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SERUM HEPCIDIN AND INTERLEUKIN-6 ELEVATE WITH SIGNIFICANT LINEAR CORRELATION IN ANEMIC HYPERTENSIVE PATIENTS

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ABSTRACT

The anemia with or without iron deficiency, occurring in response to inflammation has been recognized as an important risk factor in hypertension and related cardiovascular disorders. Hypothesis in current proposal was that inflammation via interleukin-6 (IL-6) may stimulate hepcidin (Hp) and plays role in causing anemia in patients with or without hypertension (HPT; n: 251; age: 55-69 years). Non-obese HPT (male: HPTm; female: HPTf) subjects along with normal controls (NC; male: NCm; female: NCf) were consulted. Patients with anemia (AN; male: ANm; female: ANf) and anemic hypertension (AN-HPT; male: AN-HPTm; female: AN-HPTf) were properly diagnosed. Laboratory evaluations comprised the estimation of serum levels of IL-6 and Hp for the present research project. Other clinical and laboratory tests were carried out for diagnostic purpose. The IL-6 and Hp serum levels in the present study showed highly significant difference mainly for subjects with AN and AN-HPT against other subjects. Association/correlation of age and serum IL-6 in ANm, ANf, HPTm, HPTf, AN-HPTm and AN-HPTf subjects; and age and serum Hp in ANm subjects showed significant positive linear relationship. All groups showed highly significant positive linear correlation for serum IL-6 and Hp. The present study is hence, a landmark for uncovering the potential role of IL-6 and Hp in nonobese anemic hypertensive patients on one hand, and for providing a new approach for further studies for introducing possible novel drugs with specified mode of action.

Key Words: Hypertension, anemia of inflammation, anemic hypertensive patients, non-obese subjects, serum hepcidin, serum interleukin-6

INTRODUCTION

The 'anemia of inflammation' (AI or AOI), or anemia of chronic inflammation (ACI), (previously called as anemia of chronic disease (ACD) with or without iron deficiency anemia (IDA), is a complex condition that has been recognized as an important risk factor in hypertension (HPT) and other diseases (Liberale *et al*., 2021). The pathophysiology of AI is not completely known, and hence, it is challenging to manage it properly. It is the second most prevalent anemia worldwide (after IDA) (Kassebaum *et al*., 2014). The role of decreased oxygenation due to decreasing hemoglobin (Hb) and hematocrit (HCT) has been investigated (Goel *et al*., 2021). However, the precise role of anemia on clinical picture in hypertension and other cardiovascular disorders, i.e., manifestations, severity and disorders, is still needed to be further clarified (Goel *et al*., 2021), though comprehensive studies have been conducted in AI for understanding the association of anemia and inflammation (Ganz, 2019; Weiss *et al*., 2019).

There are a variety of factors considered as having involvement in anemic hypertensive patients. Two of those factors are serum hepcidin (Hp) and interleukin-6 (IL-6), especially in patients having IDA with inflammation (Gkamprela *et al*., 2017; Ramakrishnan *et al*., 2018; Yacoub *et al*., 2020).

Hypothesis in the present proposal is that inflammation is involved in the development and progression of anemia. As immune activation leading to inflammation and causing anemia appears to be associated with the outcome and complications of the patients, it seemed necessary to study whether patients with anemia originating from various factors varied for risks and disordered events. The present study hopefully provides information about whether the involvement of inflammation/inflammatory processes as cause of anemia has a significant influence in patients with hypertension.

The present study is hopefully a landmark for uncovering the potential role of IL-6 and Hp (Gkamprela *et al*., 2017; Yacoub *et al*., 2020) in non-obese anemic hypertensive patients on one hand, and providing a new approach for introducing possible novel drugs with specified mode of action.

MATERIALS AND METHODS

The ethical approval of Umm Al-Qura University (UQU) was obtained before starting the present study. All instructions and regulations regarding the procedure for collecting the subjective and objective data of the normal

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control (NC) subjects and anemic (AN) patients, hypertensive (HPT) patients and anemic hypertensive (AN-HPT) patients were followed as established by Biomedical Ethics Committee of the College of Medicine at UQU.

The subjects and patients who participated in the present study were fully informed in their initial visits about the nature and objectives of collecting the required data for present work. The subjects/ patients were fully agreed and hence, filling the forms (Questionnaire) was initiated in the very start of the work while they participated in the current study with their own personal willingness/ agreement. It is important to mention that no any subject/ patient was inclined by force for providing data.

