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Journal

1. **Van den Berghe G, Wouters P, Weekers F, et al.** Intensive insulin therapy in critically ill patients. *N Engl J Med* 2001; 345:1359-1367.

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2. **Falk SA, ed.** *Thyroid Disease: Endocrinology, Surgery, Nuclear Medicine, and Radiotherapy.* 2nd ed. Philadelphia: Lippincott-Raven, 1997.

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Journal Of The Egyptian Society of Endocrinology, Metabolism & Diabetes

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Letter From The Editor

Dear Colleague,

In this issue, we have included an interesting Editorial Article dealing with change from patient – centeredness to community orientation as a step ahead in the developing world.

We are nearly finished with the initiation of a website for our journal, which will be published in the next issue.

Once again, we hope to meet your expectations, and until we meet in our next issue, deepest regards and best wishes.

The Editor

Prof. Samir Helmy Assaad Khalil

Cover of the Journal: The gods and goddesses of the ancient Egyptian civilization including: Isis (goddess of prosperity and female fertility), Min (god of male fertility), ect… Designed by: S.H. Assaad Khalil

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Editorial Article

From Patient-Centeredness to Community-Orientation: One Step Ahead in the Developing World.

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Introduction:

Patient centeredness and patient empowerment has been accepted as an integral part of family medicine for long[1]. In modern diabetology, too, patient-centered approach and patient-centered professionalism have been advocated by various authors[2, 3]. It is now generally accepted that a patient-centered approach is necessary in diabetes management. Till recently, however, patient-centered care seemed to be relegated to the periphery of diabetology. Experts and practitioners tended to debate more on the merits and demerits of various treatment modalities, based on meta-analysis and statistical jugglery, rather than considering the patient's viewpoint.

The latest guidelines for the management of diabetes mellitus, released by the American Diabetes Association and European Association for Study of Diabetes, strongly stress upon the need for a patient centered approach[4]. The choice of the title of this guideline, including the words "a patient-centered approach" has now brought the concept of patient-centered care (PCC) to center-stage in diabetes. But is this enough, especially for patients from the developing world, whether from Africa, the Middle East, or Asia?

Do we need to do more?

Constituents of the Community:

A person with diabetes mellitus is not an isolated entity. Rather, she or he lives as part of a community[5]. In most eastern cultures, the community has a strong influence on an individual's health. She or he is surrounded by family, friends, neighbors, colleagues and acquaintances, all of whom exert varying influences on her or his attitudes and behavior. The patient is also exposed to opinions and utterances of community leaders, religious leaders, political figures, and public personalities such as film stars and sportspersons. These people, too, have a bearing upon the behavior of the person with diabetes. Even more importantly, perhaps other doctors and paramedical personnel, such as pharmacists and nurses, as well as practitioners of complementary or alternative systems of medicine, exert a significant effect or the patient's psyche.

The collective influence of all these members of the community is too important to be ignored. The term Eco sensitivity has been utilized by the authors to explain this phenomenon. The concept of Eco sensitivity is equally relevant for all health and disease states, for all people. However, it is, perhaps, more important for people with chronic disease, living in traditional cultures such as Asian, African and Latin American communities.

Impact of the Community:

The community modifies the presentation and management of diabetes in multiple ways.

Attitudes towards, and perceptions of modern medicine determine the ease with which a patient approaches a qualified diabetes care professionals. Negative attitudes towards modern diabetology, and / or positive feelings about traditional or complementary medical systems, create a situation where people present during the later stages of disease, with severe (yet, avoidable) complications. These negative impressions may be created by prior unhappy experiences with the medical care system, with the diabetes care system, or with a particular diabetes care professional. These experiences may be personal, or may have happened to other community members.

While some negative experiences may be due to actual deficiency on part of the health care provider or system, others may be because of unrealistic expectations (" I went to the doctor

with slight blackening of the heel; that too, painless; and they cut off my foot"). At other times, hostile opinions are purposefully created or implanted by certain stakeholders with vested interests, such as practitioners of alternative medicine, who seek to limit the influence of qualified medical personnel, whom they view as competitors or encroachers into their sphere of influence.

Once the person with diabetes does consult an appropriate care provider, her or his interaction is limited to a few minutes, at best. Whatever interaction takes place is subjected to rigorous scrutiny and criticism by family and friends[6]. This criticism includes (well meaning) (uninformed) opinions about the relevance of suggested investigations and appropriateness of prescribed therapy. The most accurately written prescription of modern insulin may fail to pass what the authors colloquially term the "housemaid litmus test": "My housemaid told me that taking insulin causes blindness." This form of Eco sensitivity is especially prevalent, and important, is eastern cultures, where sharing personal information about one health and disease is an accepted part of social interaction.

