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Iron deficiency anemia (IDA) and anemia of inflammation (AI), Is Hepcidin-25 a test with value in clinical decision making?

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ABSTRACT

Hepcidin-25, the key regulator of iron metabolism that incorporates signals of infection and iron deficiency, may present new resolutions to detect and diagnose iron deficiency anemia (IDA) even in complicated situations.

The target of our study was to evaluate the effectiveness of hepcidin-25 as a new biochemical test to distinguish efficiently iron deficiency anemia from anemia of inflammation (AI) and in the detection of concurrent iron deficiency anemia in patients suffering from anemia of inflammation (IDA/AI) in hospital cohorts.

Sera from 60 anemic patients with common disorders (21 males and 39 females; 15 to 85 years) were tested for hepcidin-25 by using ELISA technique. Patients were categorized according to iron parameters and CRP as: IDA (N=20), IDA/AI(N=20), AI(N=20) and controls (N=10). Differences in hepcidin levels were processed with nonparametric methods.

Comparing groups with each other revealed significant differences ($p=0<0.0001$). Use a cutoff level (4.07ng/ml) of hepcidin-25 permitted the discrimination of IDA from IDA/AI. Furthermore, the distinguishing of IDA/AI from AI required cutoff level of (12ng/ml) (sensitivity of 90% and a specificity of 78.9%); in accordance with the receiver operating characteristic (ROC) curves.

In conclusion, hepcidin levels can be very useful in differentiating IDA, AI and AI with coexisting iron deficiency anemia, thus providing a noninvasive substitute to bone marrow iron in the near future.

KEY WORDS: Iron deficiency anemia, Anemia of Inflammation, Hepcidin-25.

1. INTRODUCTION

Iron deficiency (ID) and iron deficiency anemia (IDA) are health problems globally and public medical states encountered in daily clinical practice, that affect young children and women in premenopausal age in both low-income and developed countries (Camaschella, 2015).

Iron deficiency anemia arises when the balance of iron intake, iron stores, and the body's loss of iron are inadequate to maintain proliferation of erythrocytes (Miller, 2013). Cause of anemia is frequently difficult to be determined even after extensive investigations including bone marrow examinations. It is reported as nutritional (34%), renal insufficiency (12%), chronic diseases (20%) and unexplained (24%) (Rasheed, Ali, 2013). Measurement of serum ferritin, transferrin saturation, serum soluble transferrin receptors, and the serum soluble transferrin receptors–ferritin index are more precise than traditional red cell indices to diagnose iron deficiency anaemia (Lopez and Cacoub, 2016).

Anemia of inflammation (AI)): is the second most prevalent anemia subsequent to iron deficiency anemia, takes place in patients who experience acute or chronic immune activation. Advances in the comprehension of the pathophysiology of AI, including disturbances of iron homeostasis, impaired production of erythroid progenitor cells, and a weakened erythropoietin reaction to anemia, have made possible the emergence of new therapeutic strategies (Weiss and Goodnough, 2005). In AI serum iron concentrations are low in spite of sufficient iron stores, as proved by serum ferritin that is normal (Nemeth and Ganz, 2014).

The detection of concurrent iron deficiency anemia (IDA) in patients with anemia of inflammation (AI): Is of clinical important, as iron deficiency anemia is able to be treated, and the detection of IDA can preclude more investigations to know the cause of the anemia. Finally, it may prevent unnecessary prescription of iron supplementation. Unfortunately, diagnosis of iron deficiency becomes more complicated when concomitant inflammatory conditions are present (Van Santen and Van Dongen-Lases, 2011). Morphologically, the patients with AI/IDA have microcytic cells, if compared with patients who have AI alone, and their anemia is more severe. Clinically, assessing the amount of iron in bone marrow macrophages (iron stores) is considered the ''gold standard'' for the diagnosis of iron deficiency. However, bone marrow aspiration it is an invasive procedure. in addition, the distinguishing of iron within macrophages from artifacts is not minor matter, as it needs substantial experience and skills to get exact results (Cheng & Jiao, 2011). Indeed, traditional methods based on ferritin and transferrin saturation, have limitations owed to instability in response to various non-iron related conditions, in particular inflammation.

