

DECREASED FERRITIN AND INCREASED HEPcidIN SERUM LEVELS IN PERIODONTITIS PATIENTS WITH DIABETES MELLITUS TYPE 2: A CONTROLLED BEFORE-AND-AFTER STUDY

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ABSTRACT

Objectives: The aim of the present work is to estimate the level of ferritin & hepcidin in serum of patients suffering from periodontitis and diabetes mellitus type 2 T2DM before and after non-surgical periodontal treatment.

Subjects and methods: Our study included 60 patients, categorized into 3 groups. 20 individuals with stage II grade B generalized periodontitis with T2DM (**Group I**), 20 others with stage II grade A generalized periodontitis (**Group II**) and 20 healthy volunteers (**Group III**). We obtained two serum samples from periodontitis patients before and after treatment in addition to one sample from each control subject. Serum ferritin & hepcidin levels were determined by using Enzyme linked immunosorbent assay technique (ELISA).

Results: The ferritin level was significantly higher in periodontitis groups than control in contrast to hepcidin that was elevated in control group. A significant decrease in ferritin concentration but increase in hepcidin was noticed after 3 months. Receiver Operating Characteristic (ROC) curve analysis demonstrated more significant accuracy for ferritin than hepcidin on comparing both periodontitis groups with control.

Conclusions: As a prognostic marker for periodontitis, we could use ferritin but not hepcidin.

KEYWORDS: Periodontitis, type 2 diabetes mellitus, ferritin, hepcidin, non-surgical periodontal therapy.

INTRODUCTION

Periodontitis is a bacteria-induced inflammatory disease that damage tooth supporting structure consequently to interactions between the bacteria

and host immune response¹. A bidirectional relation has been proved between periodontitis & diabetes mellitus; periodontitis has been accepted as a risk factor for diabetes mellitus considering periodontitis a chronic inflammatory disease that may alter

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insulin resistance. On the other hand diabetes mellitus assists in the development of periodontitis due to impaired immune response, altered oral microflora, disordering in collagen formation & microangiopathy^{2,3}.

Most of periodontopathogens possess haemolytic activity; carried out by haemolysins which are toxins that disturb the structural integrity of the erythrocyte, leading to the liberation of hemoglobin and other intracellular metabolites⁴. Haemolysis causes increase in local iron concentration which eventually results in iron disorder that may be a risk for systemic diseases. Iron will deposit in the heart, liver and other tissues, causing heart failure, liver cirrhosis, hepatocellular carcinoma, arthritis, T2DM and pigmentation⁵. Serum ferritin level can reflect the iron load; it possesses a significant role in storing, recycling and releasing of iron. Additionally ferritin is an acute phase reactant, its serum level increases in systemic inflammation^{6,7}.

Hepcidin, the major known regulator of iron homeostasis is a key hormone synthesized in the liver; its main function is the control of iron metabolism. Ferroportin, the iron exporter which is found in the plasma membranes of most body cells and present at high concentration in duodenal enterocytes, hepatocytes and macrophages, is the target of hepcidin as it can prevent iron efflux from cells into the plasma by inducing internalization and degradation of the ferroportin in these cells. Based on that, hepcidin serum level is considered an indicator of iron load. Moreover its synthesis is increased during chronic inflammation and is regulated by various stimuli as proinflammatory cytokines⁸⁻¹⁰.

Studies explored that increased body iron stores is a risk factor for T2DM. Oxidative stress injury in hepatocytes and pancreatic β cells caused by increased iron store, may lead to insulin resistance and decrease in insulin secretion, thus development of T2DM. In addition this insulin disorder can

lead to inadequate hepcidin level^{11,12,13}. Moreover evidence has suggested that insulin resistance accompanied by insufficient hepcidin level can develop to overt diabetes through more dysfunction of cells via iron overload that may be aggravated by inadequate hepcidin concentration in T2DM¹⁴.

