ORIGINAL ARTICLE

Relationship of Growth Differentiation Factor 15 to Functional Iron Deficiency in Hemodialysis Patients

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ABSTRACT

Background: Functional iron deficiency is a disease remained under diagnosed and undertreated because the available diagnostic method serum ferritin is not predictive of disease severity and it is false positive in complex cases. Out of many considered biomarkers like hepcidin, Mean corpuscular volume MCV, mean corpuscular hemoglobin MCH, reticulocyte hemoglobin concentration (CHr) and many others, serum GDF 15 is a new marker for this disease

Aim: To compare the levels of serum GDF-15 in hemodialysis patients with and without Functional iron deficiency.

Methods: This study was cross-sectional comparative study and was conducted in Department of Physiology, Shaikh Zayed FPGMI Lahore and Sheikh Zayed hospital nephrology department after taking permission from concerned departments. Informed consent was taken from patients also. We measured serum GDF 15, serum ferritin, serum iron, Total iron binding capacity, complete blood count, hemoglobin in 140 hemodialysis patients. The patients were divided into 2 groups .Group A had functional iron deficiency and Group B did not have functional iron deficiency. Serum samples were taken, processed and assessed for GDF-15 and ferritin levels using commercially available ELISA kits. Mean GDF-15 of both groups was determined and compared. P value less than ≤ 0.05 was considered statistically significant.

Results:. Serum GDF-15 levels are significantly elevated in functional iron deficiency patients on hemodialysis. Mean GDF-15 levels in functional iron deficiency patients were 2759±2709 pg/ml whereas mean GDF -15 levels in patients without functional iron deficiency were 783±1258 pg/ml (p value <0.001).

Conclusion: We conclude that serum GDF-15 levels are raised in Functional iron deficiency hemodialysis patients as compared to hemodialysis patients without functional iron deficiency . GDF-15 seems to be a new promising tool to detect FID.

Keywords: Functional iron deficieny, growth differentiation factor 15, ferritin

INTRODUCTION

It is important for doctors to develop an understanding of functional iron deficiency in hemodialysis patients. About 20 -25% of hemodialysis patients suffer from functional iron deficiency disease. The incidence is increasing[1]. Iron is stored in reticuloendothelial cells of spleen, liver and bone marrow as ferritin[2]. Normal serum ferritin concentration is 18 to 200ng/ml[3]. Functional iron deficiency is distinguished by the existence of sufficient iron stores in the body, but lack of ability to mobilize this iron from the liver and other storage sites to carry on erythropoiesis even with the erythropoietin treatment[4]. it is block of iron transport into erythroid precursors[5]. Patients suffering from Functional iron deficiency have transferrin saturation less than 25%, ferritin levels more than 200ng/ml and Hb less than 11g/dl[6]. Clinically patients with Functional iron deficiency have anemia which does not respond to erythropoietin treatment and iron therapy[7]. Various clinical trials have shown that I/V vitamin C improve anemia of functional iron deficiency.

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Pathogenesis of Functional iron deficiency is still under research. It is postulated that increased pro inflammatory cytokines such as interleukins 1 & 6 in hemodialysis patients increases serum levels of hepcidin. Increased hepcidin levels inhibits iron absorption in the intestines, iron transport in macrophages and liver cells by degrading ferroportin, which are membrane channels in cell membrane through which iron molecules move. Thus a diabetic like picture of iron deficiency develops despite adequate iron stores in the body[8]. On the other hand Absolute (TRUE) iron deficiency is classic iron deficiency when serum ferritin concentration is less than 100ng/ml[9]. This type of iron deficiency is caused by iron deficient diet or by increased blood loss .Intravenous iron is effective treatment for absolute iron deficiency patients[10].

Traditional indices used to diagnose functional iron deficiency are MCV, MCHC, MCH, transferrin saturation% and ferritin. Various new variables are under research. These are hepcidin, hemoglobin density (HD), soluble transferrin receptor (s-TfR), reticulocyte hemoglobin concentration(CHr)[11]. There is a need for biomarker for diagnosis of Functional Iron deficiency because

1. Nephrologists do not agree on the definition of

functional iron deficiency. There is no consensus on the upper level of ferritin in the nephrology community[9].

