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Research Article

Impact of Long Term Metformin Therapy on Hepcidin and Iron Status in Type II Diabetic Patients

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ABSTRACT

Background /Aims: Diabetes and its complications are considered as a major public health problem in the world and the prevention of these complications has become a public health priority. The recent discovery of hepcidin, the central regulatory molecule of systemic iron homeostasis, changed the view of iron metabolism, which is long known to be linked with insulin resistant status in type 2 diabetes mellitus. Thus, the aim of the current study was to elucidate the impact of metformin administration for long time on iron regulator hepcidin and iron metabolic parameters (Hb, ferritin, TIBC) in Type 2 diabetic patients. Subjects Methods: This study included 45 subjects of both sexes, the age between 35-60 years. Thirty cases with type 2 diabetes who divided into two groups: L-group, treated with metformin for less than 5 years and A-group, treated with metformin for more than 5 years. These groups were compared with the healthy sex and age matched control group (n=15). Fasting blood glucose (FBG), serum insulin, blood hemoglobin (Hb), serum hepcidin, ferritin, Iron and total iron binding capacity (TIBC) have been carried out. Results: The present results recorded highly significant increase in serum hepcidin level in A-group and significant increase in L-group versus the control group. Serum insulin group as well as serum insulin, ferritin and TIBC levels showed highly significant increase in A-group with insignificant increase in L-grouprelative to the control group. Significant depletion in blood Hb as well as in serum iron levels were demonstrated in both A-and L-group with respect to the controlgroup. Hepcidin showed a significant positive correlation with FBG, insulin and ferritin in both diabetic groups (A-and L-group), while it showed a negative correlation with HB and serum iron. Conclusion: The current study provided an evidence for that metformin administration in Type 2 diabetic patients for long time (more than 5 years) caused anima, heperferritinemia, iron deficiency as well as marked increase in serum hepcidin indicating iron overload in liver of these patients.

Keywords: Diabetes mellitus, hepcidin, ferritin, iron and total iron binding capacity.

INTRODUCTION

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion or insulin action, or both. Type 2 diabetes mellitus (T2DM) presents mild chronic inflammation and insulin resistance (IR) in tissues such as adipose, muscle, pancreas, and liver[1]. Diabetes and its complications are considered as a major public health problem in the world and the prevention of these complications has become a public health priority[2]. The International Diabetes Foundation (IDF) and the World Health Organization (WHO) in their 2006 report have been provided an estimate (171 million in the year 2000) for the prevalence of diabetes mellitus (DM) worldwide, which is expected to increase to 366 million by 2030[3].

Iron is a strong prooxidant that catalyses several cellular reactions leading to the formation of reactive oxygen species (ROS) which produce an elevation in the oxidative stress that interferes with the insulin secretion. Thus iron is proposed to contribute to the increased risk of Type 2 DM[4] and play a potential role in the pathogenesis of Type 2 DM[2]. In animal models, excess iron resulted in β-cell oxidative stress and a decrease in insulin secretary capacity[5]. Apart from direct tissue damage, epidemiological studies have reported an association between iron overload and peripheral insulin resistance[6]. Ferritin is a widely used marker of iron status in epidemiological studies and accurately it reflects body iron stores in healthy individuals[7]. The recent discovery of hepcidin, the central regulatory molecule of systemic iron homeostasis, changed the view of iron metabolism, which is long known to be linked with insulin resistant status, in Type 2 DM[8]. Hepcidin, a 25-amino acid peptide hormone, is mainly produced by the liver in response to increased plasma or tissue iron to homeostatically down regulate absorption and recycling of the metal[9]. At the molecular level, hepcidin acts by binding and inactivating its cell membrane receptor ferroportin, the only known cellular iron exporter. Ferroportin is particularly expressed by cells critical for iron homeostasis, like absorbing duodenal enterocytes, reticuloendothelial macrophages (involved in iron storage and recycling),

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and hepatocytes (involved in iron storage and endocrine regulation)[10]. Hepcidin concentrations are decreased in situations that require increased concentrations of circulating iron. In case of increased erythropoiesis, for example in response to hypoxia, anemia, iron deficiency, or conditions characterized by ineffective erythropoiesis (such as thalassemia major and intermedia), a decreased hepcidin concentration will result in the release of stored iron and an increase in the dietary iron absorption[11]. On the other hand, infection and inflammation cause an increase in hepcidin synthesis[12], resulting in decreased availability of circulating iron, which is considered as a defense mechanism of the human body against extracellularly proliferating (iron dependent) pathogens[9]. Hepcidin is also upregulated by inflammatory cytokines, a response believed to contribute to host defense by subtracting iron from invading pathogens[13]. Also, hepcidin concentration is increased in situations of iron overload[14], except for situations in which mutations in genes encoding hepcidin or its upstream positive regulators are responsible for the surplus of iron by preventing hepcidin up-regulation[15].