Hepcidin-25: a peptide hormone with 25 amino acids that created and secreted mainly by liver, and is excreted by the kidneys (Konz & Montes-Bayon, 2014). It was first described as an antimicrobial peptide in a mass spectrometry during looking for cysteine-rich defensin-like peptides in blood and in urine (Zhao & Zhang, 2013). In iron deficiency, down regulation of hepcidin allows enteral iron resorption, while upregulation in case of chronic inflammation blocks it (Rohrig & Rappl, 2014). Hepcidin restricts the accumulation of extracellular iron by binding ferroportin (the only known iron export protein) and mediating its degradation, therefore impeding iron exit from intracellular sources (Michels & Nemeth, 2015). The major factors that are implied in regulation of hepcidin include iron stores, hypoxia, inflammation and erythropoiesis (Rishi & Wallace, 2015). Hepcidin has distinctive diagnostic characteristics from ferritin and outperforms zinc protoporphyrin, though ferritin and hepcidin are both induced by iron stores and inflammation, but only hepcidin is directly suppressed by bone marrow demand for iron. This demonstrates the nonequivalence of hepcidin and ferritin as indices of iron status and the added sensitivity of hepcidin as an indicator of erythropoietic activity (Pasricha & Atkinson, 2014). Since the clarification of hepcidin role in iron homeostasis, hepcidin has been suggested as a promising biomarker for diagnosing iron-related disorders (Konz, Montes-Bayon, 2014). Hepcidin has been verified effective for detecting ID in blood donors (Pasricha & McQuilten, 2011) and in Korean children (Choi & Song, 2012). The utility of basal hepcidin level to guide best track of iron administration has been lately showed in patients with IDA receiving oral iron therapy (Bregman & Morris, 2013), chronic rheumatic anemia (Van Santen & Van Dongen-Lases, 2011) and in patients suffering from anemia due to chemotherapy medicated with intravenous iron and darbepoetin (Steensma & Sasu, 2015). using iron isotopes, erythrocyte iron incorporation was predicted using low serum hepcidin in African children, and hepcidin was proposed as a leader for choosing individuals safe to receive iron (Prentice & Doherty, 2012; Girelli & Nemeth, 2016). Because hepcidin assays are costly and not habitually accessible, Kemna has suggested a formula to prognose hepcidin concentrations: transferrin saturation (%) – sTfR (mg/L) + C-reactive protein (mg/L) (Kemna & Kartikasari, 2008). Hepcidin is a hopeful diagnostic toolkit to be supplemented to the available iron status tests, particularly in ID, where it has the capability to discriminate IDA from AI (Girelli & Nemeth, 2016).

Our aim: was to study whether serum Hepcidin-25 levels might serve as diagnostic parameter to differentiate between IDA and AI and to diagnose coexisting IDA and AI among anemic patients, and to evaluate correlations between serum levels of Hepcidin-25 and some biomarkers of anemia and inflammation in these groups.

2. MATERIALS AND METHODS

This study was performed between June 2015 & June 2016 at two university hospitals in Damascus, Syria. Informed consent was obtained for enrollment in the study and Questionnaire interview was applied. Eligibility for this study comprised patients with hemoglobin ≤11 g/dL (for women and men) and TSAT ≤20% (Sasu & Li, 2010; Bregman & Morris, 2013). A total of 60 anemic patients (21 males/39 females. Aged between 15 - 85 years), classified according to iron biomarkers and CRP levels in three groups:20 patients with IDA(CRP< 10 mg/l, ferritin< 30 ng/ml), 20 patients with IDA/AI (CRP≥ 10mg/l, ferritin 30-100 ng/ml) and 20 patients with AI(CRP≥ 10mg/l, ferritin> 100 ng/ml) (Pasricha & Flecknoe-Brown, 2010), in addition to 10 age-matched controls. The exclusion criteria included :Hematological malignancies, cancer patients currently receiving chemotherapy or who received chemotherapy in the last 6 months, hemolytic anemia, liver or renal insufficiency, suspected vitamin B12 or folate deficiency, acute blood loss, blood transfusion in the last two months, receiving iron supplementation within 2 weeks. More than one cause of anemia was present in some patients. Among the 40 patients with AI:17.5%were diagnosed with (acute and chronic) Viral and Bacterial Infections, 15%with Autoimmune diseases, 20% with cancer and 47.5% suffered from chronic diseases (COPD, Diabetes Mellitus, Chronic Heart failure and Coronary Artery Disease). Meanwhile, among IDA patients: 30% were diagnosed with dietary iron deficiency and iron malabsorption, and 70% suffered from blood loss (e.g. peptic ulcers with Helicobacter, and menorrhagia).