2. Mean corpuscular volume (MCV), Mean corpuscular hemoglobin (MCH) , Mean corpuscular hemoglobin concentration (MCHC) are not capable to diagnose acute changes in iron availability[11].
3. Assessing iron stores by bone marrow biopsy is not reasonable and has complications such as bleeding & pain[12]
4. Percentage Transferrin saturation value is affected by nutritional status and shows diurnal variation.[13]
5. Serum ferritin levels fluctuate throughout the day and are elevated in acute inflammation also.

'GDF -15' is also found to be a new marker. Growth differentiation factor-15 is a unique member of the transforming growth factor beta super family which has greater than 40 members[14]. GDF-15 is synthesized as a 62 kilo Dalton intracellular proprotein. After breakdown by a protease it is secreted as a 25 Kilo Dalton disulfide-linked dimeric protein[15]. The GDF15 promoter contains motifs for several transcription factors such as Sp1 and p53 so increase levels of GDF-15 reflect cellular stress[16]. GDF-15 fulfills most of the requirements of an ideal biomarker of FID. GDF-15 has nearly same concentrations among citrated plasma, EDTA-treated plasma and serum[17] .GDF-15 is stable at room temperature for 2 days and it is unaffected by up to 4 freeze thaw cycles. Measurement of GDF15 concentration is not influenced by anticoagulants, albumin, bilirubin or hemoglobin[18].

Wollert et al. (2017) reported that in response to acute tissue injury, GDF-15 levels increase acutely. Cardiac muscle cells secrete GDF-15 following ischemic/ reperfusion injury[19]. Cultured macrophages, adipocytes, Endothelial cells and smooth muscle cells also secrete GDF-15 in stress. Immature erythroblasts also secrete GDF-15. In many types of tumors such as breast cancer, colon cancer[20] and thyroid carcinomas and ovarian cancer[21] GDF-15 is overexpressed. Upon secretion mature GDF-15 rapidly diffuses into circulation. GDF-15 levels are elevated in the conditions associated with inadequate erythropoiesis such as thalassemia and pyruvate kinase deficiency [22]. So measuring GDF-15 levels may help in the diagnosis of various diseases. Normal levels of GDF-15 levels in healthy population with no kidney disease are reported as 460 – 920 pg/ml by Lukaszyk et al. in 2016. [23]

MATERIALS AND METHODS

This study was conducted in National institute of Kidney diseases (NIKD) Sheikh Zayed hospital complex after getting approval from Nephrology department and Sheik Zayed Postgraduate Medical Institute. It was a Cross-sectional, Comparative study conducted from December 2017 to December 2018. (1 Year). 140 hemodialysis patients of National institute of Kidney disease Sheikh Zayed Hospital were selected. All these patients were on regular hemodialysis for 4-5 hours 3 times a week .The blood flow was between 200 and 300ml/min with the dialysate flow of 500 ml/min. Ultrafiltration varied according to the patient weight. All patients were dialyzed using low-flux polysulphone membrane and low- flux modified cellulose membrane with a bicarbonate –buffered dialysate.
Group A: composed of 70 hemodialysis patients who had functional iron deficiency. (transferrin saturation less than 25% ,hemoglobin less than 11 mg/dl ,and serum ferritin above 200 ng/ml)
Group B: composed of 70 hemodialysis patients who did not had functional iron deficiency. They had Transferrin saturation more than or equal to 25% and serum ferritin 100ng/ml to 200ng/ml .The inclusion criteria were: patients undergoing hemodialysis for at least 6 months, Adults (Age more than 18 years). The exclusion criteria were from Positive for HIV, Hepatitis B, Hepatitis C, Evidence of significant bleeding during last 6 months, Sepsis, Hemolytic anemia, Thrombosis, Acute cardiovascular complications like acute heart failure, Absolute iron deficiency, Iron replacement/supplementation during last 6 months, Blood transfusion during last 6 months.

The study was started after taking approval from the Ethical Review Board of Federal Post Graduate Medical Institute Sheikh Zayed Medical Complex Lahore. Total of 140 hemodialysis patients were taken .The patients were divided into two groups. Patients were asked for their written informed consent for participation in the research .A demographic questionnaire recorded patient name, age, gender, hospital registration number, length of time receiving hemodialysis. Pulse, temperature, RR was recorded to rule out any acute infection.

