Diagnostic Implication of Iron Deficiency in the management of Toxicity Associated with Chronic Diseases

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Abstract: Objective: The current investigation focuses on the differentiation between true iron deficiency anemia (IDA)**,** anemia of chronic diseases (ACD) and coexistence iron deficiency anemia in patients with chronic inflammatory disorders (ACD/IDA), in order to avoid toxicity and complications resulting from serious effects of therapeutic iron supplementation to patients with anemia of chronic diseases (ACD). Therefore, the accurate iron therapy could be given safely to those patients. To achieve these goals, 64 anemic patients (males and females), in addition to 10 healthy normal subjects were chosen. Individual parameters in blood serum related to iron and inflammation besides, bone marrow examination for the presence of stainable iron were estimated. **Patients and Methods:** The anemic patients were classified into three groups according to the presence or absence of stainable iron in bone marrow, also according to clinical data. **I**- True iron deficiency anemia (IDA) Group**:** patients had no stainable iron in the bone marrow. **I**-Anemia of chronic disease (ACD) Group**:** patients had stainable iron in bone marrow with chronic disorders. **III**-COMBI (ACD∕ IDA) group**:** patients had **no** stainable iron in bone marrow together with an infectious diseases, chronic inflammatory diseases or non- hematological malignancy, in addition to patients who had a C-reactive protein (CPR) above 20mg/l. **Samples and analysis: 1**-Bone marrow aspiration: was collected from the sternum bone or iliac crest by using bone marrow aspiration needle under local anesthesia, and many thin smears from bone marrow were done immediately. **2**-Blood analysis: included; complete blood count with the assessment of RBCs indices. **3**-Serum iron profile: included; serum iron concentration, total iron binding capacity (TIBC) **(**an indirect measurement of transferrin concentration**)** and serum ferritin level. **4**-Estimation of C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) as indicators for the presence of inflammation.**5**-Determination of serum transferrin receptors level (sTfR) **6**-Measurement of serum hepcidin hormone concentration. **Results**: Regard to**, sex and age distributions:** there were insignificant differences between the studied groups. **Hemoglobin concentrations:** showed significant decrease between three patient groups (IDA, ACD and ACD/ IDA) with normal group. **MCH & MCV values:** indicated significant decrease between IDA, ACD/ IDA groups with normal groups, but there was insignificant difference between ACD group with normal group**.** Regard to **RBCs count,** there was significant decrease between the three patient groups (IDA, ACD and ACD/ IDA) with normal group. **Considering the reticulocytes count,** there was significant increase between the three patient groups (IDA, ACD and ACD/ IDA) with normal group**.** Regard to **serum iron concentrations**, there were significant decrease between the three patient groups with normal group. Regard to **TIBC values,** There were significant increase differences between IDA, ACD/IDA groups with normal group, but there were significant decrease between ACD group and normal group**,** whereas there was significant increase between IDA with ACD/IDA groups. Regard to **ferritin levels,** showed significant decrease between IDA group with normal group and significant increase between ACD and ACD/IDA groups with normal group. Considering **CRP levels,** there were insignificant differences between IDA group with normal group, but there were significant increase between ACD and ACD/IDA groups with normal group. Considering **serum transferrin receptors level,** there were significant increase between IDA and ACD/IDA groups with normal group**,** but insignificant differences between ACD group with normal group and between IDA with ACD/IDA. As regards **hepcidin level,** there was significant decrease between IDA group with normal group, but significant increase differences between ACD and ACD/IDA groups with normal groups. Also, there was significant increase between ACD group with ACD/IDA group. **In conclusion:** the present data suggested that the sTfR could be used as a credible differentiating marker in the diagnosis of true iron deficiency anemia accompanied by chronic disorders (ACD/IDA). As well as, serum hepcidin might be worthy diagnostic gadget to recognize between ACD and ACD/IDA to appraise the ability to respond to oral iron therapy and stimulating the development of new diagnostic and therapeutic methods for these disorders. Therefore**,** the proper iron supplementation could be given safely, to avoid toxicity resulting from the growth-promoting effect of iron on microorganisms**,** as well as the negative effect of iron on innate immune functions.

1. Introduction

According to the *World Health Organization, (2011)* iron deficiency is thought to be the most common cause of anemia worldwide. Anemia is a condition in which the number of red blood cells and their oxygen-carrying capacity is insufficient to meet the body's physiologic needs.