Methods: Blood samples were obtained for hematological and biochemical tests after using3 types of vacutainers, one of which contained ethylene diamine tetra acetic acid (EDTA), the second with Sodium citrate, while the third without any anticoagulant. Visibly hemolyzed, icteric or lipemic samples were not used for the study. Sera for Hepcidin-25 measurements were separated and stored at -20°C until assay.

Hematological markers: EDTA-tubes were used for measuring hematological markers: complete blood count (CBC) including Hb concentration, MCV, MCH, MCHC and RDW using hematology autoanalyzer (Medonic, Sweden). Blood smear was evaluated by (Olympus CX21) microscope.

Iron profile: Iron and TIBC were analyzed photometrically Using (Biosystems- kits, Spain) by (Chem Well -USA) autoanalyzer. Transferrin saturation was calculated by using this equation: [TS= (Iron/TIBC)*100%]. Whilst for serum ferritin, ELISA-Kit (DiaMetra, Italy) was used.

Inflammatory indices: Serum C-reactive protein (CRP) was done by latex agglutination test using kit CRP Latex (Biosystems, Spain) with automated analyzer (Chem Well, USA). ESR was done by automated analyser (Dragon, China).

Serum Hepcidin-25 measurement: Hepcidin-25 levels were measured by using a commercial ELISA kit (DRG Instruments, USA) which is a solid phase enzyme-linked immunosorbent assay based on the principle of competitive binding. The automated plate washer (ApDia, A.D.Wash) and the microtiter plate calibrated reader (HUMAREADER ^{plus}) were used .The results have been calculated automatically using a 4PL (4 Parameter Logistics) curve fit.

Statistical analyses: SPSS, version 20; was used for data analysis. Values were expressed as means ± SD. Simple distribution of the study variables was applied. Calculations for statistical differences between the groups were done by nonparametric Kruskal–Wallis test, and between groups by Mann-Whitney U test. Correlation between variables was assessed by Spearman's correlation coefficient. A P-value ≤ 0.05 was considered as statistically significant. To evaluate the clinical performance of the Hepcidin-25, a series of potential cutoffs were assessed using Receiver Operating Characteristic (ROC) curves, and AUC_{ROC} values were calculated. Sensitivities and specificities, Youden indices [which seek to identify the point where these are simultaneously optimal, that is (sensitivity/100 + specificity/100) −1), positive and negative predicting values were calculated for these cutoffs.

3. RESULTS

Subject demographic and clinical characteristics: The characteristics of all patients are shown in Table.1.

Table.1. Baseline characteristic of the Groups

Variable	IDA (n=20) (Mean±SD)	IDA/AI (n=20) (Mean±SD)	AI (n=20) (Mean±SD)	P-value
AGE(years)	36 ± 12.04 ▲Δ	48.2±16.55	54.45 ± 19.93	0.004*
WBC(×10^3/µl)	6.25 ± 1.92 Δ	7.33 ± 2.31 Δ	11.3 ± 6.77	0.002*
RBC(M/µl)	3.57 ± 1.41	3.22 ± 1.63	3.89 ± 0.71	0.677
HCT(%)	30.8 ± 2.3	29.83 ± 4.77	30.03 ± 3.78	0.923
Hb(g/dl)	10.22 ± 0.6	10.07 ± 1.02	9.89 ± 1.2	0.852
MCV(fl)	74.36 ± 9.25	76.56 ± 6.03	78.32 ± 8.24	0.335
MCH(pg)	24.91 ± 3.76	25.77 ± 2.68	25.71 ± 3.4	0.576
MCHC(g/dl)	32.89 ± 1.83	33.8 ± 2.93	32.81 ± 1.71	0.796
CRP(mg/l)	4.46 ± 5.63 ▲Δ	34.96 ± 28.84	36.47 ± 27.35	0.000**
ESR(mm/hour)	23.20 ± 18.85 ▲Δ	61.1 ± 35.51	54.7 ± 36.31	0.000**
FE(µg/dl)	37.44 ± 20.61	42.55 ± 11.27	36.37 ± 22.72	0.284
TIBC(µg/dl)	412.85 ± 106.96 ▲Δ	331.39 ± 116.25	292.28 ± 87.3	0.002*
TSAT(%)	9.8 ± 5.72	13.46 ± 3.10	12.18 ± 6.17	0.058
FERR(ng/ml)	15.75 ± 8.31 ▲Δ	49.33 ± 13.32 Δ	271.9 ± 92.08	0.000**
HEP	2.45 ± 1.19 ▲Δ	8.66 ± 5.46 Δ	31.8 ± 19.73	0.000**