The NC subjects and AN, HPT and AN-HPT patients were consulted and included in the present study. A comprehensive Questionnaire comprising clinical, physiological, biochemical, anthropometric, hematological, immunological and cardiological estimations were prepared. Considering World Health Organization (WHO) records, anemia was considered having Hb less than12.0 g/dL and less than 13.0 g/dL respectively in women and men (Beutler and Waalen, 2006; Yoon *et al*., 2018).

The main target subjects in the present study were HPT patients with or without AI. Hence, it was essential to first carry out the diagnosis for HPT. The BP of about 115/75 mmHg has been suggested to be the best for healthy normal population (Basile, 2008). However, around 120/80 mmHg was considered as a satisfactory level of BP. The systolic BP (SBP) and diastolic BP (SBP) ranges for HPT patients considered for the present study were 140/ 90 mmHg or exceeding these values (de Faria *et al*., 2014), but the subjects having too high or too low BP were not considered for the present investigation.

The second step was to diagnose another group of HPT patients having AI, to further verify that the subjects had inflammatory response in the form of elevated serum IL-6 but not having the deficiency of serum iron (Weng *et al*., 2011), since deficiency of serum iron occurs in IDA (Weng *et al*., 2011). There were occasions while both inflammation response and iron deficiency occurred in certain complicated cases. Such cases were not included in the present study as they showed concomitant IDA and AI in patients with HPT. Estimation of serum ferritin was involved to further clarify whether the subject had IDA or AI (Schubert *et al*., 2005; Capocasale *et al*., 2008; Ali *et al*., 2019).

The subjects selected finally for the current study were all non-obese (the body mass index (BMI) in the range of 18-22.9 (kg/m^2) for non-obese population (Jafar *et al*., 2004). Hence, the subjects/ volunteers having higher or lower BMI values than the mentioned levels were not taken for the present work. For the estimation of BMI, body weight (kg) was divided with squared body height (kg/m^2) (Shah and Braverman, 2012).

Total number of subjects in four groups in the present study were 251 (age range: 55 to 69 years), out of which male subjects were 125 (49.80%) and female subjects were 126 (50.20%). Blood sample was then divided into number of aliquots according to requirement. Serum was used for the estimation of IL-6, Hp, ferritin, iron, serum total iron binding capacity (TIBC), and other hematological/ immunological measurements.

Laboratory tests for the present research study were: age and sex matched serum levels of IL-6 and Hp in eight groups of NCm, NCf, ANm, ANf, HPTm, HPTf, AN-HPTm and AN-HPTfs subjects/ patients. Kit methods as well as chemical methods were employed for comparing the results obtained in initial pilot studies to know about the accuracy, and inter assay/intra assay variations. The ELISA-kit (96T; Catalogue-No. ELH-IL6; RayBio, USA) was employed for measuring serum IL-6.

For determination of serum Hp, ferritin, iron and TIBC, transferred 3 ml to gel vacutainers. For the estimation of Hb and HCT, remaining 2 mL was transferred to the ethylenediaminetetraacetic acid (EDTA) vacutainer. Automated Hematology Analyzer Sysmex XT-1800i was used for CBC. Spectro-photometrically at 560 nm using a commercially available kit of Randox on Metrolab 1600, was employed for measurement of Serum iron and TIBC. Commercially available kit of Ferritin reagent pack (Eciq Vitros Immunodiagnostic system) was used for the measurement of Serum ferritin. Biorad-680 Microplate reader, USA (employing Human Hepcidin ELISA kit from Creative Diagnostics, USA) was used for measuring serum Hp level.

The mean± SD (standard deviation) values were calculated and presented. Further analysis for comparison between two variables was done using students ' t ' test. The ' p ' value, either equal to 0.05 (p: 0.05), or less than 0.05 ($p<0.05$) was considered/employed as statistically significant. Coefficient of determination R^2 was evaluated for correlation. Paired and unpaired t-tests were employed respectively for paired and unpaired comparisons. For the comparison of more than two variables, one way ANOVA was employed. Comprehensive analysis was done using SPSS software version 23. The results were analysed/ compared statistically applying general concepts/ statistical principles (Zahir *et al*., 2014).