A total of 7ml of venous blood sample was collected prior to dialysis session and before heparin administration. Samples were allowed to clot at room temperature overnight. Serum was separated from blood by centrifugation at the rate of 5000 revolution per minutes for 10 minutes and frozen at -20C till the test was performed. From this 2ml was used in a separate EDTA anticoagulated viol to measure complete blood count including haemoglobin .It was measured by a Automatic cell counter (Abacus 380 Diatron. Budapest, Hungary). 2ml was used to measure Growth differentiation factor 15 by ELISA at National health Research centre (NHRC). Test was performed according to manufacturer instruction (R & D systems, Minneapolis, MN, USA, CAT no DGD150) .1 ml was used to calculate serum iron and total iron binding capacity at Biochemistry laboratory Sheikh Zayed Hospital by Autoanalyzer. Transferrin saturation was calculated by IRON/TIBC X100[25].

1ml was used to measure serum ferritin by ELISA Kit. (Chemus bio Science USA, human Ferritin ELISA kit Cat No 10601) 1ml was used to measure Parathyroid hormone (PTH) by ELISA Kit. (Diametra, Italy ,Ref DK0157, lot 4424) . Serum PTH was measured to rule out secondary renal anemia due to Hyperparathyroidism.

Statistical Analysis: The data was entered and analyzed using IBM-SPSS version 20.

- Mean ± SD and median with inter-quartile range was given for quantitative variables i.e., age, GDF15, ferritin concentration, transferrin saturation, duration of disease, serum iron, total iron binding protein, hemoglobin, mean corpuscular hemoglobin, mean corpuscular volume and mean corpuscular hemoglobin concentration.
- For qualitative variables like age Frequency and percentage was taken.

- The data was checked for normal distribution by Shapiro Wilk test .Data was not normally distributed. So non parametric Mann Whitney U test was used to compare the mean difference in quantitative variables between groups. Chi square test was to determine the gender difference between groups. Spearman correlation coefficients were used for correlation analysis between GDF-15, age, ferritin, transferrin saturation, hemodialysis duration , serum iron, total iron binding capacity, hemoglobin, mean corpuscular hemoglobin, mean corpuscular volume ,mean corpuscular hemoglobin concentration.
- The cutoff of GDF-15 for detection of functional iron deficiency was identified by a receiver-operating characteristic (ROC) curve and the area under the ROC curve (AUC) was calculated.
- A p-value of ≤ 0.05 was considered statistically significant.

RESULTS

In this study hemodialysis patients were selected into two groups. 70 patients who suffered from Functional iron deficiency were in Group A and 70 patients in Group B who did not have functional iron deficiency. There was no difference in terms of age and gender between both groups. Data was found to be not normally distributed. There was significant difference in median GDF15 between the groups. Group A had higher GDF15. The mean GDF15 of group A was 2759.7 ± 2709.2pg/ml and mean GDF15 of group B was 783.1 ±1258.9pg/ml. Group A had significantly higher mean ferritin concentration. For group A it was 487.2±316.2ng/ml and for group B it was 260.8±268.4 ng/ml. There was no significant difference in median time duration on hemodialysis of patients between the groups. There was no significant difference in median serum iron, Total iron binding capacity ,hemoglobin, MCH, MCV,MCHC between both the groups.

No significant correlation was found between GDF15 and age, transferrin saturation, duration in years on hemodialysis and serum iron in both groups. However, the GDF15 levels significantly correlated with ferritin only in group B ($r = -0.622$, $p < 0.001$). No significant correlation was found between GDF -15 & TIBC, Hemoglobin, MCH, MCV,MCHC in both group A and Group B.