▲when comparing with AI/IDA group, Δ when comparing with AI group.

Hepcidin-25 results: In our study, mean Hepcidin-25 concentration in AI patients (31.8 ± 19.26ng/ml) was significantly higher than that in AI/IDA patients (8.66 ± 5.32ng/ml), controls (7.63 ± 5.09 ng/ml) and IDA patients (2.42 ± 1.077ng/ml)(P=0<0.0001) respectively (Table2).

Table.2. Serum Hepcidin-25 levels among the study population

Group	n	Hepcidin-25(ng/ml) mean± SD
IDA	20	2.42±1.077
IDA/AI	20	8.66 ±5.32
AI	20	31.8±19.26
Control	10	7.63±5.09

Hepcidin-25 distinguishes iron deficiency anemia (IDA) from (IDA/AI): Mean Hepcidin-25was significantly lower in patients with iron deficiency anemia compared with those with (IDA/AI) (2.42 ng/ml versus 8.66 ng/ml, P < 0.0001). We evaluated the clinical performance of Hepcidin-25 for distinguishing anemic patients in the two groups by plotting ROC curve. The area under the receiver operating characteristic curve AUC_{ROC} for Hepcidin-25 to distinguish iron deficiency anemia from mixed anemia was 0.986 (95% CI 0.96–1.00) (P=0 < 0.0001). Optimal Youden indices for distinguishing iron deficiency anemia from (IDA/AI) were seen at cutoff of 4.07ng/ml, with Sensitivity 94.7%, Specificity 94.7%, Positive predicting Value PPV=94.7%, and Negative predicting Value NPV=95%.Hepcidin-25 was found to be highly significant in differential diagnosis (figure.1).

Hepcidin-25 distinguishes (IDA/AI) from anemia of inflammation: Mean Hepcidin-25 was significantly lower in patients with (IDA/AI) compared with those with anemia of inflammation (8.66 ng/ml versus 31.84 ng/ml, P < 0.0001). The AUC_{ROC} for serum Hepcidin-25 in the combined group IDA/AI versus the AI group was 0.897 (95% CI 0.791–1.00) (P=0 < 0.0001). The optimal cutoff value for Hepcidin-25 was 12ng/ml, as calculated by the

Youden index. A Hepcidin-25 level lower than or equal to this cutoff value distinguished the IDA/AI patients from the AI patients with a sensitivity of 90%, a specificity of 78.7%, PPV=81.8% and NPV=88% .Hepcidin-25 was found to be highly significant in differential diagnosis.

Correlation Studies (Table.3):

Hepcidin-25 levels in relation to age among the study population: We found a significant positive correlation with age (r=0.372, p=0.003), that correlates with the fact that incidence of AI increases with age, thus indicating a multi-factorial etiology (Madu & Ughasoro, 2016).

Hepcidin-25 levels in relation to hematological parameters among the study population: By investigating the correlation between serum Hepcidin-25 and tested hematological parameters, we found that no correlations with statistical significance were detected between serum Hepcidin-25 and RBC, Hb, MCH, MCHC, RDW in our study; Meanwhile serum Hepcidin-25 levels exert a significant positive correlation with MCV values when all the groups were put together (r=0.297, p=0.021).

Hepcidin-25 levels in relation to iron parameters and inflammatory markers among the study population: There was a significant positive association between serum Hepcidin-25 and serum ferritin levels in the three groups and in IDA group (r=0.842, p=0 and r= 0.538, p<0.05, respectively). Hepcidin-25 showed significant negative correlation with TIBC (r=-0.415, p< 0.001). Acute phase reactants (ESR, CRP) were evaluated, and positive correlations were observed between Hepcidin-25 and inflammation parameters used for disease activity: ESR (r=0.371, p<0.003), CRP(r=0.516, p=0) and WBC(r=0.388, p<0.002) when all the groups were put together.