The quality of interaction with the diabetes care provider depends upon the cultural competence[7] and cultural skills of the professional. While the physician may not necessarily be from the same community as the patient, her or his cultural knowledge of the patient's background will impact acceptance of suggested interventions, and therapeutic outcomes

Another source of 'interference' either positive or negative, in diabetes care, is religion[8], or, rather, religious leaders. Certain religions are thought to be passive in nature ("Turn the other cheek" "Your troubles stem from sins incurred in your previous incarnation: it is your duty to suffer them."). Such teachings are misused by certain people to advocate a pessimistic approach to diabetes and its complications, and to avoid timely interventions such as insulin.

The impact of the community does not end even after appropriate diabetes care has been sought, received, and accepted.

The patient is continuously exposed to unwanted stimuli which encourage unhealthy dietary and physical activity patterns. Advertisements for high calorie foods, entice people with diabetes to commit dietary indiscretions which worsen glycemic control. The community at large tends be unhelpful, as it continue to serve unhealthy foodstuffs at parties, functions and events, and then expects people with diabetes not to eat them. This sadistic culture has been termed' culinary cruelty' or 'dietary draconism'[9], exhibited by self-styled ' diabetes police'.

The overall literacy level of the community, which may be suboptimal in many developing countries, also impacts attitudes towards diabetes care[10]. It becomes challenging to improve diabetes related literacy in culturally and linguistically diverse communities with limited formal literacy.

Availability of energy-saving devices such as escalators, lifts, vehicles, treadmills and other automated machines discourages people with diabetes from following 'old fashioned' healthy lifestyles. Cars and televisions have been found to promote sedentary behavior, and thus, increase the risk of myocardial infarction [11]

Positive Power of the Community:

Not all influences exerted by the community are negative, however. Peer education and peer support is an effective method of propagating, and ensuring, diabetes education. In resource challenged societies which lack qualified diabetes nurses, peer or by educators may be the only vehicle for dissemination of knowledge. This community support has been proven to be effective across a wide and diverse spectrum of clinical settings[12].

The family contributes in a major way to diabetes care[13] in the developing world. The choice of food and cooking practices, time and facilities available for physical activity, and choice/acceptance of diabetes care provider, are aspects of health care which are decided by the family.

Religious leaders and non-governmental organization often take up the mantle of improving diabetes care-related behaviour. In many communities, the word of the local imam or priest is taken as law. A few words in support of proactive diabetes management,

e.g. regular glucose monitoring or acceptance of injectable therapy, may go a long way in creating a positive impact with respect to glycemic control. Religion can be used as a motivational tool to help people accept therapy such as insulin [14].

Non-governmental organizations (NGOs) such as the Rotary club also influence attitudes towards diabetes through their activities. Other NGOs working in public health, though not related to diabetology directly, do improve health care-related behaviour in general.

Harnessing the Community:

Knowledge of community dynamics, and understanding of the process diffusion of innovations (diffusion research) [15], can help stakeholders working in diabetes in multiple ways. It is necessary to ensure sensitization of the community towards the need for diabetes care, create awareness of all available investigation and treatment modalities, and emphasize the potential of these modalities in preventing morbidity and mortality. This helps create a positive opinion, which surrounds the patient when she or he returns to the community. Harnessing the potential of the community facilitates an improved compliance and persistence with therapy.

It also frees the diabetes care professionals from much of the effort expended on motivating patient to accept appropriate diabetes care. This allows the care provider to focus on the actual prescription, and upon secondary or tertiary aspects of diabetes education, rather than primary ones.

The work of motivation is done by the community in general, and by lay educators[16] or “diabetes evangelists” in particular. These diabetes evangelists are patients with diabetes who spread the message of modern diabetes care, including the need for diet, physical activity, investigations and appropriate management. With improved compliance and persistence to better quality care, a community-oriented approach is able to achieve enhanced glycemic control and overall outcome. In a Jamaican study, for example,[16] lay educators were able to help achieve a sustained improvement in glycemic control. This provides objective.

Conclusion:

Recent guidelines for the management of diabetes emphasize a patient centered approach. While this in itself is laudable, we need to move a step forward. What is required, in the developing world especially is a community-oriented or a community-inclusive approach to ensure optimal diabetes control in all individuals with the disorder. A community-oriented approach is the only practical way to ensure diffusion of awareness and knowledge, and improve attitudes and practices related to diabetes care.