True iron deficiency anemia (IDA) considers the most severe consequence of iron reduction and it seemed to be the most common nutrition deficiency globally recognized. However, the causes of true iron deficiency anemia (IDA) having many features, it generally results when iron demand by the body are not met by iron absorption, despite of the reason *(Hempel and Bollard, 2015).*

Anemia of chronic disease (ACD) is the most common anemia in hospitalized patients and develops in subjects with diseases involving acute or chronic immune activation, such as patients with infections, malignancies, or autoimmune disorders *(Poggiali et al., 2014).* Sometimes, true iron deficiency is accompanied by chronic inflammatory disorders (ACD/IDA)**,** therefore differentiation between ACD and ACD/ IDA is clinically important**,** because iron supplementation is of benefit for ACD/IDA patients, but might be hurtful for ACD patients, particularly if they have infections or malignancies *(Nielsen et al., 2015).*

The absence of stainable iron in the bone marrow still the gold standard for the diagnosis of iron deficiency, but it cannot be performed regularly because it requires technical expertise, painful, expensive and time consuming *(Johnson and Graham, 2011).*

New markers are needed to evaluate iron for erythropoiesis and to estimate the accurate iron therapy when serum ferritin and transferrin **(**the main iron transporter protein in the blood**)** are insufficient without requiring bone marrow aspiration *(***Stein and Dignass, 2013).** Serum transferrin receptors (sTfR) reflect erythropoiesis and inversely the amount of iron available for erythropoiesis. Unlike serum ferritin**,** sTfR concentrations are not affected by the presence of inflammation *(Abitboletal., 2015).*

Recently, hepcidin **(**a liver-produced peptide hormone**)** was found to be a link between the immune system (cytokines) and the movement of iron to and from the storage depots *(D'Angelo, 2013).* Hepcidin is commonly present in chronic inflammatory conditions due to infectious or cancer *(Weiss, 2015 & Wang and Babitt, 2016).* The induction of hepcidin by inflammation is primarily mediated by cytokine (IL-6) to stimulate liver to synthesis and produce hepcidin to control the iron status in condition of ACD patients with chronic disorders and leads to hypoferremia due to ferroportin degradation and iron retention in iron store *(Ludwiczek et al., 2003).* Hypoferremia is a protective response against infection by depriving bacteria or microorganisms from iron**,** as well as may also be reinforced by antimicrobial activities of hepcidin. The persistence of hypoferremia restricts iron availability for erythropoiesis *(Ganz and Nemeth, 2015).*

The role of hepcidin in iron homeostasis came by *(Zhao et al., 2013)* in 2oo4 with the fact that hepcidin acts systemically to lower iron in the serum by binding to ferroportin **(**iron exporter**)** instead of iron. Ferroportin (FPN1) is a protein that located in iron stores and carries iron from its stores into plasma to be transported by transferrin **(**the major iron transporter protein in the blood) to the bone marrow. Hepcidin binds to ferroportin (FPN1) forming hepcidin - ferroportin (FPN1) complex**,** then both proteins disintegrated leading to retention of iron inside the iron stores *(De Domenico et al., 2007).*

Aim of the Present Study

The present investigation focuses on the differentiation between true iron deficiency anemia (IDA)**,** anemia of chronic diseases **(**ACD**)** and coexistence iron deficiency in patients with chronic inflammatory disorders **(**ACD/ IDA**),** to avoid toxicity resulting from detrimental effects of therapeutic iron supplementation on patients with anemia of chronic diseases (ACD)**,** because clinically iron supplementation will be of benefit for the ACD/IDA patients**,** but may be deleterious for ACD patients. To achieve these goals, 64 anemic patients (males and females), and ten (10) healthy control subjects were chosen. As well as, individual parameters in blood serum related to iron and inflammations, in addition to bone marrow examination for the presence of stainable iron were estimated.

Study design:

This is a randomized clinical study that was conducted on 64 anemic patients, in addition to ten (10) healthy subjects serving as healthy control group admitted to Tanta Al-Fadaly hospital.

The anemic patients were categorized into three groups based on the presence or absence of stainable iron in bone marrow and according to the clinical data.