Table.3. Variables correlated with serum Hepcidin-25 levels in the studied groups (only significant correlations shown)*

		Hepcidin-25
Variable	All Groups	
	r	P<
Age(years)	0.372	0.003
MCV(fl)	0.297	0.021
TIBC(µg/dl)	-0.415	0.001
WBC($\times 10^3$/µl)	0.388	0.002
CRP(mg/l)	0.516	0.000
ESR(mm/h)	0.371	0.003
FERR(ng/dl)	0.842	0.000
*Spearman correlation analysis		

DISCUSSION

An area of interest these last years has been the potential value of Hepcidin-25 to discriminate between iron deficiency anemia IDA(low Hepcidin), AI(high Hepcidin) and the combined state of AI and IDA (Konz & Montes-Bayon, 2014). There are currently more than 20 available Hepcidin-25 assays, for which results correlate well, although absolute values vary (Pasricha & Atkinson, 2014).

To our knowledge, this is the first paper describes levels of Hepcidin-25 in such anemic cohorts and determine cutoffs for Hepcidin-25 in differential diagnosis of anemia of inflammation and iron deficiency anemia in Syria.

Hepcidin-25 is effective in diagnosing IDA and differentiating it from IDA/AI: In the present work, serum Hepcidin-25cutoff levels≤4.07ng/ml (sensitivity 94.7%, Specificity 94.7%) enabled to diagnose and distinguish IDA from IDA/AI. Whereas, Svenson (2015), targeting 77 adult patients with gastrointestinal related disorders associated with anemia, defined diagnostic cutoff values for Hepcidin-25<8ng/ml (sensitivity:73%, specificity :72%) for this purpose (Svenson & Patmore, 2015; Svenson & Patmore, 2015). for Iron deficiency anemia diagnosis, Hepcidin-25 concentrations have been found to be very low, often below the lower limit of detection of the employed assays (Ganz & Olbina, 2008; Kroot & Tjalsma, 2011). Both the c-ELISA and the IC-TOF-MS differentiated anemic RA patients with IDA from those with IDA and AI (Kroot & Laarakkers, 2010). In a Hepcidin-25 plot for the identification of different states of iron deficiency among 155 anemic patients, Use of Hepcidin-25 cutoff levels ≤11.16ng/ml (4nmol/l) allowed the differentiation of IDA from AI and AI/IDA (Thomas & Kobold, 2011). Hepcidin-25 measurements appear to have potential utility in diagnostic algorithms to distinguish IDA from AI and the combined presence of IDA/AI (Kroot & Tjalsma, 2011). However, In a study including 339 Kenyan infants aged (6±1.1) months, There were small, but significant differences in serum Hepcidin-25 comparing (IDA) infants to(IDA/AI) infants (1.2 ±4.9 vs. 3.4 ±4.9 ng/ml; P, 0.001) in male infants only. Again, These findings suggest that the inflammation mediated stimuli (through IL-6) are to a large extent overruled by iron demand and erythropoiesis stimuli down regulating Hepcidin-25 synthesis (Jaeggi & Moretti, 2013).

Hepcidin-25 is effective in discrimination between AI and AI/IDA: We found that serum Hepcidin-25 levels in AI were higher significantly than that in AI/IDA group (P < 0.0001). Similar results were documented in other reports