To the best of our knowledge whether hepcidin concentrations are associated with T2D remains controversial. elucidate the impact of metformin administration for long time on iron regulator hepcidin and iron metabolic parameters (Hb, ferritin, TIBC) in Type 2 diabetic patients.

SUBJECTS AND METHODS

This study included 30 type 2 diabetic patients of both sexes, the age between 35-60 years who are the outpatients of the Diabetes Clinic in The Center of Excellence of Medical Research at National Research Centre, Giza, Egypt. These patients were divided into two groups: L-group, treated with metformin for less than 5 years and A-group, treated with metformin for more than 5 years (5-10 years). These groups was compared with the healthy sex and age matched control group (n=15). The present study was approved by the Medical Ethical Committee of the National Research Centre, Egypt. After taking a written informed consent from the all subjects enrolled in the study, a detailed medical history and physical examination were performed.

Biochemical analysis

Blood samples were withdrawn from cases and controls after an overnight fasting. Each blood sample was divided into two portions, the first one was collected in EDTA containing tube for Hb concentration analysis and the other portion was collected in EDTA free tube for separation of serum. Sera were separated for analysis of fasting blood glucose (FBG), serum insulin, hepcidin, ferritin, iron and total iron binding capacity (TIBC). FBG was determined by enzymatic colorimetric method using a kit provided by the Human Diagnostica Liquicolor Test (Germany) according to the method of Trinder[16]. Serum insulin was measured quantitively by enzyme linked immunosorrbent assay (ELISA) using kit provided by Biosource (Europe) according to the method of Temple et al.[17]. Serum hepcidin levels were detected using ELISA kit purchased from Glory Science Co., Ltd, USA according to manufacture instructions. Ferritin levels were determined in serum using Immunospec Corporation ELISA kit following the method of White et al.[18]. Serum Iron was determined using colometric determination kit of Reactivos GPL. Barcelona, Espana according to Perrotta[19]. Total iron-binding capacity (TIBC) was determined according to the method Baadenhuinisen et al.[20] using saturation-precipitation kit of Reactivos GPL, Baracelona, Espana. Blood Hemoglobin determinations were performed by an automated cell counter from a tube of well-mixed EDTA-anticoagulated blood filled to a predetermined level according to Walker et al.[21].

Statistical analysis

Data were collected, revised, coded and entered to the Statistical Package for Social Science (IBM SPSS) version 14 Data were analyzed by one way analysis of variance (ANOVA) followed by least significant difference (LSD) to compare significance between groups. Spearman correlation coefficients were used to assess the relation between each two studied parameters in the same group. Receiver operating characteristic curve (ROC) was used to assess the best cut off point with sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV). The confidence interval was set to 95% and the margin of error accepted was set to 5%. The p-value was considered significant at $P < 0.05$

RESULTS

Characteristics and biochemical measurements of the studied groups are shown in (Table 1). Mean age of control group was 46.93±6.99 years; they were 7 males and 8 females. Mean age of diabetic (L-group) was 48.4±6.45 years and they were 10 males and 5 females. Mean age of diabetic (A-group) was 52.8±11.72 years and they were 11 males and 4 females. Statistically significant increase in diabetic groups (L- and A-group) versus the control group was found in FBG (p=0.00 and p=0.000 respectively) and serum hepcidin (p=0.003 and p=0.031 respectively). Significant increase in serum insulin (p=0.000), ferritin (p=0.000) and TIBC (p=0.000) was recorded in the A-group as compared to the control group. But insignificant increase in serum insulin (p=0.089), ferritin (p=0.061) and TIBC (p=0.412) was detected in the L-group when compared with the control group. Significant depletion in blood hemoglobin (p=0.000 and p=0.000 respectively) as well as serum iron (p=0.000 and p=0.001 respectively) was observed in both A-group and L-group with respect to the control group (Table 1).