It is apparent from the literature that disorder in iron load which could be represented by ferritin and also hepcidin and their relation to periodontal diseases and T2DM still has lacunae for intensive research. Hence, the present study was undertaken to assess concentrations of ferritin and hepcidin in serum from periodontitis patients with and without T2DM before and after non-surgical periodontal therapy.

SUBJECTS AND METHODS

All enrolled subjects has signed an informed consent form that has been approved by Research Ethical Committee and followed the Declaration of Helsinki after explaining to them the study procedures including the clinical examination, sampling procedures, treatment modality and follow-up appointments needed.

This clinical trial has been registered in U.S. National Institutes of Health Clinical Trials Registry, ClinicalTrials.gov, Identifier: NCT03964428.

Study Population:

60 subjects were recruited in our controlled before-and-after study. The subjects were categorized into three main groups:

Group I (Periodontitis with T2DM): 20 patients with stage II grade B generalized periodontitis with T2DM (12 males & 8 females; age range: 35-50 years with mean age 42.5 years).

Group II (Periodontitis Group): 20 patients with stage II grade A generalized periodontitis (10 males and 10 females; age range: 35-50 years with mean age 42 years).

Group III (Control group): 20 periodontally and systemically healthy volunteers who served as control subjects (9 males & 11 females; age range: 35-50 years with mean age 41.6 years).

Periodontitis participants were collected from the Outpatient Clinic, Department of Oral Medicine, Periodontology and Diagnosis, Faculty of dentistry, Fayoum University, between September 2018 and January 2019. The periodontitis included subjects were diagnosed according to the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions¹⁵, patients with more than 30% of the sites had clinical attachment level (CAL) 3-4mm with T2DM were diagnosed with stage II grade B generalized periodontitis, (Group I). Systemically healthy subjects suffering from periodontitis with more than 30% of the sites had CAL 3-4mm were diagnosed with stage II grade A generalized periodontitis (Group II). Diabetic patients were enrolled in this study according to the criteria of American Diabetes Association¹⁶. The control group (Group III) was selected from healthy subjects who attended the restorative dental clinic and had apparently clinically healthy gingiva with ≤ 3 mm pocket probing depth (PPD), nearly zero plaque index (PI), gingival index (GI) and zero CAL.

Exclusion criteria for enrollment in our study

(I) Pregnancy or lactation, (II) History of receiving professional periodontal treatment during the past 6 months; (III) Treatment with any type of medications and/ or antibiotics during the past 3 months and (IV) Current or former smokers.

Evaluation Parameters

The evaluation in our investigation included the assessment of ferritin and hepcidin in serum of Group I and II at baseline and 3 months after scaling and root planning. For Group III assessment were performed at beginning of the study only.

Clinical Parameters

At baseline, all participants underwent a periodontal clinical examination including the following parameters: PI, GI, PD, and CAL by a single calibrated examiner (S.H) who registered these measurements at six periodontal sites in all teeth. PI was established according to description of **Silness & Loe**¹⁷, marginal gingival bleeding was registered with GI¹⁸. PD was measured from the free gingival margin to the base of the periodontal pocket¹⁹ and CAL is the distance from cemento-enamel junction to the base of the periodontal pocket²⁰. All obtained readings were rounded to the highest whole millimeter by using of manual periodontal probe, the Michigan 0 probe with Williams' markings.

Blood sample collection

Blood samples were collected from all participants at beginning of our study. 3 months after treatment samples were collected again from group I & II patients. Blood samples were obtained from the antecubital fossa through venipuncture by using a 20-gauge needle. All collections were performed by well-trained nurses. The blood samples were collected in vials, and anticoagulant was later centrifuged in order to collect the serum. The serum was stored at -70°C for further analysis.

