasma in times of iron sufficiency. Then, hepcidin acts as a negative regulator of intestinal iron absorption and macrophage iron release. Our data demonstrated that the elevated hepcidin in Type 2 DM patients may be due to those elevated ferritin levels and elevated inflammatory proteins.

Circulating sTfR has been proposed as a novel marker of iron status that is less affected by the presence of inflammation. Increased sTfR may be the most sensitive serum biochemical marker for the identification of iron-deficient erythropoiesis in patients with chronic disease [24]. The sTfR concentration begins to rise early in iron deficiency with the onset of iron-deficient erythropoiesis, and continues to rise as iron-deficient erythropoiesis progressively worsens, prior to the development of anemia. The sTfR level reflects total body TfR concentration, which seems to be less subject to within-person variability than ferritin. In vitro data suggested that insulin is capable of redistributing TfR to the cell surface leading to increased cellular iron uptake in adipose tissue and the liver, it has been hypothesized that insulin-mediated glucose transportation may lead to increased transferring receptors on the cell surface, resulting in increased uptake of extracellular iron [9]. This effect of insulin on the up-regulation of iron uptake occurs concurrently with its effect on glucose uptake [25]. Negative correlation between pro-hepcidin and sTfR was found in studies on non-anemic pregnant females as well as healthy individuals [26]. We also found sTfR levels decreased in diabetic group with increased hepcidin, although we could not find the relationship had significant meaning. Decreased sTfR as an indicator of tissue iron overload, our data suggested that the change of sTfR mediated by insulin disturbance may affect hepcidin expression in Type 2 DM.

The present study demonstrated a strong positive correlation between serum hepcidin and the ferritin in diabetic patients, suggested that iron load could regulate hepcidin expression in Type 2 DM. Increased hepcidin in turn leads to down-regulation of FPN on enterocytes and macrophages and consequent intracellular sequestration of iron, decreased iron intake. Iron excess might resulted in β-cell oxidative stress and decrease insulin secretary capacity [3]. Research also indicated that depletion of iron stores via phlebotomy has been shown to reduce postprandial hyperinsulinaemia in healthy volunteers [27]. So, the confirmation of an increased risk of diabetes with moderately elevated ferritin levels could have clinical and public health consequences, since persons at high risk could be targeted for more intensive screening and preventive interventions [8]. From these points, the elevated hepcidin concentrations may have adaptive value through down-regulated iron absorb in patients with Type 2 DM.

Although the liver has been considered as the main site of hepcidin expression [28], there was research pointed out that the pancreatic islets were an additional source of the peptide hepcidin and expression of hepcidin in β-cells was regulated by iron, suggested that pancreatic β-cells might be involved in iron metabolism in addition to their genuine function in blood glucose regulation. These data may provide an insight into the phenomenology of intriguing mutual relationships between iron and glucose metabolisms [29]. Hepcidin expression has been reported in different models of infection, this can be construed as evidence for a possible new role for hepcidin beyond innate immunity, or indeed immunity, into regulation of cell proliferation [30]. On the other hand, addition of hepcidin to macrophages results in enhanced production of inflammatory cytokines, indicated that hepcidin could be considered to have pro-inflammatory effects in addition to its well-known role in iron homeostasis. Hepcidin may contribute to a vicious cycle that perpetuates the inflammatory state and, therefore, blocking hepcidin expression or function could have double meaning to type 2 DM patients: help to reduce inflammation [31] as well as preventing or ameliorating their iron overload. From these points, hepcidin concentration may be useful to predict which patients could benefit form it although the potential use of hepcidin targeted therapeutics are yet available.

Conflict of interest

The authors declare that they have no conflict of interest.

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