Spearman rank correlation was used to evaluate the correlation between hepcidin and iron metabolic parameters (iron, Hb, Ferritin, TIBC) in the studied groups. Serum hepcidin levels were high among diabetic groups (A-group and L-group) with statistical significant difference between the patients and the control subjects as shown in Table (1). Table (2) illustrated the correlation between serum hepicidin and the studied biochemical

Table 1: Characteristics and l*aboratory biochemical parameters of the studied groups*

	Control group	Diabetic group n=30			
		L-group	P value	A-group	P value
N (Male/Female)	15 (7/8)	15 (10/5)	---	15 (11/4)	----
Age (Y)	46.93±6.99	48.4±6.45	0.647	52.8±11.72	0.072
Wt (kg)	84.6± 7.56	87.13±11.02	0.41	86.2± 5.42	0.6
FBG (µg/dL)	80.35±7.85	194.8±85.05**	0.000	184.73±34.37**	0.000
Insulin (µIU/mL)	33.73±4.83	40.66±10.99	0.089	49.85±14.57**	0.000
Hemoglobin (g/dL)	13.47±1.46	10.78±1.33**	0.000	10.62±0.94**	0.000
Hepcidin (µg/L)	478.12±65.7	624.16±158.05*	0.031	835.11±259.42**	0.000
Ferritin (ng/mL)	208.03±46.61	250.0±56.69	0.061	371.66±73.11**	0.000
Iron (µg/dL)	107.04±22.31	80.73±20.97**	0.001	78.06±19.68**	0.000
TIBC (µg/dL)	290.04±67.432	313.86±83.59	.412	368.4±83.98**	0.009

Data are presented as mean ± SD. *: Significance change at P < 0.05 in comparison with control group; **: Significance change at P < 0.01 in comparison with control group

Table 2: *Spearman correlation between serum hepicidin and the measured biochemical parameters in diabetic groups.*

	L-group		A-group	
	r	p-value	r	p-value
FBG (µg/dL)	0.450*	0.013	0.551**	0.002
Insulin (µIU/mL)	0.371*	0.043	0.586**	0.001
Hemoglobin (g/dL)	-0.261	0.163	-0.492**	0.006
Ferritin (ng/mL)	0.499**	0.005	0.684**	0.000
Iron (µg/dL)	-0.024	0.898	-0.319	0.086
TIBC (µg/dL)	-0.008	0.967	0.532**	0.002

** correlation is significant at p-value <0.01; * correlation is significant at p-value <0.05

measurements. Highly significant positive correlation was found between serum hepcidin level and ferritin in L-group (r=0.499, p=0.005) and A-group (r=0.684, p=0.000) as shown in Fig. (1d &2d) respectively. Highly significant positive correlation was found between serum hepcidin level and FBG in A-group (r=0.551, p=0.002), serum insulin (r=0.586, p=0.001) and TIBC (r=0.532,p=0.002) as shown in Figs. (1a, 1b, 1f) respectively. Significant positive correlation was found between serum hepcidin level and FBG in L-group (r=0.450, p=0.013) as well as serum insulin (r=0.371, p=0.043) as shown in Figs. (2a, 2b) respectively. Highly Significant negative correlation was recorded between serum hepcidin and hemoglobin in A-group (r=-0.492 and p=0.006) (Fig. 1c). Moreover, insignificant negative correlation was found between serum hepcidin and hemoglobin in L-group (r=-0.261 and p=0.163) and TIBC (r=-0.008,p=0.967) as shown in Figs (2c, 2f) respectively. Also, insignificant negative correlation was found between serum hepcidin and iron in both A-group (r=-0.319,p=0.086) and L-group (r=-0.024 and p=0.898) as shown in Figs. (1e & 2e) respectively.

Figs. 3&4 showed the receiver operating characteristic curve (ROC-curve) for hepcidin versus FBG as well as insulin and ferritin to identify the diabetic with higher serum hepcidin level and higher FBG as well as higher levels insulin and ferritin for each L- and A-group. The area under curve (AUC^{ROC}) was AUC^{ROC}=0.188 for FBG, AUC^{ROC}= 0.24 for insulin and ferritin AUC^{ROC}=0.016 for A-group. On the other hand, the area under curve (AUC^{ROC}) was AUC^{ROC}=0.188 for FBG, AUC^{ROC}= 0.308 for insulin and ferritin AUC^{ROC}=0.183 for L-group. The optimal cut-off for hepcidin derived from the ROC-curve was 405 µg/l. Using this cut-off, hepcidin sensitivity and specificity estimates to detect hepcidin with high FBG as well as insulin and ferritin were 100% in both as shown in Fig. (3&4).

