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ORIGINAL RESEARCH

Iron Metabolism Markers and Lower Extremity Arterial Disease in People with Type 2 Diabetes

Hua Jin, Peihong Chen, Shan Zhang, Ping Wu, Xuemei Yu

Department of Endocrinology and Metabolism, Fengxian Central Hospital, Shanghai, 201404, People's Republic of China

Correspondence: Xuemei Yu, Department of Endocrinology and Metabolism, Fengxian Central Hospital, No. 6600, Nanfeng Road, Nanqiaoxincheng, Fengxian District, Shanghai, 201404, People's Republic of China, Tel +86 21-57413468, Email yuxuemeiyxm@163.com

Objective: To determine the levels of serum iron, ferritin, total iron-binding capacity, and hepcidin in patients with type 2 diabetes mellitus (T2DM), and to elucidate the relationship of these biomarkers with lower extremity arterial disease (LEAD).

Methods: Three hundred fifteen patients with T2DM were selected for the study and divided into non-LEAD (n = 119) and LEAD groups (n=196) based on the ankle-brachial index (ABI) results. Demographic data and clinical test results were collected from all patients. Serum iron, ferritin, total iron-binding capacity, and hepcidin levels were measured, and the transferrin saturation was calculated.

Results: Hepcidin levels were substantially higher in the LEAD group (19.17 ± 8.66 ng/mL) than the non-LEAD group (15.44±7.55 ng/mL, $P < 0.001$), and there was a negative correlation between the ABI and serum lecithin level ($r = -0.349$, $P < 0.001$). There were no other correlations with the other iron metabolism indicators. The results of dichotomous logistic regression with LEAD as the dependent variable revealed that smoking history (OR = 4.442, $P = 0.008$), hypertension history (OR = 3.721, $P = 0.006$), cardiovascular disease history (OR = 11.126, $P < 0.001$), diabetes duration (OR = 1.305, $P < 0.001$), age (OR = 1.056, $P = 0.021$), hs-CRP level (OR = 1.376, $P = 0.002$), HbA1c concentration (OR = 1.394, $P = 0.001$), and hepcidin level (OR = 1.097, $P = 0.003$) were independent risk factors for LEAD in T2DM patients.

Conclusion: Serum hepcidin levels were elevated in the LEAD group compared with the non-LEAD group, and elevated hepcidin levels were associated with the development of LEAD in T2DM patients, suggesting that hepcidin may be involved in the occurrence and development of LEAD in T2DM patients.

Keywords: hepcidin, type 2 diabetes mellitus, lower extremity arterial disease, ankle-brachial index

Introduction

Type 2 diabetes mellitus (T2DM) is a chronic metabolic condition with numerous consequences that can affect multiple organs. According to the most recent epidemiologic census, the prevalence of diabetes among adults in China was as high as 11.2% in the national sample from 2015–2017.[1] In 2019, the global diabetes prevalence is expected to reach 9.3% (463 million people).[2]

Lower extremity arterial disease (LEAD), a potentially fatal consequence of diabetes, is characterized by atherosclerotic stenosis or occlusion of the lower extremities (lower extremity atherosclerosis). Diabetic patients have a higher incidence and prevalence of LEAD than non-diabetic adults.[3] LEAD, with atherosclerosis as the most prevalent pathologic alteration, is one of the most common consequences of T2DM. Indeed, T2DM patients have a 2-fold higher risk of LEAD than the general population.[4] Of approximately 5000 newly-diagnosed T2DM patients in the UK Prospective Diabetes Study, 1.2% had LEAD at the time of diagnosis.[5]

The ankle-brachial index (ABI), a ratio of the systolic pressure of the ankle artery to the brachial artery, is commonly used to test for LEAD. Iron is a powerful oxidant that has been linked in vivo to the development of atherosclerosis. Diabetes mellitus is linked to iron overload (IO). IO may cause vasodilatory dysfunction and atherosclerosis by altering lipid profiles, sustaining endothelial activation, elevating inflammatory mediators, altering vascular permeability, and

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decreasing nitric oxide availability, thus resulting in vasodilatory dysfunction and atherosclerosis. A number of studies have verified the positive link between serum ferritin (SF) expression and the incidence of diabetes, yet there are unresolved issues: 1. The link between SF and LEAD. Is a high SF level the cause of LEAD or a consequence of the pathogenesis? The specific link and interaction concept requires additional investigation. 2. It is necessary to determine whether SF is an independent risk factor for LEAD. 3. The sites of iron and low-density lipoprotein-cholesterol (LDL-C) interaction and the implications for treatment are unknown.[6]