Periodontal Therapy

Periodontitis diagnosed patients were given detailed instructions and motivation on self-performed plaque control measures using soft tooth brush and interdental cleansing devices. Patients received a full mouth supra and subgingival scaling and root planning by the same operator (S.H & N.T) in a quadrant-wise approach from two to six sessions. Subgingival debridement under local anesthesia included use of periodontal Gracey curettes (Lustra Gracey periodontal curettes, Dentsply, Surrey, UK). The outcome of our treatment included removal of all subgingival calcified deposits to achieve a smooth and hard tooth surface. One month after

the end of periodontal treatment, patients received further oral hygiene instructions, motivation, and supragingival plaque control. For all periodontitis patient reassessment was performed after 3 months for the previous included clinical parameters in addition to collecting of following up serum samples.

Laboratory assays

A) *Human ferritin detection*

The level of ferritin was measured by using ELISA kit that was provided by Thermo Fissure scientific; USA. The Human Ferritin solid-phase sandwich ELISA is designed to measure the amount of the target bound between a matched antibody pair. A target-specific antibody has been pre-coated in the wells of the supplied microplate. Samples, standards, or controls are then added into these wells and bind to the immobilized (capture) antibody. The sandwich is formed by the addition of the second (detector) antibody, a substrate solution is added that reacts with the enzyme- antibody-target complex to produce measurable signal. The intensity of this signal is directly proportional to the concentration of target present in the original specimen.

B) *Human Hepsidin detection*

Hepsidin level was detected by ELISA kit provided by MyBiosource, USA. This experiment use double sandwich ELISA technique and the ELISA Kit provided is typical. The precoated antibody is human Heps25 monoclonal antibody and the detecting antibody is polyclonal antibody with biotin labeled. Samples and biotin labeling antibody are added into ELISA plate wells and washed out with PBS. Then Avidin peroxidase conjugates are added to ELISA wells in order; Use TMB substrate for coloring after reactant thoroughly washed out. TMB turns into blue in peroxidase catalytic and finally turns into yellow under the action of acid. The color depth and the testing factors in samples are positively correlated.

Statistical analysis

Turkey's contrasts for multiple comparisons of means were used to statistically analyze obtained data to compare the means of patient and control groups. Statistical work was done using R statistical software (version 3.3.3) ²¹ using R Commander Package (version 2.5) ^{22,23}.

Receiver Operating Characteristic curve (ROC) has been made to demonstrate the cut-off values of serum ferritin & hepcidin for distinction between studied groups. ROC curve analysis was performed using MedCalc Version 11.3 for Windows (MedCalc Software bvba)

Sample size calculation

Sample size was calculated at a power of 80% using 5% alpha (α) level and 20% beta (β) level Using the G*Power (Version 3.1.9.2) software. 60 individual's to be divided into three equal groups were needed.

RESULTS

The changes in clinical parameters throughout our study period in addition to comparison between the mean serum levels of ferritin, hepcidin and their ratio in all studied groups are demonstrated in table (1).

The results of the current study before treatment showed statistically significant different of serum ferritin level when comparing all studied groups mean levels. Concerning hepcidin levels and ratios of both markers only significant difference is showed between control group and the other groups.

As regards to the changes of both markers levels and their ratio before and after treatment within the same group of patients (Group I & II), it was evident that there was a statistically significant decrease in mean levels after 3 months in ferritin level, accompanied with statistically significant increase in hepcidin level in addition to their ratios (Table 1).

The comparison of treated groups together regarding markers levels and their ratios after treatment revealed statistically significant difference between the mean level of ferritin and their ratios. However hepcidin level showed non-significant difference between groups.

ROC curve analysis revealed

Differentiation between group I and II [Table 2, figure 1(A)]

At cut-off value of 93ng/ml; Ferritin showed 84% diagnostic accuracy while at a cut-off value of 1.15ng/ml; Hcpicidin showed 57.1% diagnostic accuracy for differentiation between them. Pair-wise comparison between areas under the ROC curve (AUC) of the two markers showed that Ferritin showed statistically significantly higher AUC than hepcidin ($z = 3.751, P\text{-value} < 0.001$).