DISCUSSION

The present results recorded highly significant increase in serum hepcidin level in A-group and significant increase in L-group versus the control group. Serum insulin group as well as serum insulin, ferritin and TIBC levels showed highly significant increase in A-group with insignificant increase in L-grouprelative to the control group. Significant depletion in blood Hb as well as in serum iron levels were demonstrated in both A-and L-group with respect to the controlgroup. Hepcidin showed a significant positive correlation with FBG, insulin and ferritin in both diabetic groups (A-and L-group), while it showed a negative correlation with HB and serum iron. Iron may contribute to the pathogenesis of Type 2 diabetes mellitus (DM). It has been suggested the insulin resistance and compensatory hyperinsulinism characteristic of these patients may contribute to iron overload[22]. Body iron overload is frequently observed in patients with type 2 diabetes mellitus (DM2)[23] or patients with impaired glucose tolerance (IGT)[24]. Iron overload has been confirmed as an independent factor contributing to the development of DM2 by causing oxidative stress

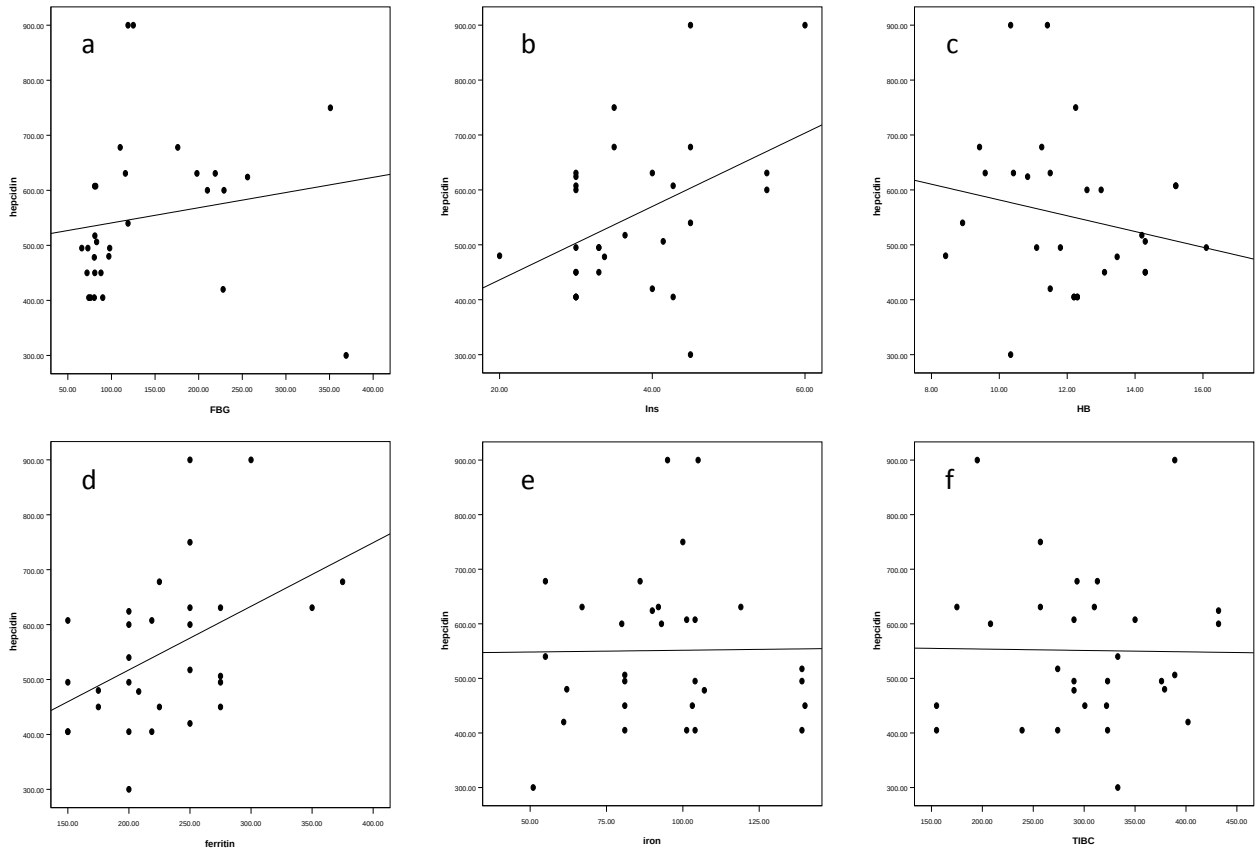

Fig. 1: *Spearman correlation between serum hepicidin level and different measured biochemical parameters in diabetic L-group a: FBG (r*=0.450, *p=0.013),* b: *insulin (r*=0.371, *p*=0.043*),* c: *HB (r*=-0.261, *p*=0.163*),* d: *Ferritin (r*=0.499, *p*=0.005*),* e: *iron (r*=-0.024, *p*=0.898*) and* f: *TIBC (r*=-0.008, *p*=0.967*).*