Every diabetes care professional should be sensitized to the need for community-oriented approaches, is tandem with patient centric professionalism, while managing diabetes. It is also necessary to train professionals in the fine intricacies of diffusion research and community modulation. This will help ensure healthy diabetes care - seeking behavior, and correct self-management behavior in all people with diabetes.

As every community in unique, local guidelines will be necessary to help professionals practice appropriate **community oriented diabetes care** Sustained emphasis on the importance of community oriented diabetology is required to ensure better care for people with diabetes. This will be a small step forward for diabetes care professionals, but a giant leap for our patients.

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Original Article

Diagnostic and Prognostic Utility of a Multiple Metabolic Biomarkers Panel in Critically ILL Patients with Acute/Chronic Decompensated Heart Failure: BILLIARD Study.

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Abstract:

Introduction: The progression of heart failure is complex and is driven by multiple biological processes. As such, a single biomarker is unlikely to be sufficient for risk stratifying patients with HF. We studied the additive diagnostic and prognostic value of a panel of multiple new biomarkers implicated in the different pathophysiological aspects of the development of the disease; B-type natriuretic peptide (BNP) (Myocyte Stress/Stretch), high sensitivity troponin (hsTnT) (Myocyte Injury), soluble ST2 (sST2) (Myocyte stretch / inflammation) and Galectin-3 (Gal-3) (Inflammation & Extracellular Matrix Modeling), Parathormone hormone (PTH 1-84) (neurohormonal activation). **Methods:** The study was conducted on 60 patients with ADHF, as well as 20 healthy volunteers. The primary endpoints were the improved specificity and sensitivity for the diagnosis of ADHF, and the prediction of short term mortality combining the panel of biomarkers to the ADHERE and EFFECT clinical short term mortality risk scores. The secondary endpoint was the prediction of a composite of major adverse cardiac events (MACEs) in the ADHF group. **Results:** BNP had the best accuracy for the diagnosis 98.75% and the largest ROC derived AUC=0.998 at 100 pg/ml, followed by ST2 91.25% (AUC=0.948) at 2917 pg/ml. Gal-3 and hsTnT have shown similar diagnostic accuracy of 83.75%, (AUC= 0.895, 0,894, respectively) at a cutoff point of 4.9 ng/ml for Gal-3 and 0.015 ng/ml for hsTnT. PTH1-84 accuracy for the diagnosis was 56.67%, AUC=0.733. The levels of BNP, hsTnT, ST2 and Gal-3 were significantly higher in non-survivors compared to survivors. While there were no significant difference in the level of PTH 1.84. The use of the proposed panel of multiple biomarkers (BNP+sST2+hsTnT+Gal-3) significantly improved the prediction of short term mortality in the ADHF patients group compared to the clinical scores alone; improving the AUC for the ADHERE in-hospital mortality score from 0.697 to 0.962 (p=0.006) and for the EFFECT 30 days mortality risk score from 0.734 to 0.954, p=0.027 and for the EFFECT 1 year mortality score from 0.643 to 0.931, p=0.005. **Conclusion:** There is a synergistic value of combining a panel of multiple biomarkers (BNP, hsTnT, ST2, Gal-3) to existing clinical score for the prediction of short term mortality and MACEs in severe ADHF patients. While the additive diagnostic value of this multiple biomarkers study was much less valuable, with little effect on the accuracy of the diagnosis of ADHF compared to BNP.

Keywords: Acute decompensated heart failure – multiple biomarkers strategy – BNP – sST2 – Galectin 3 – hsTnT – Parathormone PTH – Diagnosis – Prognosis.

Introduction:

Heart failure is becoming a major public health problem worldwide. Despite improvements in medical therapy, outcome remains poor, with a 5-year mortality approaching 50% in symptomatic patients and a reported in-hospital mortality up to 19%.[6]

Although HF is increasingly encountered in medical practice, its prompt diagnosis can be challenging, even for experienced clinicians. Furthermore, when the diagnosis of HF is made, it often remains challenging to risk stratify patients.

The progression of heart failure is complex and is driven by multiple biological processes[1] As such, a single biomarker is unlikely to be sufficient for risk stratifying patients with HF and with the development of new assays there has been growing interest in the measurement of a diverse biomarker profiles and panels, reflective of the underlying

biology of heart failure, as a means to improve the accuracy of diagnosis and the precision of risk stratification.[7–9]

The proposed panel in our study included multiple new biomarkers implicated in the different pathophysiological aspects of the development of the disease; B-type natriuretic peptide (BNP) (Myocyte Stress/Stretch), high sensitivity troponin (hsTnT) (Myocyte Injury), soluble ST2 (sST2) (Myocyte stretch / inflammation) and Galectin-3 (Gal-3) (Inflammation & Extracellular Matrix Modeling), Parathormone hormone (PTH 1-84) (neurohormonal activation).