IDA group:

Twenty-seven patients (15women and 12 men) who fulfilled all the morphologic criteria of iron deficiency and had no stainable iron in the bone marrow, they were classified as IDA patients.

ACD group:

Twenty-seven anemic patients (10 women and 17 men) were classified as ACD patients, who had stainable iron in the bone marrow, in addition to acute or chronic infections, chronic diseases and other inflammatory diseases.

COMBI group (ACD/IDA**):**

Ten(10) anemic patients who had **no** stainable iron in the bone marrow together with an infectious diseases, or a chronic inflammatory disorder (such as rheumatoid arthritis or colitis ulcerosa) or a non-hematological malignancy. In addition to, patients who had a C-reactive protein (CRP) value above 20mg/L were placed in a COMBI group.

►**Inclusion criteria:**

* Adults aged from 39 to 60 years old.
* Clinically, healthy subjects with no history of anemia and diseases.
* Patients with history of anemia (IDA, ACD and ACD/IDA).

►**Exclusion criteria: including;**

* Hemolytic anemia,
* Defined vitamin B12 or folate deficiency,
* Blood transfusion within the past 3 months,
* Hematologic malignances, trauma, cancer patients currently receiving chemotherapy or who received chemotherapy in the last 3 months, "since chemotherapy and/or radio therapy as well as bone marrow infiltration by tumor cells might alter the pathophysiology of the anemia compared with subjects with ACD on the basis of autoimmune or infectious disease".
* Patients who currently taking iron supplements or receiving recombinant such as erythropoietin *or* mycophenolate mofetil therapy.

All patients were subjected to:

1-Full history taking with emphasis on:

* Age, gender, occupation, level of education.
* Time elapsed before hospital admission.
* History of medical diseases that include active infections.

2-Compelete physical examination including:

* Regular monitoring of vital signs (pulse, blood pressure, respiratory rate and temperature).
* General clinical examination.

►**Samples and analysis:**

Samples

♦**Bone marrow aspiration**

The bone marrow was collected from the sternum bone or iliac crest with the help of the bone marrow aspiration needle under local anesthesia.

Immediately, many thin smears from bone marrow were done. The remaining of the sample was placed in EDTA tube for further investigations.

♦**Blood**

Ten milliliters (10mL) of freshly venous blood were withdrawn from each subject under complete aseptic precautions. Two ml of blood were placed in EDTA tube for complete blood count. The remaining of the blood was placed in sterile tubes without use of any anticoagulants, and left to clot for 30 minutes. Serum was then separated by centrifugation at 3000g for 15 minutes. Serum was subdivided immediately into three Eppendorf tubes; one for immediate assay of Iron and TIBC. The second Eppendorf tube was for immunoassay determination of CRP and Ferritin The last was frozen at -20°C for further specific investigations of serum transferrin receptors and hepcidin. Before analysis, frozen samples were allowed to thaw at room temperature. Hemolysed and lipaemic samples were discarded, also repeated freezing and thawing was avoided.

►**Analysis and Methods**

Analysis included:

* Complete blood count and assessment of RBCs indices (mean cellular hemoglobin [MCH]**,** mean corpuscular hemoglobin concentration [MCHC], and mean corpuscular volume [MCV]**.**
* Reticulocytes count**.**
* Serum iron concentration**.**
* Total iron binding capacity [TIBC]**,** (an indirect measurement of transferrin concentration)**.**
* Serum ferritin level**.**
* Prussian blue stain bone marrow aspiration films
* Erythrocytes sedimentation rate [ESR] and C-reactive protein [CRP]**.**
* Serum transferrin receptor [sTfR]**.**
* Serum hepcidin hormone.

►**Methods:**

I.   Bone marrow aspiration films stain for iron store by using Sigma –Aldrich Iron Stain**.** Reticulo-endothelial iron, usually seen as blue particles on the bone marrow film**.**

II.   Complete blood counts were determined using automated hematology analyzer (ABX Micros 60 analyzer)**.**

III.   Reticulocytes count**:** manual reticulocytes count was carried out using Brilliant Cresyl blue stain *(Lewis et al.,2006)*.