Hepcidin controls the quantity of iron in the blood by regulating the amount of ferroportin on the surface of enterocytes, hepatocytes, macrophages, and enterocytes.[7] Recent evidence suggests that hepcidin, by delaying or blocking iron mobilization from macrophages, may cause atherosclerosis and is linked to an increased risk of cardiovascular disease.[8] The onset and progression of T2DM is influenced by iron metabolism, either directly or indirectly. The soluble transferrin receptor-to-ferritin ratio is inversely associated with the incidence of T2DM, and high serum ferritin is one of the risk factors for T2DM.[9]

Recent evidence suggests that insulin therapy, as well as other diabetic medications, impacts hepcidin synthesis, hence changing the iron burden in cells. Iron load correction via hepcidin expression might be a unique and crucial mechanism of action for diabetic medications.[10]

Few clinical studies have been reported on the relationship between in vivo iron metabolism levels and LEAD in the T2DM population. Numerous cellular and metabolic functions, such as oxygen transport and energy metabolism, depend on iron. Hepcidin, a master regulator of iron homeostasis, maintains intracellular and circulating iron levels. The only known "iron exporter" that moves ferrous iron from within cells to the plasma is the transmembrane protein, ferroportin.[11] Inflammatory cytokines, particularly IL-6 and IL-1, increase the expression of hepcidin.[12] With an emphasis on hepcidin, the main regulator of body iron homeostasis, more recent in vitro and animal investigations have clarified the complicated signaling pathways controlling iron.[13]

This study aimed to evaluate the association between serum iron (Fe) metabolic indices and LEAD in individuals with T2DM and to offer novel insights into the processes underlying LEAD formation.

Subjects and Methods

Study Subjects

Three hundred fifteen patients with T2DM hospitalized in the Endocrinology Department of Fengxian District Central Hospital in Shanghai from January 2016 to October 2019 were selected. According to the ankle-brachial index (ABI) values, the patients were separated into two groups: non-LEAD (n = 119); and LEAD (n = 196). The T2DM inclusion criteria were as follows: meeting the diagnostic criteria of diabetes mellitus established by WHO in 2011 (fasting glucose ≥ 7.0 mmol/L, glycosylated hemoglobin [HbA1c] ≥ 6.5%, and/or 2 h postprandial glucose ≥ 11.1 mmol/L). The exclusion criteria were as follows: type 1 diabetes; special types of diabetes; acute complications of diabetes; acute and chronic infections; stress state; anemia; hematologic disorders; surgery; tumor; pregnancy; history of liver and kidney disease; alcoholism; no severe bleeding within 6 months; no history of iron administration; cardiac, hepatic, renal insufficiency, and other endocrine diseases; and an ABI >1.3. The study was approved by the Ethics Committee of Shanghai Fengxian District Central Hospital and the procedures followed were in accordance with the Declaration of Helsinki. All patients signed an informed consent form.

General Indexes

The cases studied were all inpatients and the medical histories, including histories of smoking, hypertension (blood pressure ≥ 140/90 mmHg measured 3 times at different times at rest), cardiovascular and cerebrovascular diseases, and duration of diabetes, blood pressure, height (m), and weight (kg), were recorded and the body mass index (BMI, weight/height^2) was calculated. HbA1c, total cholesterol (TC), triglycerides (TG), high-density lipoprotein-cholesterol (HDL-C), LDL-C, hemoglobin (Hb), white blood cell (WBC) count, and hypersensitive C-reactive protein (hs-CRP) were measured.

Serum Iron Metabolism Indicators

Serum hepcidin and SF were measured by double-antibody sandwich ABC-ELISA. An enzyme-linked immunosorbent assay (ELISA) was used to detect Fe and total iron-binding capacity (TIBC). The transferrin saturation (TS) was calculated as Fe/TIBC*100%. The kits were purchased from the Shanghai Xitang Company. The assay was performed according to the kit instructions and the absorbance was measured at 450 nm (Denley Dragon Wellscan MK3; Thermo Fisher Scientific, Waltham, MA, USA).