Methods:

The study was conducted on 60 patients admitted the Alexandria University Main Hospital (AUMH) ICUs presented with acute decompensated heart failure (ADHF), as well as 20 healthy volunteers, selected to match the patients group's age and gender after exclusion of heart failure.

The primary endpoints were the improved specificity and sensitivity for the diagnosis of ADHF, and the prediction of short term (in-hospital / 30 days) and 1 year mortalities. A second endpoint was determined including a composite of major adverse cardiac events (MACEs): in-hospital hemodynamic instability (cardiogenic shock) with subsequent need for vasopressor and inotropic IV drugs, in-hospital life threatening arrhythmias – defined as new onset arrhythmia during hospitalization responsible of instability or worsening of hemodynamic status –, need for mechanical ventilation (for severe pulmonary congestion or cardiogenic shock – Killip class III/IV) and re-hospitalization for decompensation of heart failure during a 1 year follow up period.

On ICU admission, serum levels of BNP have been measured using the Dxl® BNP (Beckman Coulter, Alere reagents). R&D Systems® (Abingdon, United Kingdom) ELISA kits were used for the measurement of sST2 and Gal-3. hsTnT serum level has been measured using Roche Diagnostics® (Indianapolis, Indiana) reagents, while serum PTH 1-84 has been measured using the LIAISON® PTH 1-84 assay (DiaSorin).

Clinical risk score were calculated for each patient in the ADHF group; ADHERE tree algorithm for in-hospital mortality and the EFFECT 30 days and 1 year mortality risk score.

Results:

Patients' characteristics and outcome are fully illustrated in table 1. The levels of the studied biomarkers were significantly higher in the ADHF patients group in comparison with the control group. For each marker a ROC curve derived cut off point was calculated for achieving the best sensitivity, specificity and accuracy for the diagnosis of ADHF. BNP had the best accuracy for the diagnosis 98.75% and the largest ROC derived AUC=0.998 at 100 pg/ml, followed by ST2 with an accuracy for the diagnosis with 91.25% (AUC=0.948) at 2917 pg/ml. Gal-3 and hsTnT have shown similar diagnostic accuracy of 83.75%, (AUC= 0.895, 0,894, respectively) at a cutoff point of 4.9 ng/ml for Gal-3 and 0.015 ng/ml for hsTnT. While PTH1-84 showed the least accuracy for the diagnosis with 56.67%, AUC=0.733.

The levels of BNP, hsTnT, ST2 and Gal-3 were significantly higher in non-survivors compared to survivors. While there were no significant difference in the level of PTH 1.84. The optimal cut-off points for the prediction of mortality were 1278 pg/mL for BNP, 0.04 ng/mL for hsTnT, 3788 pg/mL for sST2 and 11.16 pg/mL for Gal-3.

The use of the proposed panel of multiple biomarkers (BNP+sST2+hsTnT+Gal-3) significantly improved the prediction of 1 year mortality in the ADHF patients group compared to the routinely used BNP alone, with a larger ROC derived AUC of 0.924 versus 0.822, p=0.03.

Patients with higher ADHERE risk tree score showed a higher -but not statistically significant - trend for in-hospital mortality (p=0.063), while patients with higher EFFECT risk score showed a statistically significant higher 30 days mortality (p=0.028). Adding the proposed panel of multiple new biomarkers (BNP+hsTnT+ST2+Gal3) to these clinical scores, improved their performance for the prediction of short term mortality, significantly improving the ROC derived AUC for the ADHERE score from 0.697 to 0.962 (p=0.006) and for the EFFECT 30 days risk score from 0.734 to 0.954, p=0.027. The same results were reproduced for the EFFECT 1 year mortality risk score that showed a modest performance in our study, which significantly

improved when combined to the proposed panel of biomarkers, (AUC 0.643, 0.931 respectively, p=0.005).

While hsTnT, ST2 and Gal-3 were significantly higher in patients who showed at least one of the MACEs (p<0.001), BNP levels were not significantly higher (p=0.07). The ROC curve analysis showed an AUC for the prediction of all events of 0.636 for the BNP, which significantly improved when combining all biomarkers proposed in our panel (BNP+hsTnT+ST2+Gal-3) into 0.958, with a strong statistical significance between both AUCs (p<0.001).