IV.   Serum Iron determination using ELTECH diagnostic kit**.**

V.   Serum total iron binding capacity [TIBC] determination using ELTECH diagnostic kit**.**

VI.   The quantitative determination of ferritin concentrations in human serum by ELISA *(Tietz, 1999)***.**

VII. Measurement of serum transferrin receptors [sTfR] by ELISA *(Lee et al., 2002).*

VIII. Measurement of human hepcidin by ELISA *(Jordan et al., 2009).*

IX. Determination of serum C –reactive protein [CRP] concentration by ELISA *(Ridker et al., 2007).*

►**Data analysis**

Data were presented as mean ± SE. Results were analyzed using the computer program of SPSS. All statements of significance were based on probability of P≤0.05 was considered to be significant.

4. Results:

The current study has been conducted to investigate the biochemical changes accompanying true iron deficiency anemia (IDA), anemia of chronic diseases **(**ACD) and coexist iron deficiency in patients with chronic inflammatory disorders (ACD/IDA), in order to differentiate and distinguish between the three kinds of anemia. Therefore iron supplementation could be given safely.

In the present study all the 74 subjects (healthy **and** patients) were underwent a bone marrow aspiration as a gold standard to examine iron status. Classification of the patients into three groups was based on presence or absence of stainable iron in bone marrow and inflammation. Thirty seven patients showed absence of stainable iron (negative) in bone marrow, included 27 patients with true iron deficiency classified as IDA group and 10 patients with chronic inflammatory disorders classified as ACD/IDA group. On the other hand, 37 subjects showed presence of stainable iron (positive) in bone marrow, included all of 27 patients with chronic disorders, classified as ACD group and 10 healthy normal subjects classified as normal control group.

The present results demonstrated that there were insignificant differences between **sex and age distributions** *P*>0.05. The data are graphically illustrated in **(**Figs. 1&2**)**.

Hemoglobin concentrations:

There were significant decrease differences between three patient groups with normal group *P*<0.001**(**Fig.3**)**.

Hematocrite values:

There were significant decrease differences between the three patient groups with normal group *P*<0.001(Fig.4).

MCH values & MCV values:

There were significant decrease differences between IDA and ACD/IDA patient groups with normal group *P*<0.001, and with ACD group *P*<0.001, whereas there was insignificant difference between normal group with ACD group *P*>0.05 and, also between IDA group with ACD/IDA *P*>0.05 **(**Figs.5&6**)**.

RBCs values in the human blood:

The data recorded showed significant decrease differences between the three patient groups with normal group *P*> 0.001, but there was insignificant difference between ACD/IDA group with ACD group *P*>0.05 **(**Fig.7**)**.

Reticulocytes count (Retix):

There were significant increase between the three patient groups with normal group P<0.001and also between IDA and ACD/IDA groups with ACD group *P*<0.001. On the other hand there were insignificant difference between IDA group with ACD/IDA group *P*>0.05 **(**Fig.8**)**.

Iron concentrations:

There were significant decrease differences between the three patient groups with normal group *P*<0.001, but there were insignificant changes between IDA group with ACD/IDA group *P*>0.05 (Fig.9).

TIBC values:

There were significant increase differences between IDA, ACD/IDA groups with normal group *P*<0.001, but there were significant decrease between ACD group and normal group *P*>0.05**,** whereas there was significant increase between IDA with ACD/IDA groups **(**Fig.10**)**.

CRP levels:

There were significant increase differences between ACD and ACD/IDA groups with normal group *P*<0.001**,** but there were insignificant differences between IDA group with normal group *P*>0.05**,** as well as between ACD group and ACD/IDA group as *P*>0.05 (Fig.11).

Ferritin levels:

There were significant decrease differences between IDA group with normal group *P*<0.001. On contrast, there was significant increase between ACD and ACD/IDA groups with normal group *P*<0.001 and *P*<0.05 respectively. The IDA group showed the lowest ferritin level between all groups, while the ACD group showed the highest level **(**Fig. 12**)**.

Transferrin receptors levels in human serum:

Data demonstrated significant increase differences between IDA and ACD/IDA groups with normal group *P*<0.001, but there were insignificant differences between ACD group with normal group *P*>0.05, also there were in significant differences between IDA with ACD/IDA group (Fig.13**)**.