RESULTS

Age of hypertensive, anemic and anemic hypertensive subjects

The mean ± SD for age (years) of various subject groups of normal control males (NCm) and females (NMf), anemic males (ANm) and females (ANf), hypertensive males (HPTm) and females (HPTf) & anemic hypertensive males (AN-HPTm) and females (AN-HPTf); and one-way analysis of variance (ANOVA) showed non-significant differences.

Serum interleukin-6 levels in hypertensive, anemic and anemic hypertensive subjects

The mean ± SD values for serum IL-6 of various subject groups, NCm, NMf, ANm, ANf, HPTm, HPTf, AN-HPTm and AN-HPTf were 3.25 ± 2.07, 3.42 ± 2.18, 10.32 ± 9.37, 11.39 ± 10.34, 4.36 ± 2.51, 4.56 ± 2.69, 9.33 ± 7.15 and 12.58 ± 11.28, respectively that showed highly significant difference of Serum IL-6 for AN-HPTm vs. HPTm (p: 0.0006); and AN-HPTf vs. HPTf (p: 0.0003). The Tukey Kramer test further showed significant variations for: NCm vs. ANm (p: 0.0001), NCm vs. AN-HPTm ($p<0.0001$), NCf vs. ANF ($p < 0.0001$), NCf vs. AN-HPTf ($p < 0.0001$), ANm vs. HTPm (p: 0.0013), and ANf vs. HTPf (p: 0.0007). All other comparisons were found statistically non-significant. The one-way ANOVA showed highly significant variation (F: 9.566; $p < 0.0001$) of serum interleukin-6 among the various subject groups.

Serum hepcidin levels in hypertensive, anemic and anemic hypertensive subjects

The mean ± SD values for serum Hp of various subject groups, NCm, NMf, ANm, ANf, HPTm, HPTf, AN-HPTm and AN-HPTf gave 10.58 ± 6.63, 6.99 ± 6.49, 15.27 ± 11.66, 11.39 ± 10.34, 7.52 ± 4.98, 7.05 ± 4.14, 15.52 ± 9.01 and 10.54 ± 5.93 respectively. Serum Hp showed highly significant difference for AN-HPTm vs. HPTm ($p< 0.0001$), and AN-HPTf vs. HPTf (p: 0.0091). Tukey Kramer test further showed significant variations for: NCm vs. NCf (p: 0.0324), ANm vs. ANf (p:0.0422), ANm vs. HPTm (p: 0.0014), NCm vs. ANm (p:0.0534), NCm vs. AN-HPTm (p: 0.0152), NCf vs. ANf (p: 0.0639, not quite significant statistically), NCf vs. AN-HPTf (p: 0.0257), ANm vs. HTPm (p: 0.0014), AN-HPTm vs. AN-HPTf (p:0.0112) and ANf vs. HTPf (p: 0.0357). All other comparisons were found statistically non-significant. One-way ANOVA showed highly significant variations among the groups (F: 6.757; $p < 0.0001$).

Association of age and serum interlukin-6

Significant association (positive linear correlation) of age against serum IL-6 was found for ANm, ANf, HPTm, HPTf, AN-HPTm and AN-HPTf (Table 1).

Association of age and serum hepcidin (Hp)

Association of age and serum Hp showed significant positive correlation in only NCm (Table 1), though the positive linear association was present in all groups of subjects.

Association of serum interleukin-6 and hepcidin

Association of serum IL-6 and Hp showed significant positive correlation for all groups of subjects (Table 1).

Table 1. Association of age and serum interlukin-6; age and serum Hp and serum IL-6 and Hp in subjects with anemia and anemic hypertension.

Subject Groups	Age and Serum interlukin-6		Age and serum Hp		Serum interlukin-6 and Hp	
	R^2	P	R^2	P	R^2	P
NCm	0.6070	0.0089	0.4500	0.0191	0.5354	P < 0.0001
NCf	0.1870	0.0573	0.9220	0.0003	0.7575	p < 0.0001
ANm	0.0001	0.3964	0.0084	0.2164	0.6782	p < 0.0001
ANf	0.0002	0.3848	0.0780	0.1030	0.4164	P < 0.0001
HPTm	0.0270	0.1634	0.0910	0.0987	0.7070	P < 0.0001
HPTf	0.0054	0.2379	0.1630	0.0661	0.7797	P < 0.0001
AN-HPTm	0.0110	0.1985	0.3180	0.0332	0.5947	P < 0.0001
AN-HPTf	0.00005	0.4282	0.1360	0.0725	0.4263	P < 0.0001