Discussion:

Our findings demonstrate the powerful diagnostic value of BNP for the diagnosis of ADHF, as previously demonstrated in many previous studies. [10] In a systematic review of 20 studies, using a cutoff value of 52 pg/ml for BNP achieved a high diagnostic sensitivity; values below this cutoff point were able to exclude the diagnosis of heart failure.[11] These findings led to a Class I LoE A recommendation for the use of this marker in the diagnosis of heart failure, especially in case of diagnosis uncertainty.[1]

Interestingly, analysis from Breathing Not Properly (BNP) Multinational Study,[12] showed an add on benefit of the measurement of BNP levels for the diagnosis of congested heart failure (CNF), even in patients with clinical certainty for the diagnosis; at an 80% cutoff level of certainty for the diagnosis of CHF, adding BNP to the clinical judgment would have enhanced diagnostic accuracy from 74% to 81%.

The use of the proposed panel of multiple biomarkers BNP+ST2+Gal-3+hsTnT had a modest additive diagnostic value, with a non-significant improvement of the ROC derived AUC for the diagnosis of ADHF compared to the routinely used BNP alone (p=0.498), accordingly we dis-recommend their sole use for the diagnosis of ADHF.

Yet, in special situation, their combined use may be useful for the diagnosis of ADHF, in case of uncertainty, especially in the presence of any of the known confounding factors for the natriuretic peptides are present; female sex, old age, absence of sinus rhythm & high BMI.

In our study, only one patient had an admission BNP level below the calculated cutoff point of ≤100 pg/ml**,** but had the levels of ST2, Gal3 and hsTnT above the calculated cutoff point for the diagnosis of ADHF. Further studies with larger number of patients are needed to show the utility of the combined biomarker strategy for the diagnosis of ADHF in cases where BNP levels are low. The answer for this question is important because a prompt diagnosis of ADHF and consequently the early management has been shown in many studies to be associated with better outcome and to be cost effective; decreasing the length of hospital stay and the rate of re-hospitalization. [13] [14] [15]

After establishing the diagnosis of ADHF, assessing the patient's risk is, at times, a challenging proposal. Early risk stratification may help identify patients who are likely to receive the greatest benefit from early resources-intensive strategies. There are few data on short-term risk stratification for ADHF patients.[3,4,16–20] Most of these scores has been derived and validated before the era of the recently described new HF biomarkers, even before the widespread routine use of natriuretic peptide measurement for patients hospitalized with ADHF. For example, the derivation cohort of the ADHERE model - using the CART statistical method that tends to prioritize available data over missing data - only 25% of the patients had BNP levels available in their records. This limitation may suggest that more sensitive and specific variables have been rejected just for their unavailability according to the retrospective design of the study.

In our opinion, with the availability of the new HF biomarkers reflect the different pathophysiological processes of HF and the current data about their ability to predict worsened outcome, as demonstrated in our study and other pilot studies,[21][22][23][24][25][26][27][28,29][30] new prediction models will be developed associating multiple biomarkers with the existing clinical risk scores, to improve the accuracy for the prediction of mortality in ADHF. Choosing the panel of biomarkers to deploy in clinical practice is yet to be defined

depending on its significant predictive ability cost and ease of assay.

In the present work, the use of the proposed panel of multiple biomarkers (BNP+sST2+hsTnT+Gal-3) significantly improved the prediction of 1 year mortality in the ADHF patients group compared to the routinely used BNP alone, with a larger ROC derived AUC of 0.924 versus 0.822, p=0.03.

The study by Pascual-Figal, on 136 ADHF patients showed the same significant association of elevated levels of ST2, hsTnT and NT-proBNP with short term mortality. The combination of the three biomarkers had an additive value for the prediction of short term mortality.[28]

The latest study describing the utility of a combined biomarkers strategy for the risk stratification of patients with HF was The Barcelona Bio-Heart Failure Risk Study (BCN Bio-HF Calculator) that was derived and validated on 864 patients with chronic ambulatory heart failure. The stratification for 1, 2, and 3 years mortality was better in the models containing more than one biomarker; with the highest found using the combination of ST2 and hs-cTnT.[31]

We tested the performance of two of the most widely validated clinical scores for the prediction of short term mortality in patients hospitalized with ADHF, the ADHERE risk tree for the prediction of in-hospital mortality [3] and the EFFECT risk score for the prediction of 30 days. [4] Surprisingly, the performance of these clinical scores in our study cohort was modest. ROC derived AUC of 0.697 for the ADHERE score, p=0.075, while the AUC for the EFFECT risk score was 0.734, p=0.034.