Differentiation between group I and III [Table 2, figure 1(B)]

At cut-off value of 68ng/ml; Ferritin showed 80% diagnostic accuracy while at a cut-off value of 1.25ng/ml; hepcidin showed 57.5% diagnostic accuracy for differentiation between both groups. AUC of the two markers demonstrated that Ferritin showed statistically significantly higher AUC than hepcidin ($z = 3.033, P\text{-value} = 0.002$).

Differentiation between group II and III [Table 2 figure 1(C)]

At cut-off value of 82ng/ml; ferritin showed 95% diagnostic accuracy while at a cut-off value of 1.05ng/ml; hepcidin showed 60% diagnostic accuracy for differentiation between two groups. Comparing AUC of the two markers revealed that ferritin showed statistically significantly higher AUC than hepcidin ($z = 5.195, P\text{-value} < 0.001$).

TABLE (1): Comparisons of (mean, standard deviation) clinical parameters, serum ferritin, hepcidin, H/F ratio among the study groups

Clinical parameters	Group I		Group II		Group III
	Before SRP	After SRP	Before SRP	After SRP	
PI	1.68 ± 0.52 ^a	0.44 ± 0.32 ^b	1.81 ± 0.23 ^a	0.76 ± 0.15 ^b	0.7 ± 0.06
GI	1.26 ± 0.46 ^a	0.48 ± 0.29 ^b	1.88 ± 0.14 ^a	0.85 ± 0.22 ^b	0.5 ± 0.12
PD (mm)	4.85 ± 0.58 ^a	2.98 ± 0.32 ^b	4.01 ± 0.48 ^a	2.02 ± 0.33 ^b	1.6 ± 0.43
CAL (mm)	5.02 ± 0.72 ^a	3.65 ± 0.45 ^b	4.24 ± 0.52 ^a	2.85 ± 0.49 ^b	
Ferritin (ng/ml)	100.65±4.91 ^{**}	83.10±8.06 ^b	108.05±9.70 ^{**}	99.25 ± 10.45 ^b	68.00 ±11.23 [*]
Hcpicidin (ng/ml)	0.95±0.154 ^{**}	1.06±0.147 ^b	0.944±0.165 ^{**}	1.055±0.15 ^b	1.08±0.154 [*]
H/F ratio [‡]	0.0094±0.0015 ^{**}	0.013±0.0021 ^b	0.0089±0.0017 ^{**}	0.0107±0.0017 ^b	0.0159±0.0016 [*]

Same Letters in the same group before and after is not statistically significant; * statistically significant at $P < 0.05$ compared to control subjects.

TABLE (2): Cut-off values for included markers along with the associating sensitivity, specificity, predictive values, diagnostic accuracy, AUC and 95% confidence interval (95% CI) of the AUC for differentiation between various groups

Groups	Group I / II		Group I / III		Group II / III	
	Ferritin (ng/ml)	Hepcidin (ng/ml)	Ferritin (ng/ml)	Hepcidin (ng/ml)	Ferritin (ng/ml)	Hepcidin (ng/ml)
Cut-off value	93	1.15	68	1.25	82	1.05
Sensitivity %	95	70	100	100	95	60
Specificity %	75	40	60	15	95	60
+PV %	79.2	53.8	71.4	54.1	95	60
-PV %	93.7	57.1	100	100	95	60
Diagnostic accuracy %	84	55	80	57.5	95	60
AUC	0.882	0.501	0.823	0.536	0.991	0.545
95% CI	0.741 – 0.962	0.339 – 0.663	0.669 – 0.925	0.372 – 0.695	0.896 – 1.000	0.380 – 0.703