injury in hepatocytes and pancreatic β-cells[25], which may finally lead to insulin resistance (IR) and reduction in insulin extraction and secretion[26]. Bozzini et al.[27] and Jehn et al.[28] have established a link between iron metabolism and insulin resistant states, including type 2 diabetes mellitus and the metabolic syndrome (MetS). Prospective clinical studies have demonstrated that body iron storage is positively correlated with the prevalence of DM2[29]. Besides, free Fe^{3+} and/or serum ferritin is significantly increased in DM2 patients with poor glycemic control compared with those with good glycemic control[30].

The underlying mechanisms for the frequent iron overload in type 2 diabetes mellitus (DM2) remain unclear. But the view of iron overload disorders has radically changed by the discovery of hepcidin[31]. Hepcidin, a circulatory antimicrobial peptide mainly expressed in the liver, plays a critical role in the regulation of iron metabolism by negatively regulating intestinal iron absorption and macrophage iron release and lowering the level of circulating iron[31]. Hepcidin has been found to be inappropriately low in genetic hemochromatosis (HH)[9]. The hereditary hemochromatosis is a disorder of abnormal iron absorption resulting 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. Hereditary hemochromatosis is also a disease caused by a deficiency of hepcidin which is associated with several iron related disorder[32]. Under normal circumstances, hepcidin expression and subsequent release into plasma prevent further absorption of iron from the duodenal enterocytes by counteracting the efflux of iron *via* ferroportin channels, hence reducing the amounts of iron delivery through transferrin to hepatocytes[10]. In animal studies and in response to iron loading, hepcidin expression increased to prevent the further uptake of iron[33]. A growing body of evidences indicated that 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 serum prohepcidin level was negatively correlated with insulin sensitivity evaluated by glucose clamp technique. Thus prohepcidin has been suggested to be associated with insulin resistance or impaired glucose metabolism[34]. Recently, it has been demonstrated that, hepcidin is directly regulated by insulin and it plays an important role in iron overload in DM2[35]. It has been demonstrated that iron overload caused up-regulation of hepcidin expression[36]. High hepcidin levels in diabetic patients may be due to high ferritin and IL-6 levels and the elevated hepcidin might have adaptive value through down-regulated the absorbed iron and play an important role in pathogenesis of Type 2 DM[36]. In the present study,