When comparing the performance of the two clinical scores, they showed matching, yet weak, AUC for the prediction of short term mortality risk with no statistically significant difference between both AUCs, p=0.639. This is in concordance with data published by Auble et. al, on over 30,000 patients hospitalized with ADHF, that showed similar AUCs for the ADHERE and the EFFECT for the prediction of in-hospital and 30-days mortality, (0.72, 0.74, respectively). The two models had equal ability to stratify patients at high risk, while the EFFECT score was superior to identify patients at low risk for short term mortality.[32]

The modest performance of these scores, observed in our study, may be explained by many factors concerning the study design, the characteristics of our cohort of patients as well as the process of implementation and calculation of these scores.

Our study has been concerned with the risk stratification of critically ill patients admitted to the ICU presenting with severe forms of ADHF, the mean SBP on admission was 107 mmHg, 40% needed resources-intensive support strategies including support with mechanical ventilation (25%) and/or use of vaso-active drugs for hemodynamic instability (31.67%). In reviewing the patients' characteristics deriving and validating the ADHERE and EFFECT clinical scores for the risk stratification of patients with ADHF, we found that most of the patients cohorts are lacking or under-presenting critically ill ADHF patients with such severe forms of heart failure.

In the ADHERE study, only 30% of the 65 thousands studied patients had severe heart failure at presentation, only 23.4 % received vaso-active drug therapy – 60% of these drugs were vasodilators, the mean SBP on admission was 144.7 mmHg and 47% of the patients had an LVEF above 40%. As for the EFFECT study cohort including 4031 patients, only 47.7% had an LVEF below 40%, the mean systolic blood SBP was 148 mmHg, clearly a less sick cohort of patients than our group of interest.

Similarly critically ill patients with severe ADHF were under presented in derivation cohorts for other available clinical scores. The PROTECT 7 days in-hospital outcome score [33], derived and validated on a cohort of 2033 patients, while 77% of these patients had NYHA class III-IV, those on inotropic therapy or mechanical ventilation support were excluded from the derivation model, as well as those with co-morbidities including severe pulmonary disease, recent ischemia or cerebrovascular stroke, and significant arrhythmias.

An interesting risk model, where severe form of ADHF was well represented is the

ESCAPE risk model, derived out of 423 full data patients, mean LVEF was 20%, mean SBP was 106 mmHg and a mean hospital stay of 8.5 days, with 5% in-hospital and 18.7% six months' mortality rates. Yet the derived risk prediction model was only validated at patients' hospital discharge and not for in-hospital risk stratification.

Another point that may explain the modest performance of these clinical scores for critically ill ADHF patients may be the process of their calculation. In our experience, we found the implementation of the ADHERE score to be easy and simple through its easily retained tree algorithm design including only admission BUN, SBP and creatinine values, yet in this cohort of critically ill patients, the measure of SBP could rapidly vary during the first hours of admission, as well as the values of BUN and creatinine that could largely vary during the first days from admission, as previously demonstrated in previous studies; showing worsening of renal function especially during the first 3 days post-admission, a small increase in serum creatinine of 0.1 mg/dl or in BUN levels were linked with worsen patients outcome.[34] [35] [36] [37]This, in our opinion can be confusing; whether to include for the risk score calculation the worst SBP or renal function values early after ICU admission rather than the first measured values on admission.

The implementation of the EFFECT score model in clinical practice, was also more complex, because of the sometimes encountered difficulties to determine the presence of the co-morbidities at admission (dementia, liver-cirrhosis), required for its calculation. These difficulties have been encountered and described by other studies validating this clinical score. [32]

Lastly, in our study, these clinical scores showed a superior ability to identify less sick patients, who are at low risk for short term mortality, (none of the patient with a score of 1 showed short term or 1 year mortality). This demonstrates the stronger capability of these clinical score to identify patients at low risk, rather than high risk for mortality, emphasizing on their role for especially triaging ADHF patients who might be effectively and safely treated and monitored in intermediate care wards rather than intensive care units.

In our study, we showed that adding the proposed panel of multiple new biomarkers (BNP+hsTnT+ST2+Gal3) to these available clinical scores, improved their performance for the prediction of short term mortality, with a significant improvement of the ADHERE AUC for the prediction of in-hospital mortality (from 0.697 to 0.962, p=0.006). Also, the performance of the EFFECT risk score for the prediction of 30 days mortality has significantly improved when combined to the proposed biomarkers panel, significantly improving the AUC from 0.734 to 0.954, p=0.027. Similarly for the EFFECT 1 year mortality risk score, improving the ROC curve derived AUC from 0.643 to 0.931, p=0.005.