ABI Examination

All subjects underwent an ABI determination at rest using an ES-100 V3 handheld Doppler flow detector (Japan). The same staff followed the device instructions. The lower of the two ABIs was used for diagnosis. The 2019 edition of the Chinese Diabetic Foot Prevention and Treatment Guidelines[14] was used to determine the LEAD criterion, and the results showed that a resting ABI ≤ 0.9 was consistent with LEAD. Patients ($0.9 < \text{ABI} \leq 1.3$) who conducted an ABI exercise load test and had a post-exercise ABI > 1.10 were classified as non-LEAD.[15]

Statistical Analysis

The normally distributed measurement data are represented as the mean ± standard deviation (SD), and the statistical analysis was carried out with SPSS 22.0 software. The Student's *t*-test was used to compare measurement data between two groups, while the chi-square test was used to compare categorical data. Continuous variable correlations were investigated using Pearson correlation analysis. For risk factor screening, we employed dichotomous logistic regression analysis. In determining the required sample size to achieve a $1\text{-}\beta = 0.80$ and an $\alpha = 0.05$, a two-sample *t*-test was conducted and the statistical power results indicated that a minimum of 74 subjects were required in each group. A $P < 0.05$ two-tailed was used to determine statistical significance.

Results

Comparison of General Information Between the Two Groups

There was no statistical difference between the two groups with respect to gender and BMI ($P > 0.05$; Table 1). Compared to the non-LEAD group, patients in the LEAD group were older ($P < 0.001$), had a longer duration of diabetes ($P < 0.001$), and had a higher proportion of patients with a combined history of smoking ($P = 0.008$), hypertension ($P < 0.001$), cardiovascular disease ($P < 0.001$), and diabetic retinopathy ($P = 0.008$; Table 1).

Comparison of Laboratory and Iron Metabolism Indexes Between the Two Groups

The *t*-test between the two groups revealed that the diastolic blood pressure, TC, and LDL-C levels in the non-LEAD group were higher than the LEAD group, while the Hb level was significantly lower and hepcidin was significantly

Table 1 Basic Characteristics Between Two Group (Mean ± SD)

	Total	Non-LEAD	LEAD	*P*
Number of cases	315	119	196	
Sex (male/female)	200/115	82/37	118/78	0.12
Smoking history, n (%)	136 (43.17)	40 (33.61)	96 (48.98.)	0.008
History of hypertension, n (%)	147 (46.67)	21 (17.65)	152 (77.55)	<0.001
History of cardiovascular and cerebrovascular disease, n (%)	123 (39.05)	10 (8.40)	113 (57.65)	<0.001
Diabetic retinopathy, n (%)	88 (27.94)	23 (19.33)	65 (33.16)	0.008
Duration of diabetes mellitus (years)	7.81 ± 7.23	3.78 ± 1.92	8.43 ± 6.47	<0.001
Age (years)	57.63 ± 12.073	48.85 ± 10.92	62.92 ± 9.32	<0.001
BMI (kg/m^2)	24.57 ± 4.36	23.91 ± 4.86	24.37 ± 4.03	0.290

Abbreviation: BMI, body mass index.

higher in the LEAD group ($P < 0.001$). The differences between the two groups in the remaining test indexes were not statistically significant (Table 2).

Correlation of Serum Iron Metabolic Index Levels with ABI

Pearson correlation analysis revealed that ABI was negatively correlated with Fe, TS, and hepcidin, but not with ferritin and TIBC (Table 3). The results of partial correlation analysis with Fe, ferritin, TIBC, and TS as covariates showed that the serum hepcidin level was still negatively correlated with the ABI ($r = -0.349$, $P < 0.001$), but other iron metabolism indicators were not correlated with the ABI.

Logistic Regression Analysis of LEAD Risk Factors

LEAD grouping variables were used as dependent variables, and the risk factors for diabetes mellitus complicated by LEAD included histories of smoking, hypertension, and cardiovascular disease, and gender, duration of diabetes, age, systolic blood pressure, diastolic blood pressure, BMI, TG, TC, HDL-C, LDL-C, WBC count, hs-CRP, HbA1c, Hb, Fe, SF, TIBC, TS, and hepcidin as independent variables for dichotomous logistic regression analysis.[14] The results showed that histories of smoking, hypertension, and cardiovascular disease, and duration of diabetes, age, hs-CRP, HbA1c, and hepcidin were independent risk factors for the development of LEAD in patients with T2DM (Table 4).

Table 2 Comparison of Laboratory and Iron Metabolism Indexes Between the Two Groups (Mean ± SD)