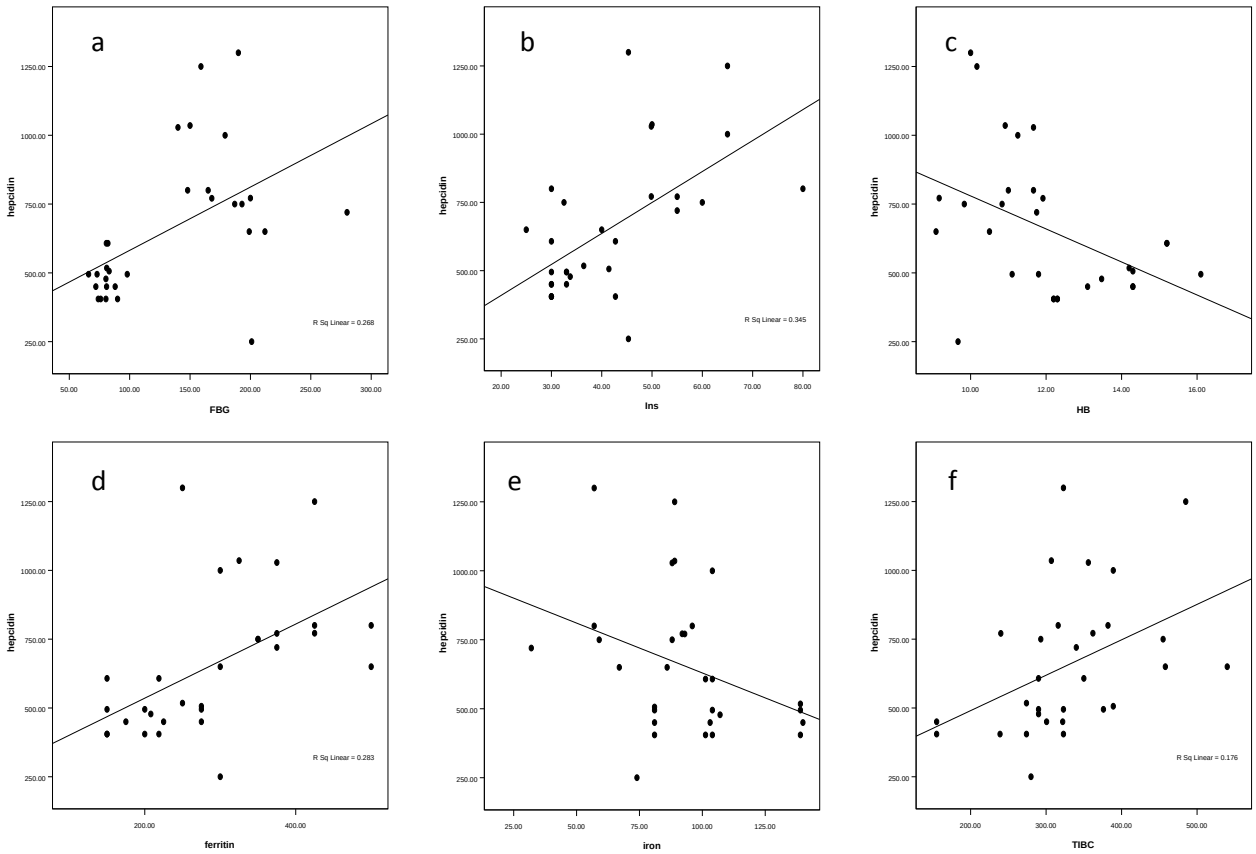

Fig. 2: *Spearman correlation between serum hepicidin level and different measured biochemical parameters in diabetic A-group a: FBG (r*=0.551, *p=0.002),* b: *insulin (r*=0.586, *p*=0.001*),* c: *HB (r*=-0.492, *p*=0.006*),* d: *Ferritin (r*=0.684, *p*=0.000*),* e: *iron (r*=-0.319, *p*=0.086*) and* f: *TIBC (r*= 0.532, *p*=0.002*).*

serum ferritin was shown to be the most important correlate of serum hepcidin concentration. It was found that increased serum ferritin concentration is associated with increased serum hepcidin concentration[37]. Iron, TIBC, and transferrin saturation (TS) only showed moderate association with serum hepcidin. This indicates that TS determines hepcidin concentration, confirming hepcidin's proposed role in counter regulation of the increased body iron concentration *via* decreasing iron absorption and macrophage iron release[38].

High correlation between serum hepcidin and serum ferritin concentration in healthy persons has been documented[37]. Ackerman and Gems[39] have revealed a complex interplay between insulin/IGF-1 signaling and ferritin expression. On the other hand, some prospective studies have shown a positive association between baseline levels of ferritin and development of Type 2 diabetes[28]. Several studies have linked elevated ferritin levels with DM[40]. In diabetes, metabolic abnormalities may lead to increased ferritin levels through a variety of mechanisms. In certain insulin-sensitive cells such 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 transferrin receptors on the cell surface, resulted in increased uptake of extracellular iron[41]. Moreover, 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 necrosis factor-α (TNF)-α, interleukin-1 (IL-1) and interleukin-6 (IL-6) induce the expression of ferritin in cultured hepatic cell lines[42]. Type 2 DM is closely correlated with chronic inflammation with increased circulating concentrations of IL-6 and TNF-α[43]. So, the increased ferritin levels in type 2 DM is probably induced by the elevated inflammatory cytokines in these patients[36]. It has been found a relationship between ferritin and insulin resistance only in women but not in men[44]. The largest prospective study showed that ferritin is an independent predictor of future development of type 2 diabetes mellitus patients[45]. It has been found in the present study that serum hepcidin concentrations correlated with Hb and serum ferritin concentrations. Serum ferritin concentration is elevated in individuals with diabetes and is positively correlated with both Hb concentrations and T2D risk factors. It has been suggested that serum ferritin concentration correlated with T2D risk factors, while serum hepcidin concentration is positively associated with Hb and serum ferritin concentrations in T2D[46]. Factors that contribute to the development of T2D such as obesity, inflammation and the metabolic syndrome have also been demonstrated to be significantly associated with serum ferritin concentration[47]. Previous studies have also detected association between serum ferritin concentration and both urinary[48] and serum[49] hepcidin concentrations. Hepcidin