Hepcidin level:

Considering the hepcidin levels, the recorded data showed significant decrease difference between IDA group with normal group *P*<0.001**,** whereas significant increase differences between ACD and ACD/IDA groups with normal group *P*<0.001. Also, there was significant increase between ACD group with ACD/IDA group *P*<0.001 **(**Fig. 14**)**.
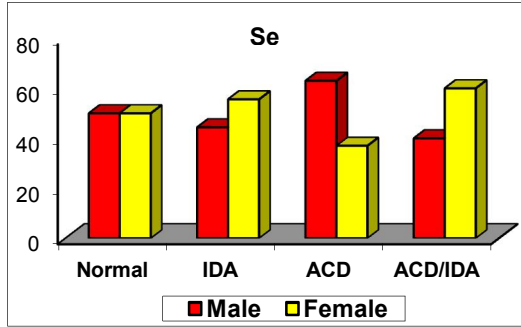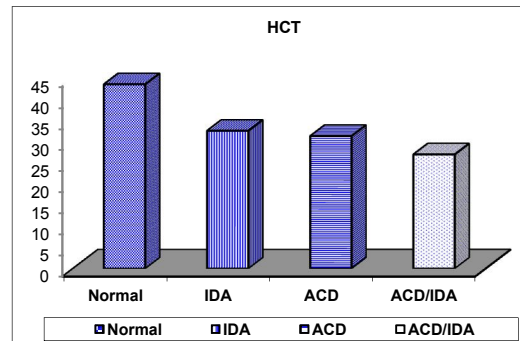
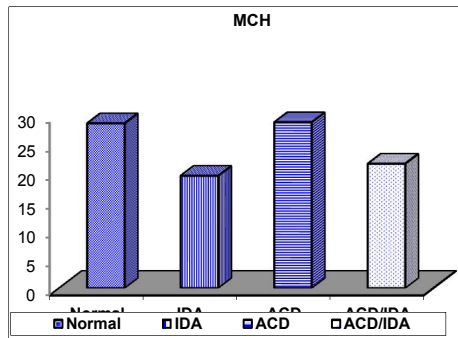**Fig (1):** Sex distribution in the anemic patients and normal group.
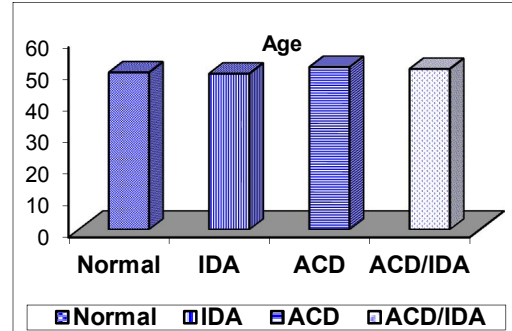
Fig (2): Age distribution in the anemic patients and normal group.
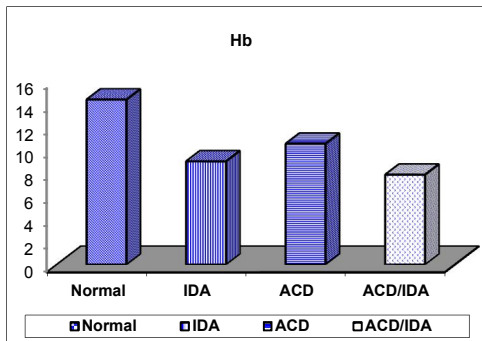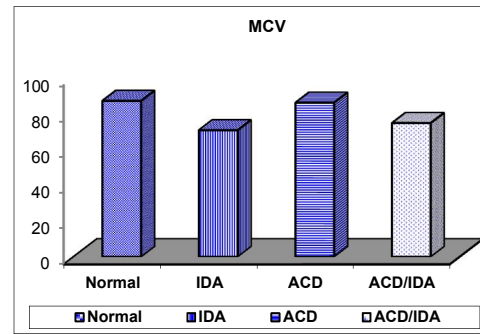

Fig (3): Hemoglobin concentrations in the anemic patients and normal group
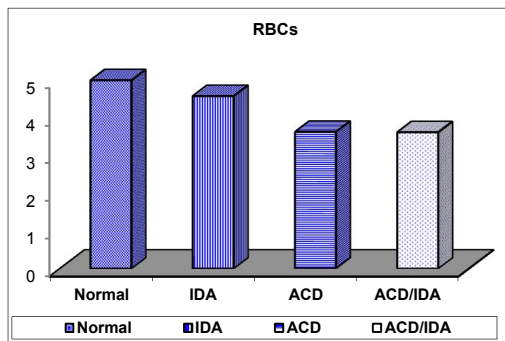