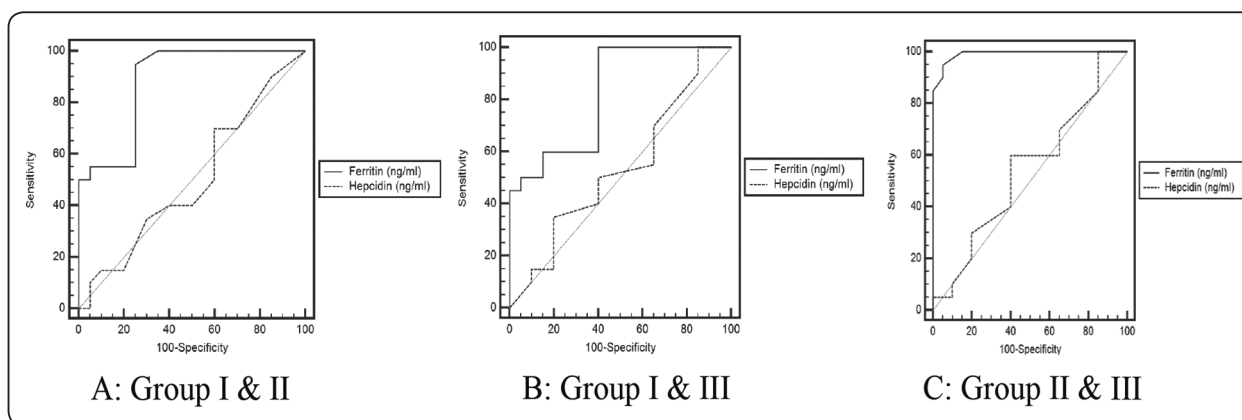


Fig. (1) ROC curves of ferritin & hepcidin to differentiate between different enrolled groups

DISCUSSION

Since research in the last 2 decades considered the relationships among inflammation, inflammatory mediators, systemic diseases and periodontium, our study aimed to explore the role of ferritin as an acute-phase reactant, elevated in chronic infections as periodontitis, In addition to hepcidin, as an essential regulator of systemic iron homeostasis, in periodontitis and T2DM.

Key finding of our results is that significant higher ferritin serum level was found in both periodontitis groups than control group which was decreased after treatment, associated with improvement in clinical parameters. However hepcidin serum level was higher in control compared to studied groups in spite of its increasing significantly after treatment in the periodontitis groups as well as their ratios were also increased significantly.

The demonstrated level of serum ferritin in the current investigation was in accordance with former study where **Chakraborty et al.**²⁴ had found that serum ferritin level raised in patients with chronic periodontitis and decreased post-treatment. They suggested that inflammation may up regulate ferritin expression in serum of patients with chronic periodontitis via inflammatory mediators, interleukin-6 (IL-6) & tumor necrosis factor- α (TNF- α). Other contributing factors could increase serum ferritin level such as bacterial virulence factors and lipopolysaccharides. Consequently non-surgical periodontal treatment improved periodontal inflammation in addition to decreasing the existing bacteria thus explaining the decreased serum ferritin after therapy.

Additionally, Guo et al.³ findings of higher ferritin level in the periodontitis patients and periodontitis with T2DM groups than in the control group suggested, as demonstrated by **Mandalunis et al.**,²⁵ that iron overload existence contributes to inhibition of alveolar bone formation which in turn, may related to the progression of periodontitis. Moreover iron overload also has been confirmed as an independent risk factor for T2DM through oxidative stress injury that it could perform to hepatocytes and pancreatic β cells¹³. So, the presented results could be attributed to these postulations.

As proved in literature, subgingival accumulation of anaerobic bacteria is the major cause of periodontium destruction subsequently periodontitis. As well it is reported that periodontitis incorporate β -haemolytic bacteria that trigger dissolution of erythrocyte accordingly increase of iron load²⁶. So reinforcing our results and hypothesis correlating ferritin to periodontitis.