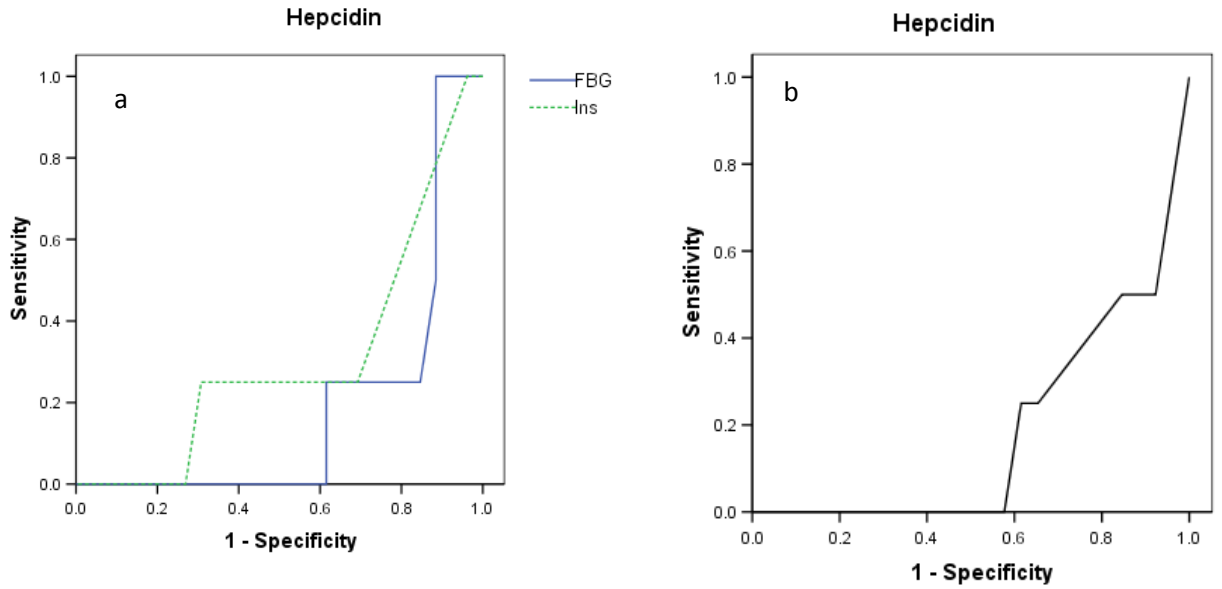

Fig. 3 a: *ROC curve between hepicidin as well as FBG0.188 and insulin0.308 in the diabetic A-group.;* b: *ROC curve between hepicidin as well as ferritin (0.183)in the diabetic A-group.*