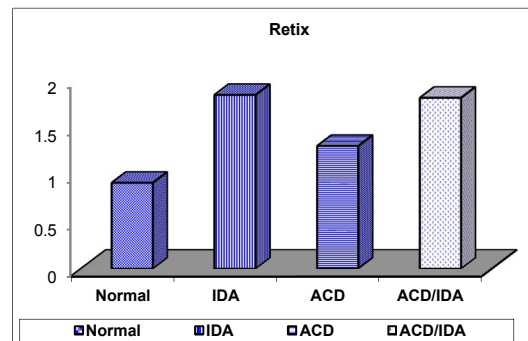**Fig (4):** Hematocrite values (HCT) in the anemic patients and control group

Fig (5): MCH values in the studied anemic patients and normal group.
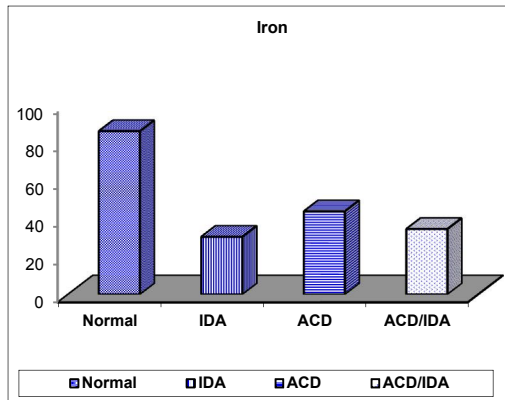

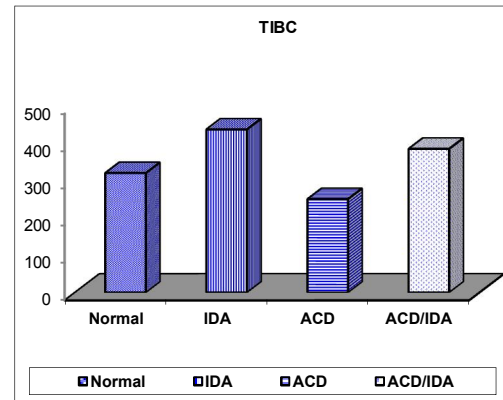**Fig (6):** MCV values in the studied anemic patients and normal group.

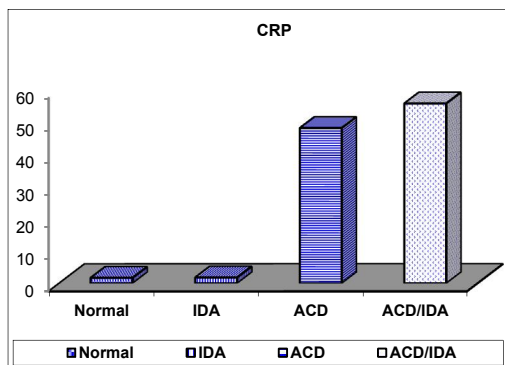**Fig(7):** RBCs count in the studied anemic patients and control group

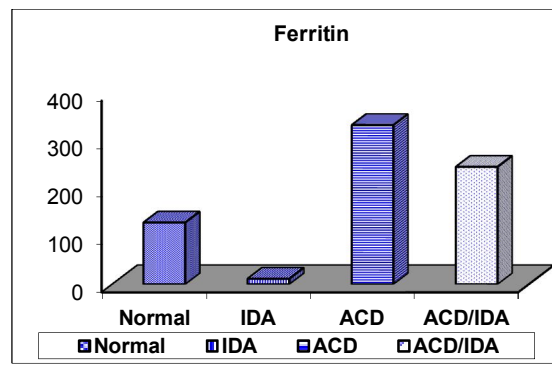**Fig(8):** Reticulocytes count in the anemic patients and normal group

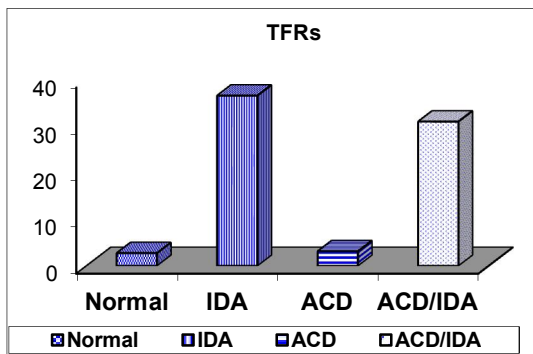**Fig (9):** Serum iron levels in the anemic patients and normal group.