Similarly, Ky et al. have recently showed that adding a more complex biomarker panel consisting of high-sensitivity C-reactive protein, myeloperoxidase, B-type natriuretic peptide, soluble fms-like tyrosine kinase receptor-1, troponin I, sST2, creatinine, and uric acid to the Seattle HF Model improves the predictive accuracy for 1-year all-cause death in chronic heart failure patients. [7]

The use of the combined biomarkers strategy of (BNP+hsTnT+ST2+Gal-3) had also a better performance for the prediction of all MACEs compared to the routinely used BNP levels alone, (AUC 0.958, 0.636, respectively, p<0.001). When analyzing each of the selected events, levels of BNP, hsTnT, ST2 and Gal-3 were significantly higher in patients who showed hemodynamic instability, need for inotropic and vasopressor IV drugs as well as mechanical ventilation (MV).

As expected, BNP as a marker of myocyte stretch, was the only marker in our proposed panel to show significantly higher levels in patients who needed high doses of IV diuretics (p=0.025). While ST2 was the only biomarker in our proposed panel to show significantly higher level and so to identify patients who needed IV vasodilator drugs, p=0.02. This is an interesting finding that could support the recently described link between sST2 and endothelial function. It has recently been described that human venous and arterial endothelial cells secrete sST2 protein.[38] Moreover, it has been proved that increased expression of sST2 is mediated, in part, by endothelin-1, a potent vasoconstrictor[39] and also in response to elevated indexes of diastolic load[40]. All these findings suggest that the vascular endothelium, sensing hemodynamic and inflammatory status, is a potential source of s ST2 levels in hemodynamic overload and heart failure,

hence the need for vasodilator treatment for patients with elevated sST2 levels.

These findings from our study might represent the first step for a biomarker guided therapy for patients with ADHF; this strategy would permit a more tailored individualised management. This hypothesis, if proven, shows that biomarkers are not only markers of severity of the disease and high mortality but also might help for guiding the therapy and the prevention of this mortality.

To our best knowledge, our study is the first to combine the use of sST2 and Gal-3 in a panel for the risk stratification of patients with ADHF. Patients with elevated Gal-3 (levels above the calculated cutoff point of 11.16 ng/ml) and low sST2 (below the calculated cutoff point of 3788 pg/ml) showed a significantly better outcome than patients with high Gal-3 and high sST2 (p=0.001). Galectin-3 is a product of active macrophages, with binding sites on cardiac-resident fibroblasts, leading to an increase in myocardial collagen expression and interstitial fibrosis and subsequent LV dysfunction in response to myocardial injury or inflammation.[41,42]

The response of healthy cardiac tissue to injury or mechanical stress involves the production and binding of IL-33 to membrane bound ST2 (ST2L), which stimulates a cardioprotective signaling cascade that defends against fibrosis and cardiac remodeling.[43][44][45] When sST2 levels are elevated, however, it acts as a as a soluble decoy receptor of IL-33, binding to IL-33, thus reducing the beneficial effect of IL-33 through the ST2L receptor, so that cardiac fibrosis starts to develop.[39] These scenarios suggest an immunoregulatory roles for sST2 and ST2L in heart failure, regulating inflammatory signals in heart failure.[46]

Our results support this hypothesis showing that despite the elevated levels of the Gal3 as a marker of inflammation, the low levels of sST2 permitted the modulation of this inflammation, attenuating the magnitude of its deleterious effect and leaded to a better outcome for these patients.

This study is mainly limited by the small sample size with mainly male patients and the single centre design, which increases the risk of a type II error. Also, each of the biomarkers proposed in our panel was a significant predictor of primary and secondary endpoints by univariate analysis, when entered into multivariate analysis including other predictors, none showed statistical significance. Only sST2 showed independent significant relation with ALL MACEs, on the multi-variant logistic regression analysis. This is likely due to the limited power of our study. Larger studies with larger patients' cohort are needed to confirm the findings of our study.

Furthermore, a major limitation of our study is the comparison of the diagnostic value of the proposed biomarkers between ADHF patients and completely healthy asymptomatic volunteers. More interestingly was to study the behavior of this panel of biomarkers in a control group of symptomatic patients admitted to the ICU after the exclusion of the ADHF diagnosis. Yet, as this panel has never been studied in the Egyptian population, and their characteristics have never been described in this population, we found it reasonable to include a group of healthy volunteers as a pilot first step study; especially that this design has previously been adopted in other studies. [30]

Lastly, we hypothesized in our study the ability of the proposed panel of biomarkers to guide therapy but further prospective studies designed specifically for the purpose to study the success of each biomarker guided intervention are needed to prove this hypothesis.