NC, AN, HPT and AN-HPT respectively represent normal control, anemic, hypertensive and anemic hypertensive subjects, m and f denote male and female subjects**.**

DISCUSSION

Since all chronic diseases are not related to anemia of inflammation (AI), anemia of inflammation (AI) is a preferrable term instead of previously used term of anemia of chronic inflammation/disease. The AI in the present project showed the elevated IL-6 and it was distinguished from anemia due to ID (Weng *et al*., 2011). The subjects selected for the present work were diagnosed by laboratory findings that they should have the normal or high serum levels of ferritin, as the ferritin remains normal or high in AI without ID, whereas ferritin is found low in IDA. (Weng *et al*., 2011).

While diagnosing the patients clinically, it became essential to characterize the laboratory diagnosis for these patients. The present study followed the general criteria for anemia in the diagnostic methods as Hb <13 and <12 g/dL respectively in men and women (Yoon *et al*., 2018). Anemia in men and women in the present study was dealt as characterized in a WHO report that was defined properly (Yoon *et al*., 2018).

Anemia and mild anemia by categorizing on the basis of Hb was revealed as an independent risk factor in cardiovascular processes in HPT subjects, where HPT was found quite more in anemic than in non-anemic subjects (Kim-Mitsuyama *et al*., 2019). Quite similar results during the diagnosis of patients in the current work were found. However, differing results were also obtained in a study wherein an association of anemia with pulse rate was revealed but not with HPT (Yoon *et al*., 2018), and evidence of the elevated BP and significantly lower diping status in anemic patients was revealed compared to those having normal levels of Hb.

The approach of selecting the subjects for the current study based on the investigation that the patients with AI initially have almost normal levels of iron store with a little disturbance in the re-cycling of iron, and hence, mild normocytic anemia develops, but reduction in iron absorbing extent occurs with the time that may lead progressively to microcytic anemia (Weiss and Goodnough, 2005; Ali, 2019). The mentioned reports further clarify the hypothesis in the present study. However, all these processes may occur further to lower the half-life of erythrocytes by erythrophagocytosis and less availability of iron to progenitor cells for producing erythrocytes for responding to anemia, resultantly causing decrease in the number of erythrocytes, and developing iron restricted erythropoiesis.

The patients consulted in the present investigation were having just mild or moderate extent of anemia along with the presence of inflammation, and with or without HPT, as has been verified that AI is a mild to moderately severe anemia (Hb is rarely less than 8 g/dL) (Nemeth and Ganz, 2014), and it is a common consequence in people suffering from infectious/autoimmune disorders, HPT, cancer, chronic kidney disease, cardiovascular diseases, and other inflammation-related diseases. Other reports present similar manifestations that AI is usually mild to moderate in extent with normocytic, and normochromic manifestations (Weiss, 2015).

Inflammatory processes also stimulate Hp and suppress ferroportin, and hence, limiting the iron supply for erythropoiesis (Pasricha *et al*., 2021). All these manifestations vary in AI in view of the increased inflammation and increase IL-6 leading to the changes as obtained in the present work. The patients with AI in the present report revealing anemia and significantly higher serum IL-6 and Hp compared to the controls was similar as found previously (Yacoub *et al*., 2020).

The data of IL-6 in the present study shows significant variations for age-based variations in ANm, ANf, HPTm, HPTf, AN-HPTm and AN-HPTf comparisons that is quite similar to the previously published data in men and women (Yacoub *et al*., 2020). The IL-6 varied significantly for men and women with AI, and AN-HPT in the present report. Studies in AN subjects and AN-HPT subjects identify the link of IL-6 with Hb and other hematological parameters and association with inflammatory conditions in HPT patients compared to NT subjects (Mtali *et al*., 2019) that helps interpreting the present report.

The present investigations can further be verified by another report identifying significant relationship for IL-6 and CVD mortality, even not depending on CRP levels (Lee *et al*., 2012). Significantly elevated levels of IL-6 is evident in the present study for AN-HPTm vs. HPTm , AN-HPTf vs. HPTf, NCm vs. ANm, NCm vs. AN-HPTm, NCf vs. ANf, NCf vs. AN-HPTf, ANm vs. HTPm), and ANf vs. HTPf since IL-6 has been found to be related with HPT and atherosclerosis; and treatment of high BP decreases the IL-6 levels in young HPT subjects (Vázquez-Oliva *et al*., 2005), AI subjects (Yacoub *et al*., 2020), and AN-HPT subjects (Mtali *et al*., 2019).