	Total	Non-LEAD	LEAD	*P*
Number of cases	315	119	196	
WBC (10^9/L)	6.48 ± 1.59	6.47 ± 1.70	6.49 ± 1.53	0.932
SBP (mmHg)	136 ± 18	131 ± 16	138 ± 18	0.001
DBP (mmHg)	80 ± 11	82 ± 12	79 ± 10	0.017
TG (mmol/L)	1.74 ± 1.16	1.88 ± 1.27	1.66 ± 1.08	0.106
TC (mmol/L)	4.93 ± 1.26	5.17 ± 1.33	4.78 ± 1.19	0.008
HDL-C (mmol/L)	1.11 ± 0.26	1.09 ± 0.25	1.12 ± 0.27	0.363
LDL-C (mmol/L)	3.20 ± 0.90	3.33 ± 0.89	3.12 ± 0.89	0.045
Hb (g/L)	136.35 ± 15.66	139.57 ± 14.34	134.39 ± 16.13	0.004
HbA1c (%)	10.15 ± 2.31	10.17 ± 2.32	10.13 ± 2.32	0.887
hs-CRP (mg/L)	2.34 (0.50, 3.20)	2.32 (0.40, 3.30)	2.34 (0.62, 2.90)	0.809
Fe (μmol/L)	12.61 ± 4.88	12.08 ± 4.52	12.93 ± 5.07	0.138
SF (ng/mL)	57.89 ± 22.80	59.03 ± 21.78	57.19 ± 23.43	0.489
TIBC (μmol/L)	63.57 ± 16.07	64.07 ± 13.76	63.26 ± 17.35	0.668
TS (%)	20.76 ± 9.22	19.58 ± 8.23	21.47 ± 9.72	0.078
Hepcidin (ng/mL)	17.76 ± 8.44	15.44 ± 7.55	19.17 ± 8.66	<0.001

Abbreviations: SBP, systolic blood pressure; DBP, diastolic blood pressure; TG, triglycerides; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; WBC, white blood cell; Hb, hemoglobin; HbA1c, glycosylated hemoglobin; hs-CRP, hypersensitive C-reactive protein; Fe, serum iron; SF, ferritin; TIBC, total iron binding capacity; TS, transferrin saturation.

Table 3 Correlation Between Iron Metabolism and ABI

Index	*r*	*p*
Fe (μmol/L)/ABI	−0.156	0.006
SF (ng/mL)/ABI	−0.021	0.712
TIBC (μmol/L)/ABI	0.103	0.069
TS (%)/ABI	−0.183	0.001
Hepcidin (ng/mL)/ABI	−0.349	<0.001

Abbreviations: ABI, ankle-brachial index; Fe, serum iron; SF, ferritin; TIBC, total iron binding capacity; TS, transferrin saturation.

Table 4 Logistic Regression Analysis of Risk Factors for LEAD in Patients with T2DM

Variables	B	S.E	Wald	OR	p	95% CI
History of smoking	1.500	0.559	7.208	4.482	0.007	1.499–13.406
History of hypertension	1.339	0.477	7.894	3.815	0.005	1.499–9.710
History of cardiovascular disease	2.392	0.684	12.209	10.930	<0.001	2.858–41.806
Duration of diabetes	0.260	0.084	9.560	1.297	0.002	1.100–1.530
Age	0.057	0.024	5.817	1.059	0.016	1.011–1.109
hs-CRP	0.292	0.105	7.682	1.340	0.006	1.089–1.647
HbA1c	0.328	0.099	10.870	1.388	0.001	1.142–1.686
Hepcidin	0.094	0.031	9.175	1.099	0.003	1.033–1.165

Abbreviations: hs-CRP, hypersensitive C-reactive protein; HbA1c, glycosylated hemoglobin.

Discussion

LEAD is a typical chronic consequence of T2DM. According to the China DIA-LEAD research, the prevalence of LEAD among T2DM patients > 50 years of age in China is 21.2%, with a rate of missed diagnoses of 55.7%.[16] LEAD is a component of peripheral arterial disease, which can result in ischemic ulcerations and lower-limb amputation. According to the findings of a 2012 diabetic foot survey in China, the proportion of patients with diabetic foot mixed with LEAD was 59%.[17] Because LEAD frequently co-exists with cardiovascular disorders, such as myocardial ischemia and cerebral infarction, LEAD may be useful in predicting the development of arterial thrombotic diseases.[18]

Iron is used as a strong oxidant and generates large amounts of oxygen radicals through the Haber-Weiss reaction, thus causing peroxidation of intracellular lipids, damage to vascular endothelial cells, and lipid deposition on the vessel wall, which leads to plaque formation and affects the stability of atheromatous plaques.[19,20] Hepcidin is a low molecular weight antibacterial agent discovered by Krause et al[21] and Park et al[22] in 2000 and 2001, respectively. Hepcidin is a low molecular antibacterial polypeptide synthesized in the liver and expressed in macrophages, adipocytes, and other systemic locations. Hepcidin inhibits intestinal iron uptake and iron release from the monocyte-macrophage system and is an important iron regulator.