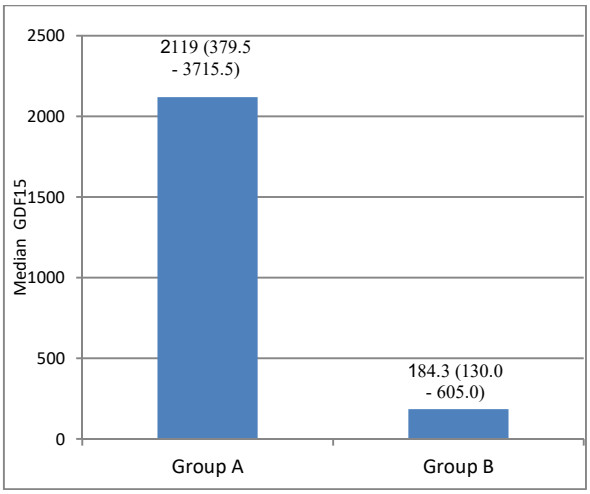

Figure 1: Comparison of serum GDF-15 between Group A & B

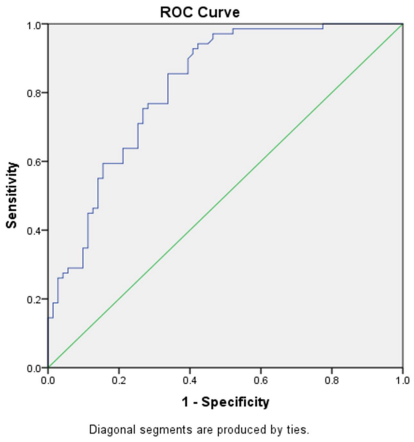

Figure 2: ROC curve showing GDF-15 is a indicator of Functional iron deficiency

To determine the cutoff values of GDF15 for group A, ROC analysis was performed. The optimal cutoff value of GDF15 was 316.0pg/mL with 81.2% sensitivity and 66.2% specificity. The area under curve (AUC) was 0.818 with Confidence interval of 0.749 – 0.887 (p-value < 0.001) which shows that GDF-15 is good marker for diagnosis of group A.

Table 1: Biochemical characteristics of hemodialysis patients with and without functional iron deficiency .

	Group A /FID (+)	Group B FID (-)	P value
Age	51 (41.0 – 62.0)	52 (37.7 - 59.3)	0.455
Gender	40/30	38/32	0.860
GDF-15	2119.0 (379.5 - 3715.5)	184.3 (130.0 – 605.0)	< 0.001
Ferritin	381(341.9 - 528.9)	190 (58.8 - 385.9)	< 0.001
Transferrin saturation %	20.0 (17.0 – 23.0)	26.1 (19.5 – 34.0)	< 0.001
Time duration on hemodialysis of patients	4.0 (1.0 – 7.0)	3.0 (1.0 – 7.3)	0.892
Serum Iron	41.0 (34.3- 49.5)	40.0 (34.0 – 45.0)	0.226
Total iron binding capacity	205.0 (189.0 - 259.5)	233.0 (198.0 – 264.0)	0.121
Hemoglobin (g/dl)	9.9 (9.1- 10.5)	9.8 (8.9 – 11.0)	0.707
Mean Corpuscular volume	75.6 (72.0 – 78.0)	77.0 (73.0 – 81.0)	0.108
Mean corpuscular Hemoglobin (Picograms)	27.6 (26.0 – 29.0)	27.0 (25.1 – 29.0)	0.263
Mean corpuscular hemoglobin concentration	35.2 (34.3 – 37.6)	35.0 (34.1 – 37.1)	0.181

Table-2: Correlation of GDF15 with age, ferritin, transferrin saturation, Duration in years on hemodialysis and serum iron in group-A and group-B

Study Groups	GDF15	Age (years)	Ferritin (ng/ml)	Transferrin saturation%	duration in years	serum iron (ug/dl)
Group A	Correlation Coefficient[a]	0.097	-0.017	0.029	-0.127	-0.072
	p-value	0.428	0.889	0.811	0.300	0.556
Group B	Correlation Coefficient	0.164	-0.622	-0.213	0.136	-0.107
	p-value	0.176	< 0.001*	0.074	0.259	0.372

Table 3: Correlation of GDF-15 with total iron binding capacity, Hemoglobin, MCH, MCV,MCHC in group-A and group-B.