The results presented in the present manuscript can further be elaborated with the help of several other relevant work. Interleukins (ILs) and mainly IL-6 in AI seem quite involved as the immune cells, in view of handling the immune system-related conditions or infection, release an important proinflammatory cytokine IL-6 that is pleotropic with a variety of functions (Raj *et al*., 2009).

Furthermore, the interleukin (IL) is specialized in regulating the inflammatory processes/immune responses, hematopoiesis, acute phase reaction, bone metabolism etc. (Raj *et al*., 2009; Ali *et al*., 2019); and production of ferritin is enhanced via the process whereby IL-6 causes inhibition of the activity of TNF-α (Capocasale *et al*., 2008). It is known that the ferritin is a marker for ID, but it is upregulated in AI (Ali *et al*., 2019), i.e., increased levels of ferritin increase the retention of iron within reticuloendothelial system cells, and hence, usually increased

level of ferritin characterizes AI. All these reports directly or indirectly verify the results mentioned in the current work, though the extent of changes vary.

Hypertension and prehypertension status studied in association with AI (Lee *et al*., 2019) agrees with the present project report. The data of age and sex-based serum Hp ranges in in the present study is similar to the previously published work in men and women (Yacoub *et al*., 2020). Association of age and Hp showed only ANm group having significant positive linear relationship in the present study.

The Hp varied significantly for men and women with AI, and HP with AI in the present study in AN-HPTm vs. HPTm, AN-HPTf vs. HPTf, NCm vs. NCf, ANm vs. ANf, ANm vs. HPTm, NCm vs. ANm, NCm vs. AN-HPTm, NCf vs. AN-HPTf, ANm vs. HTPm, AN-HPTm vs. AN-HPTf and ANf vs. HTPf that can be interpreted by the previous reports indicating role of inflammation related to Hp, and IL-6 in patients having HPT and chronic blood loss (Gkamprela *et al*., 2017; Yacoub *et al*., 2020). Hence, it is suggested that, there is further need to study the male and female HPT subjects with and without AI.

Interaction for Hp obtained in the present project relates to IL-6 levels and it is considered as an important regulator for iron homeostasis in association with AI and research on Hp and IRPs identified the deficiency of iron intracellularly in cardiovascular system as an important factor causing diseased conditions, and it provides an opportunity for developing therapeutic strategies based on influencing iron homeostasis ((Lakhal-Littleton, 2019).

The present study showed significant elevation of IL-6, and Hp in several anemic and anemic hypertensive groups, and significant positive linear correlation for serum IL-6 plotted against Hp, that indicates that increase in IL-6 stimulates the hepcidin production in liver, resultantly shutting down the ferroportin (Weiss *et al*., 2019).

The present investigation presents a significantly positive linear correlation between serum IL-6 and Hp in patient groups of AI and HPT with AI, that has been explained by considering IL-6 involved in inducing process for Hp in several inflammation-related anemic models (Wang and Babitt, 2016), and, importantly, the Hp, ultimately causing anemia (Weiss *et al*., 2019). Furthermore, the present study provides detailed information about whether the causes of anemia play significant role in HPT patients. It was found that the AI is at least in some degree different and separate from the anemia observed in kidney failure wherein anemia occurs due to decreased production of erythropoietin, or due to some drugs for HIV infection having side effect of inhibiting the erythropoiesis.

Sample size for the present study was taken from only one centre. Future studies might be carried out by taking samples from various centres for diversity and possibility of studying the role of more risk factors and manifestations. Further studies can be conducted involving multi-center study approach and obtaining large sample size that may help understanding the significant relationship existing between serum IL-6 and Hp levels in AI patients. Furthermore, the present study was done in subjects/patients of 55-69 years age range, though the findings for lower and higher than this age range are required to be carried out to have better idea of precise age and gender-based changes in IL-6 and Hp in patients with anemia or anemic hypertension.

The present study suggests a possibility of AI or ACI to be a common and strongly related condition in patients with hypertension and other cardiovascular diseases, wherein the role of IL-6 and Hp may be checked for planning and developing the novel drugs for inhibiting the effect of IL-6 and peptide Hp.

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