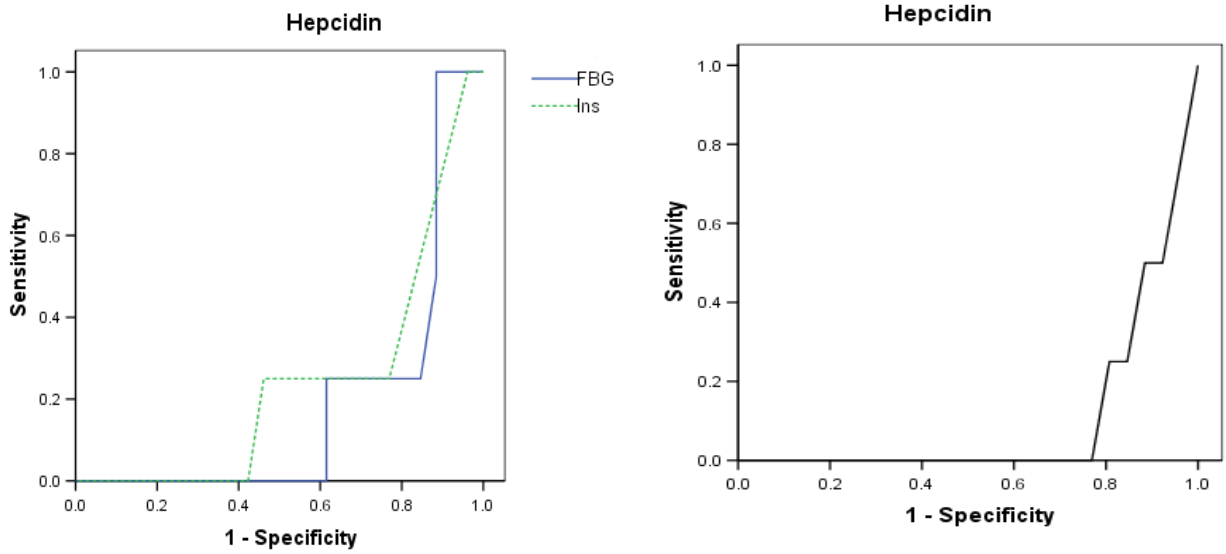

Fig. 4 a: *ROC curve between hepicidin as well as FBG0.188 and insulin0.24 in the diabetic A-group.;* b: *ROC curve between hepicidin as well as ferritin (0.016)in the diabetic A-group.*

acts as a negative regulator of iron absorption, and its positive association with serum ferritin concentration is probably due to feedback responses resulting from elevated systemic iron status[46].

In the view of the present data, TIBC showed a negative correlation with serum hepcidin, which corresponds to the observation that TIBC increases in situations of low iron status[38]. Metformin, one of the most widely used oral drugs for the treatment of type 2 diabetes, decreases hyperglycemia and has beneficial effects on circulating lipids, without affecting insulin secretion[50]. The glucose lowering effects of metformin are attributable to both an increase in muscle glucose uptake[51] and a decrease in hepatic glucose production[50]. Activation of AMPK by metformin was found to be required for the decrease in glucose production and the increase in fatty acid oxidation in hepatocytes and for the increase in glucose uptake in skeletal muscle[52]. Metformin increases hepatic insulin sensitivity without changing liver fat content[53]. The ability of metformin to increase hepatic insulin sensitivity has been documented in several previous human studies[54]. In conceptual agreement, the administration of the insulin sensitizer metformin to patients with polycystic ovary syndrome (PCOS) reduces ferritin levels in parallel with an evident increase in insulin sensitivity[55].

It was suggested that one of the key actions of metformin was to stimulate muscle glucose uptake[56]. A growing body of evidence from clinical studies and animal models suggested that the primary function of metformin is to decrease hepatic glucose production, mainly by inhibiting gluconeogenesis[57]. This preferential action of metformin in hepatocytes is due to the predominant expression of organic cation transporter 1 (OCT1), which has been

shown to facilitate cellular uptake of metformin. Consistent with this notion, deletion of the Oct1 gene in mouse dramatically reduces metformin uptake in hepatocytes, and humans who have a reduced function polymorphisms of the OCT1 gene display an impaired effect of metformin in lowering blood glucose levels[58]. It has been showed that intake of metformin in female rats with induced PCOS leads to a significant reduction in serum iron[59], which indicates that metformin could prevent the absorption of iron from intestinal lumen resulting in a decreased body iron concentration[22] and this finding is in agreement with other report[60]. Luca, and Francesca[61] have found that metformin increased insulin sensitivity *via* decreasing intestinal iron absorption in patients PCOS. Tahira et al.[62] reported that three months intake of metformin in PCOS patients caused a reduction in ferritin and improved glycemic and insulin sensitivity. It has been revealed that metformin treatment activated energy sensor AMP-activated protein kinase (AMPK) in hepatocytes in both humans and rodents[63] and the regulation of gluconeogenic gene expression by metformin is dependent on the phosphorylation of CREB-binding protein (CBP) through atypical PKCι/λ[64]. This suggests the complexity of the mechanism of metformin action. The most widely accepted mechanism of metformin action is the inhibition of transcription of key gluconeogenic genes in the liver. Metformin stimulates CRTC2 phosphorylation in response to metabolic signals such as energy stress through the LKB1-AMPK/SIK1 pathways, which promotes binding to 14-3-3 proteins, thereby sequestering CRTC2 from the nucleus to the cytoplasm[63] leading to altered CRTC2 regulation and the activation of gluconeogenic genes[22]. In conclusion, the current study provided clinical evidences for the role of hepcidin in type 2 diabetes mellitus (T2DM) patients who treated with the conventional drug for diabetes, metformin. Moreover, the current study provided a strong document for that metformin administration for Type 2 diabetic patients for long time (more than 5 years) may cause anima, heperferritinemia, serum iron deficiency as well as hyperhepcidinemia which indicating iron overload in the liver of these patients.

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