Fig (10): TIBC values in the anemic patients and normal group.
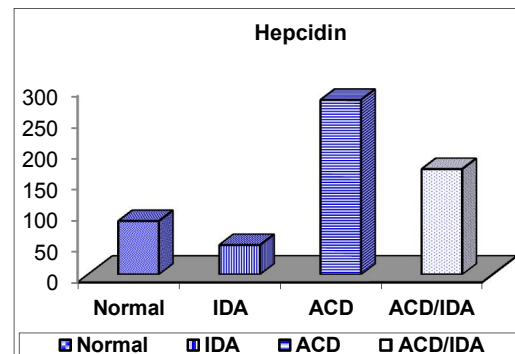

Fig (11): Serum C-reactive protein levelsin the anemic patients and normal group.

Fig (12): Ferritin levels in the anemic patients and normal group.

Fig (13): Serum transferrin receptors levels in the anemic patients and normal group.

Fig (14): Serum hepcidin levels in the anemic patients and normal group.

4. Discussion:

Iron deficiency anemia (IDA) considers the most common nutrition deficiency worldwide. Despite the fact that iron deficiency anemia (IDA) is sometimes associated with chronic diseases; however ACD is mediated by different mechanisms *(Clark, 2008)*. The anemia of chronic disease **(**ACD**)** is an inflammatory response characterized by cytokines release, abnormal mobilization of the iron from its stores and reduction in erythrocyte longevity *(Theurl et al, 2014)*. Assessment of the iron deficiency anemia in patients with inflammatory disorders appears very difficult, because the regular laboratory estimations of iron levels are powerless to differentiate between true iron deficiency anemia **(**IDA**)** and anemia of chronic disease **(**ACD**)** *(Zhu et al., 2010)*. Therefore, bone marrow examination is necessary to evaluate the exactly iron in its stores and to setup decisive

diagnoses *(Zimmermann, 2008)*. However, bone marrow examination cannot be performed regularly, because it is painful, expensive, time consuming and needs technical experts *(Ferraro et al., 2012)*.

Concerning the total iron-binding capacity (TIBC), it is known that it increases when iron stores are diminished and when iron in iron stores is elevated; the levels of TIBC are decreased *(Stein and Dignass, 2013)*. The TIBC reflects transferrin which is the main iron transporter in the blood.

In the current study the TIBC showed significant increase in patients with iron deficiency anemia (IDA group) due to the low content of iron in its stores. In contrast, the TIBC in anemia of chronic disease patients (ACD group) showed significant decrease changes with normal group as the iron stores were filled with iron. Nevertheless, there was significant difference in the TIBC value between IDA and ACD/IDA groups where IDA is higher than ACD/IDA group. As result, TIBC is not a good measurement to detect true iron deficiency in ACD/IDA group. This comes in the same line with *(Sanad and Gharib, 2011)*.

Regard to ferritin concentrations, data showed significant decrease in ferritin concentrations of all patients of IDA group with absence of stainable iron in bone marrow, while showed significant increase in patients of ACD group with presence of stainable iron in bone marrow. Although, all patients of ACD/IDA group showed absence of stainable iron in bone marrow, the mean values of ferritin concentrations showed significant increase this could be attributed **to the fact that** ferritin is considered as an acute phase protein which elevated in the presence of inflammation **(Kalantar-Zadeh and Lee 2006 & Wang et al., 2010)**.

In conclusion the present results suggested that serum ferritin still the effective marker for diagnosis of the true iron deficiency anemia (IDA) in absence of inflammation and chronic diseases, **but** it is **not** helpful parameter to differentiate true iron deficiency anemia when associated with chronic inflammatory disorders (ACD/IDA).

The C- reactive protein (CRP) was estimated as specific indicator for the presence of inflammation.

The results of the present study showed significant increase of CRP levels in the patients with ACD and ACD/IDA groups, but insignificant in patients with IDA group when compared with controls. There was insignificant difference existed between ACD and ACD/IDA patients. This could be explained in the view that, C- reactive protein (CRP) is considered specific gauge for the presence of inflammation, because it is an acute phase protein which increases in the inflammatory disorders. This is in the same line with *(Ramamoorthy,2012)* who

reported increase of C- reactive protein (CRP) levels in cases of inflammatory disorders.