However in contrast to our results **Prakash et al.**²⁷ and **Latha S et al.**²⁸ studies had showed no difference in serum ferritin levels with respect to chronic periodontitis and healthy groups. This difference may be due to different enrolled subject's criteria in addition their studies were not interventional.

Regarding hepcidin serum levels in the studied groups, our results exhibited higher level in control group than periodontitis groups with mean values, 1.080, 0.945 & 0.944 in control, periodontitis with T2DM & periodontitis patients respectively. On the other hand a study by **Carvalho et al.**²⁹ hypothesized that systemically healthy subjects suffering from chronic periodontitis showed higher level of hepcidin than control group based on the fact of releasing immunoinflammatory mediators, such as prostaglandin E2, matrix metalloproteinase, IL-6, TNF- α in addition to production of acute phase reactant as hepcidin & C-reactive protein^{30,31}.

In the same line a recent study conducted by **Guo et al.**³ who found higher hepcidin level in chronic periodontitis & chronic periodontitis with T2DM groups than control, has its plausibility based on the insight that, the state of iron overload could enhance serum hepcidin production. This contradiction in results may be owed to included subjects number and for differences in the employed ELISA kits sensitivity in both studies. Putting into consideration that, in spite of revealed difference between mean values of hepcidin in our studied groups it is a subtle change but we couldn't ignore as it is significant.

In the herein investigation, significant increase in the hepcidin serum level is demonstrated after periodontal therapy in both periodontitis groups. On the contrary previous study showed decreased of the prohormone of hepcidin (prohepcidin) after periodontal treatment, expecting that decrease as response to decreased iron overload and arresting of inflammatory process³². This difference may return to the different measured markers and the included subjects as they had treated patients with chronic kidney disease in addition to different enrolled populations.

Based on the fact that hepcidin is regulated through iron levels, the level of hepcidin only could not accurately reflect the ability of the body to regulate iron load. Thus the hepcidin/ ferritin ratio has been calculated to estimate the adequacy of

hepcidin production for a measured iron load. The decrease in this ratio means insufficient hepcidin in relation to overloaded iron in the body^{33,34}.

Back to our results a significant higher ratio in control group in comparison to periodontitis group and also significant increase was revealed within each group pre and post operatively which is a logic result consistent with increase hepcidin level. In contrast to presented results, **Guo et al.**³ declared that the decrease in the serum hepcidin/ferritin ratio in chronic periodontitis patients with T2DM suggested inadequacy of hepcidin in these patients.

It has been supposed that the increase in hepcidin level reflects the comprehensive biological responses that occur during the process of periodontitis, as multifactorial local inflammatory condition in addition to its alteration in systemic response. In the present findings, it should be taken into account it's a significant but minor increase that could be related to other underlying factors or may perhaps be due to other inflammatory changes occurred in the enrolled subjects within the interval of our study period. Since no previous investigations in the literature have studied the possible association between periodontitis, serum hepcidin & T2DM before and after periodontal treatment; so further research is recommended to clarify the exact interrelationship between them.

ROC curve analysis for the present investigation demonstrated high diagnostic accuracy of serum ferritin level between each two groups. The highest diagnostic accuracy is expressed in the systemically healthy periodontitis and the control subjects followed by the two periodontitis groups together and finally periodontitis with T2DM and control group.

Concerning the hepcidin analysis also highest diagnostic accuracy was showed within periodontitis and the control subjects then periodontitis with T2DM and control groups ending with the two periodontitis groups. In addition ferritin showed statistically significantly higher AUC than hepcidin

in all studied groups. To the best of our knowledge; this study is the first to perform ROC curve analysis for ferritin & hepcidin along with diabetes & periodontitis

Collectively, within the limits of the presented results cogitation for connection between ferritin, periodontitis & T2DM should be well thought out. However the link with hepcidin still indistinguishable so we recommend more studies to prove the clinical significance of these findings using more specific body fluid to periodontium environment as saliva or gingival crevicular fluid

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