Study Group	GDF15	Total iron binding capacity ug/dl	Hemoglobing/dl	MCH(pg)	MCV (fL)	MCHC(g/dl)
Group A	Correlation Coefficient [a]	-.210	.227	.077	-.281	.448
	p-value	.083	.061	.531	.191	.203
Group B	Correlation Coefficient [a]	.143	.067	-.233	-.283*	.097
	p-value	.234	.581	.051	.712	.421

DISCUSSION

It is clinically important to diagnose Functional iron deficiency anemia[9]. Various variables are used to differentiate between FID and absolute iron deficiency. MCV, MCH, MCHC, Hemoglobin, Transferrin saturation, serum ferritin are used traditionally.

In our study we compared the levels of serum GDF-15 in hemodialysis patients with and without functional iron deficiency. Serum GDF-15 levels are significantly elevated in functional iron deficiency patients on hemodialysis. Mean GDF-15 levels in functional iron deficiency patients were 2759±2709 pg/ml whereas mean GDF -15 levels in patients without functional iron deficiency were 783±1258pg/ml. Yilmaz et al. also showed that both GDF-15 and hepcidin levels were significantly elevated in Hemodialysis patients who had FID. Median GDF-15 levels were 1239.7pg/ml in patients with FID as compared to 452.1 pg/ml in patients without FID in Yilmaz study[7]. No difference was found in GDF-15 levels in patients with and without FID by Malyszko. (5026 pg/ml -5218 pg/ml)[9].

The study done by Yilmaz had 105 hemodialysis patients .53 has FID & 52 without FID. Malyszko study had 98 patients .75 did not had FID and 23 had FID. Our study had 140 patients. 75 had FID. 75 did not have FID.

Yilmaz defined FID as serum ferritin more than 800ng/ml, transferrin saturation less than 25%, Hemoglobin less than 11g/dl . Whereas our criteria for the definition of FID was serum ferritin more than 200 ng/ml, same as that of Malyszko definition.

By ROC analysis Yilmaz identified the cut-off value of GDF-15 as 749pgml with 96.4 sensitivity,100% specificity,100% positive predictive value (PPV). The area under curve was 0.982. In our study the cut off value for GDF-15 was 318pg/ml with 81.2 sensitivity and 66.2 % specificity .The area under curve was 0.818. Yilmaz also showed that GDF-15 is a better indicator of FID than hepcidin by ROC analysis.

In this study the mean age of functional iron deficiency patients (50.7± 13.8 years) was similar to mean age of patients without functional iron deficiency (48.7±14.4 years) . Lukaszyk et al. (2016) reported high GDF-15 in elderly patients; however in our study the mean age of both groups was similar and less than 60 years[23].

According to our study the mean duration of years on hemodialysis in FID patients was 4.1 years and without FID was 3.0 years. There was no significant difference in duration of years on hemodialysis in both groups (P= 0.892). Nair et al. (2017) reported high GDF-15 levels in advanced renal failure patients on hemodialysis for more than 10 years ; however in our study patients in both groups would probably have same level of renal failure and mean hemodialysis duration was less than 10 years[24].

In the present study the mean Transferrin saturation of patients with functional iron deficiency (19.5±4.2%) was less than the mean transferrin saturation of patients without FID (28.2±12.4%). This significance is in accordance with the definition of functional iron deficiency[7]. Both Yilmaz et al.(2016) and Malyszko et al(2012) stated that FID patients have transferrin saturation less than 25%[9].

In our study mean serum ferritin concentration of patients with functional iron deficiency (457±316ng/ml) was more than the ferritin concentration of patients without functional iron deficiency(260.8 ±268ng/ml). This significant difference in the mean ferritin concentration between two groups is also in accordance with the definition of functional iron deficiency described by Malyszko[9]. Thomas et al (2013) also stated patients with FID have serum ferritin more than 200 ng/ml[11].

Yilmaz observed a positive correlation between GDF-15 and ferritin levels (r=0.745, P <0.01) in patients with FID. Malyszko observed no correlation between GDF-15 and ferritin levels . No correlation was found between GDF-15 and ferritin in FID patients in our study too. But our study showed a negative correlation between GDF-15 levels and serum ferritin levels.(r= -0.622) in patients without FID.