Our study showed that within the T2DM population, hepcidin levels were significantly higher in the LEAD group compared to the non-LEAD group, and were negatively correlated with the ABI after excluding other influencing factors. Moreover, in a logistic regression analysis of risk factors for LEAD, elevated serum hepcidin levels were shown to be one of the risk factors for the development of LEAD in T2DM patients. Other indicators of iron metabolism in the body were not correlated with the ABI and were not influential factors for the development of LEAD. The correlation between hepcidin levels and atherosclerosis is also supported by reports of clinical studies conducted in different populations.[23–26]

Valenti et al[23] observed the correlation between hepcidin and carotid intima-media thickness in 143 patients with non-alcoholic fatty liver disease (NAFLD) and found that hepcidin levels were associated with carotid plaque formation. Elevated hepcidin levels were also found to increase carotid intima-media thickness in patients with diabetic nephropathy in a study,[24] and similar results were obtained in patients with chronic kidney disease on hemodialysis.[25] Another study showed that in a community population, elevated hepcidin levels increased carotid-femoral pulse wave velocity, suggesting that hepcidin is associated with atherosclerosis.[26] Ferritin and transferrin saturation are modestly associated with peripheral arterial disease, especially in people with high cholesterol levels.[27]

Li et al[28] reported that hepcidin is strongly associated with all-cause mortality in patients with acute coronary syndromes and may be a predictor of the risk of developing coronary artery disease in patients with hepcidin. The ratio of serum hepcidin-to-ferritin in postmenopausal women is significantly and negatively correlated with the resting ABI, suggesting that hepcidin may be involved in the development of atherosclerosis in postmenopausal women.[29] The association between hepcidin and atherosclerosis has also been found in animal studies, where some researchers fed high-fat food to hepcidin receptor gene knockout rats. The hepcidin receptor gene-deficient rats exhibited a significant reduction in atherosclerosis compared to the control group.[30]

The mechanisms by which hepcidin is involved in the development of atherosclerosis are still unclear. It has been shown that hepcidin increases iron deposition in macrophages within atherosclerotic plaques, which enhances lipid

peroxidation in macrophages, promotes foam cell formation, and makes it easier to form unstable arterial plaques.[31,32] In addition, as hepcidin production in the liver decreases, the intracellular iron content of macrophages decreases, the cholesterol efflux capacity increases, and foam cells are less likely to form, thus reducing the formation of atherosclerosis.[33] The variability of blood hepcidin levels in diabetic patients is connected to inflammatory stimulation, anemia, and nutritional status, suggesting that hepcidin might be an early predictor of inflammation in diabetes patients with peripheral artery disease. Furthermore, an increase in hepcidin is a risk factor for diabetic foot ulcers.[34]

The results of this study showed lower cholesterol and LDL-C levels in the LEAD group than the non-LEAD group, which is inconsistent with the results of a previous study on arterial lesions;[35] we consider that this finding may be related to the fact that patients in the LEAD group would more aggressively use lipid-lowering drugs. T2DM patients have impaired liver function, which affects the production of regulator proteins involved in iron metabolism. As a result, dysregulated iron metabolism may result in chronic illness, anemia, or iron-loading anemia deficiency.[36,37] Hepcidin levels are changed in T2DM patients and are disproportionately impacted by weight. Furthermore, despite having higher iron storage, individuals with T2DM present with subclinical anemia.[37]

High plasma homocysteine (Hcy) concentrations are also linked to new-onset peripheral artery disease ([PAD] OR=2.08, 95% CI: 1.08–4.03, P=0.030).[38] Endothelial dysfunction, oxidative stress, and vascular remodeling are all pathogenic processes involving Hcy.[39] Smokers with high Hcy concentrations are significantly more likely than non-smokers with normal Hcy concentrations to have new-onset PAD (OR=4.44, 95% CI: 1.77–11.12, P=0.001). Diabetes has a significant effect on PAD when combined with a high Hcy concentration (OR=3.67, 95% CI: 1.25–10.80, P=0.018).[38] In descending order of strength, a history of cardiovascular disease, smoking, and hypertension, and HbA1c levels, hs-CRP levels, duration of diabetes, hepcidin levels, and age were all connected to the risk of LEAD. The hepcidin/ferritin ratio was shown to be superior to hepcidin alone as a risk marker in a meta-analysis.[40]

Due to the limited sample size and several contributing factors that impact LEAD, it was not feasible to establish whether there was a causal link between hepcidin and LEAD in this study. To answer this, further prospective research will be required.

Conclusion

A high serum hepcidin level is one of the risk factors for the development of LEAD in T2DM patients, and it may have a role in the onset and progression of LEAD in T2DM patients.

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Disclosure

The authors declare no competing interests in this work.

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