(Sasu & Li, 2010; Abdel-Khalek, El-Barbary, 2011). Theurl have shown elevated Hepcidin-25 levels in AI individuals and intermediate levels in AI/IDA (P<.001). His observation was confirmed by decreased Hamp mRNA expression in livers of animals with AI/IDA compared to animals with AI alone. This suggests that during inflammatory anemia a concomitant true iron deficiency reduces pSMAD1/5/8 mediated transcriptional activation leading to lower Hepcidin-25 levels despite massive STAT3 activation. Importantly, these regulatory pathways appear to underlie a specific hierarchy because inflammation mediated induction of Hamp transcription can be partly reversed *in vivo* by the regulatory cascades induced by true iron deficiency (Theurl & Aigner, 2009). According to our study, the optimal cutoff value for Hepcidin-25 to diagnose AI patients and distinguish from the IDA/AI patients was 12 ng/ml (sensitivity 90%, Specificity 78.7%). Svenson, using 3 tests: CBC, RetHe and Hepcidin-25 in differentiating IDA from AI in mixed etiology cases, determined diagnostic cut off values for Hepcidin-25 >40 ng/ml (Sensitivity 25%, Specificity 91%) to differentiate AI from mixed anemia. In elderly patients, Karlsson, determined the optimal cut-off for Hepcidin-25 as 31.5 ng/ml (sensitivity and specificity of 82% and 95%, respectively) for iron deficiency (Karlsson, 2015). In this line, van Santen, in a cross-sectional study on 155 patients with rheumatoid arthritis (RA) defined that AUC_{ROC} was 0.88 with Hepcidin-25 at 6.7 ng/ml had a sensitivity of 89% and a specificity of 88% (van Santen & van Dongen-Lases, 2011). However, Thomas, reported that it was not possible to differentiate between AI patients with and without IDA based on Hepcidin-25 alone (AUC_{ROC} =0.569), attributing this discrepancy to the fact that their study considered the ferritin index plot to differentiate AI from IDA/AI (Thomas & Kobold, 2011). Also, in Chronic hepatitis C disease (CHC), no statistical difference was found between these two groups (Toima & Saleh, 2010). Less Hepcidin-25 elevation in patients with (AI/IDA) anemia despite the fact that these patients had elevated CRP levels, may be due to that erythroid demand for iron is a more powerful regulator of hepcidin expression than inflammation-induced hepcidin formation. Another intriguing explanation is differences in the half-lives of CRP and Hepcidin-25 (the half-life of CRP is 19 h, whereas the half-life of Hepcidin may be much shorter) (Abdel-Khalek & El-Barbary, 2011).

In our investigation, serum levels of Hepcidin-25 in patient groups were statistically different (P<0.0001). These results are concordant with those of previous reports indicating that Hepcidin-25 could be a potential marker for detection and differentiation of these anemias (Cheng & Jiao, 2011; van Santen & van Dongen-Lases, 2011). Another study reports similar results with serum Hepcidin-25 among geriatric patients (p=0.034) (Rohrig & Rappl, 2014).

Correlations of Hepcidin-25: In our study Hepcidin-25 showed a strong positive correlation with ferritin (r=0.842, p<0.001). This is consistent with numerous studies that have documented a positive correlation between ferritin and Hepcidin-25 (Choi & Song, 2012, Naqvi & Faizan-ul-Hassan, 2014, Gaillard & Bock, 2016). To explore whether Hepcidin-25 was associated with an inflammatory state, the relationship between Hepcidin-25 and biomarkers of inflammation were examined. Hepcidin-25 concentrations correlated well with inflammatory status, as significant correlations were observed between Hepcidin-25 and each of CRP (r=0.516, p<0.001), ESR (r=0.371, p=0.003) and WBC (r=0.388, p=0.002) when all the groups were put together. These results confirm previous assertions (Sabau & Valeanu, 2013; Rohrig & Rappl, 2014; Karlsson, 2015). No Significant correlations were observed between Hepcidin-25 levels and hematological parameters and iron profile in our patients except for a positive correlation with MCV (r=0.297), and a negative correlation with TIBC(r=-0.415). Karlsson, found such negative correlation with transferrin (r=-0.775) (Karlsson, 2015).

4. CONCLUSION

The current and all future harmonization and standardization efforts will greatly impact the development of Hepcidin-25 as a new marker by paving the way for (a) explanation of generally approved and practical reference intervals and decision limits, (b) application of consistent clinical decision limits for medical care and best practice guidelines and (c) pooling and comparison of data from various studies to facilitate medical research and its translation to the clinic (van der Vorm, Hendriks, 2016).

Determination of serum Hepcidin-25 is still a novelty in Syrian medical practice. Our study on patients with different types of anemia confirms the ability of the used immunochemical method to differentiate IDA from IDA/AI and AI from IDA/AI, but the introduction of a dependable everyday procedure for the study of Hepcidin-25 in biological fluids is a step forward in further inclusion of Hepcidin-25 in clinical practice and public health. However, more local studies with larger patient groups are needed to support our results.

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