Regard to transferrin receptors (sTfR), the numbers of sTfR on the cell surfaces reflect the iron requirement; therefore iron reduction results in the prompt induction of transferrin receptors synthesis *(Gupta et al., 2009)*.

In the current study the sTfR value in IDA patients group showed significant increase in its mean value compared with normal value. The significant increase in sTfR in IDA patients group could be attributed to the erythroid marrow hyperplasia where its concentration correlates directly with erythropoietic activity and inversely proportional with the amount of iron available for erythropoiesis. The data come in the same line with number of studies (***Bozzini et al., 2008, Skikne, 2008, Jain et al., 2010 andYokus et al., 2011)***.

The transferrin receptors (sTfR) value in ACD patients group showed insignificant change in respect to the control. While its value in ACD/IDA group showed significant increase compared to normal control value. Therefore, sTfR measurements could be of great importance to recognize iron deficiency even in cases accompanied inflammatory disorders or in patients of infectious diseases. This explanation is in accordance with number of studies *(Koulaouzidis etal., 2009 & Jain et al., 2010)* who reported that the value of the sTfR is a good indicator to distinguish between the presence or absence of iron in anemic conditions inspite of the presence of acute or chronic disorders. In the present study there was an increase of sTfR with ACD patients in the presence of IDA with **no** iron stain bone marrow. The data of the present data agreed with number of authors *(Pavai et al.,2008 Koulaouzidis et al., 2009, Jain etal., 2010 and Saboor et al., 2011)* who studied the possibility of the coexistence of IDA with ACD.

In Conclusion: the results of the current investigation concluded that the sTfR measurements could be a useful differentiating indicator in the diagnosis of true iron deficiency in anemia of chronic disorders (ACD/IDA)**.**

Hepcidin is considered a key hormone controlling mammalian iron homeostasis, as well as hepcidin-ferroporten axis forms the key mechanism to regulate systemic iron homeostasis, and its perturbation results in iron-related disorders. After the discovery of hepcidin, many trials and researches have been done to understand the biology and pathology of this hormone to get better diagnosis and effective treatment of iron disorders with existing new therapies (**Arezes and Nemeth, 2016).** Furthermore, hepcidin consideres the main regulator of iron metabolism, also a pathogenic factor in iron disorders. Hepcidin deficiency leads to iron overload**,** on the other hand

excess hepcidin contributes to the development of iron-restricted anemia in chronic inflammatory diseases**.** Under physiological conditions**,** there is a balance between activating signals and inhibitory signals that regulate the mechanisms involved in the synthesis of hepcidin (*CondeDiez et al.,* 2017).

The regulation of hepcidin by body iron acts as a feedback mechanism to allow sufficient iron to enter the plasma when the demand for iron is high but to limit iron release into the plasma in times of iron sufficiency. It is well documented that deficiencies in hepcidin are associated with several iron-related disorders *(Ganz, 2011).*

The present results demonstrated that hepcidin levels in IDA patients group showed the lowest value among all groups. This could be contributed to suppressive effects of scanty iron stores that comes in accordance with *(D'Angelo, 2013)* who reported that hepcidin production is suppressed in the case of iron deficiency.

Regard to**,** hepcidin levels in ACD group with its level in ACD/IDA group, the results indicated that serum hepcidin level in ACD patients group was significantly higher than that of ACD/IDA group. This can be attributed to the fact that hepcidin might be initially increased in ACD patients group by the excessive production of cytokines when associated with IDA *(Ganz, 2011)*.

The present results are parallel with (*Pan et al., 2010)* who reported increase hepicidin levels in ACD and ACD/IDA groups**,** but decreased in IDA group.

Conclusion

Hepcidin could be taken as a good diagnostic tool**,** as well as a potential measurement for the identification and to distinguish between the three kinds of anemia; IDA, ACD and ACD/IDA. Moreover, hepcidin could give green light for the development of new diagnostic and therapeutic methods of iron-overload and iron-restricted anemia.

The present study expected that there will be in the near future discovery channel of new effective treatments aimed to correct the defect of many iron metabolism disorders.

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3/16/2017