Our study showed there is no significant difference in mean of serum iron, MCHC, MCV, MCH, Hemoglobin and total iron binding capacity between both groups . Thus they cannot be used in the assessment and diagnosis of this disease.. Similarly Fusaro et al.(2005) Enko et al (2015), Bovy et al in 2007 found no clear cut advantage in using MCV ,MCHC ,MCH and hemoglobin as indicators of FID[25,26,27]. No difference was observed in MCV,MCHC, MCH and hemoglobin in patients between FID and without FID in study done by these studies. However a study by Gezgin et al (2019) showed that Mean hemoglobin, MCV were significantly lower in FID patients as compared to patients with absolute iron deficiency[5].

Genzin et al. (2019) showed that new red cell parameters such as hemoglobin density (LDH),red blood cell size factor can distinguish Functional Iron Deficiency (FID) from absolute iron deficiency[5]. Yilmaz said that patients with FID should have a reticulocyte hemoglobin content (CHr) less than 29 pg.[7] Fusaro et al . (2005) found no clear cut advantage in using soluble transferrin receptor(s-TfR) and reticulocyte hemoglobin content (CHr) as indicators of FID.[25] However Enko et al.(2015) stated that soluble transferrin receptor was better indicator of FID as compared to hepcidin and ferritin.[26] The problem with sTfR is that they not readily available everywhere and are costly to measure also. Interestingly Bovy et al in 2007 showed that percentage of hypochromic red cells (% hypo) was showed to be a better indicator of FID than transferrin saturation, ferritin and reticulocyte hemoglobin content (CHr) .[27]

Our study did not showed any correlation between GDF-15 and TIBC, hemoglobin, MCH,MCV,MCHC, age of patients ,transferrin saturation and duration in years on hemodialysis. Study done by Malyzsko and Yilmaz also showed no correlation between GDF-15 and these variables.

Hepcidin is a another marker under research for the diagnosis of FID[28] but it levels are also affected by acute inflammation[29] and advanced renal failure[30]. Similarly another marker Bone Morphogenetic protein 6 (BMP-6) marker seems to be unrelated to FID . Malyszco et al did not observe any difference of BMP-6 levels between hemodialysis patients with and without FID[9].

Other authors have suggested to diagnose functionally iron deficiency clinically by observing the effects of erythropoietin and iron on patient's hemoglobin levels. [31,]

The mechanisms that cause high GDF-15 in functional iron deficiency patients might be as follows: 1. GDF 15 may increases to suppress abnormally elevated hepcidin levels in FID patients .This will increase iron absorption and normalize iron absorption. Tarkun et al. stated that high amount of GDF-15 are secreted by placenta in pregnancy leading to increase iron absorption by suppression of hepcidin expression.[32] High levels of GDF-15 in thalassemia patients suppressed hepcidin mRNA in human hepatocytes. When GDF-15 was depleted by immunoprecipitation, amount of hepcidin mRNA in cells increased. 2. Chronic inflammation is present in hemodialysis patients of functional iron deficiency. This chronic inflammation/ Cellular stress may increases the expression of GDF-15 by immature erythroblasts resulting from ineffective erythropoiesis[33].

3. GDF-15 maybe induced by iron depletion in erythroblasts. Iron depletion develops in erythroblasts as a result of iron sequestration in reticuloendothelial cells (feature of FID). GDF-15 RNA & protein is strongly responsive to intracellular iron depletion[34]. Local iron depletion in rapidly dividing cancer cells causes up regulation of GDF-15 in prostate, colorectal cancers[35].

This is the first study in Pakistan to evaluate serum GDF-15 as a biomarker of Functional iron deficiency in patients with end stage renal failure .Explaining the signaling pathway of GDF-15 may help to understand the exciting puzzle of GDF-15. We conclude that Serum GDF-15 levels are elevated in Functional iron deficiency in hemodialysis patients of Pakistani Population. We recommend that prospective randomized trials and experimental studies should be carried out to determine the exact role of GDF-15 in functional iron deficiency. Serum GDF-15 can be a beneficial biomarker to monitor iron status and iron demand in hemodialysis patients . Future research should be carried out to complete understanding of GDF-15 signal transduction and functions .

This study had some limitations. First a cross-sectional study is not enough to establish a relationship between GDF-15 and functional iron deficiency. It should be supplemented with a prospective randomized trial. Second, the patients in our study were ethnically Punjabis and hence, caution should be exercised when extrapolating our results to other ethnic groups. Third ,this was a single centre study.

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