We demonstrated for the first time, the synergistic effect of a panel of multiple biomarkers (BNP, hsTnT, ST2, Gal-3) for the prediction of short term mortality and MACEs in severe ADHF patients compared to the routine use of BNP alone as well as the use of clinical scores alone. While the additive diagnostic value of this multiple biomarkers study was much less valuable, with little effect on the accuracy of the diagnosis of ADHF compared to BNP.

For better understanding of our study design and results, we estimate patient stratification and triaging like a BILLIARD game, where the used score acts like a tool (BILLIARD stick) to sort patients (balls), into different outcomes and risks (pockets). Adding the multiple biomarker score to the clinical scores, not only adjusted the precision of the patients' risk stratification, but also added the number of predictable events (pockets), of interest specially in the cohort of critically ill patients, such as the need for MV, need for inotrope and need for vasopressor drug therapy, which may guide patients' management and consequently improve outcome.

Disclaimer

This study complied with the Declaration of Helsinki and was approved by ethical committee of the Alexandria Faculty of Medicine Main University Hospital. There is no conflict of interest to disclaim.

Table I: ADHF patients group characteristics

	Mean ± SD.	
Age	59.7 ± 12.6	
BMI	26.6 ± 3.1	
NYHA	3.3 ± 0.7	
EF	31.7 ± 7	
ICU stay	5.1 ± 3	
Hospital Stay **Routine laboratory tests**	9.0 ± 3.4	
Hb (g/dl)	12.2 ± 2.2	
WBCs (103c/mm3)	12.0 ± 4.6	
Sodium (Na) (meq/L)	133.4 ± 5.4	
Potassium (K) (meq/L)	4.5 ± 0.74	
Urea (mg/dl)	85.6 ± 55	
Creatinine (Cr) (mg/dl)	1.53 ± 1	
ASAT (Ul/l)	114.2 ±288	
ALAT (Ul/l)	101.02 ± 243	
RBS (mg/dl)	184.1 ± 88	
	n	**%**
Male patients	43	71.7
Primary outcome		
Short term mortality **(In-hospital/30 days)**	8	13.3
1 year mortality	3	5
ALL mortality	11	18.3
Secondary outcome		
ALL MACEs	30	50
HD Instability	19	31.6
Serious in-hospital Arrhythmias	10	16.6
Mechanical Ventilation	12	20
Re-Hospitalization for ADHF	12	20
Initial line of **ICU support treatment**		
Need for IV Vasopressor	14	23.33
Need for IV Inotrope	17	28.33
IV Diuretic Shots	37	61.67
IV Diuretic Infusion	8	13.33
High Dose IV diuretic	16	26.67
Need for IV nitrates	12	20.00
ATB	19	31.67

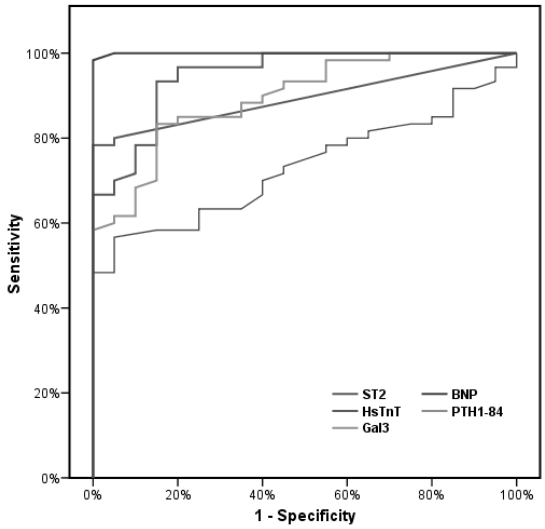

Figure (1): ROC curve for ST2, Gal3, PTH1-84, BNP and hsTnT for the diagnosis of ADHF

Table II: Comparison between AUCs for each mortality score and its biomarkers combination

	AUC	p	Difference between AUCs
ADHERE in-hospital mortality risk score	0.697	0.075	0.006*
ST2,BNP,hsTnT,Gal3 + ADHERE risk	0.962*	<0.001	
EFFECT 30 days mortality risk score	0.734*	0.034	0.027*
ST2,BNP,hsTnT,Gal3 + EFFECT 30d risk	0.954*	<0.001	
EFFECT 1 year mortality risk score	0.643	0.141	0.005*
ST2,BNP,hsTnT,Gal3 + EFFECT 1 year Risk	0.931*	<0.001	

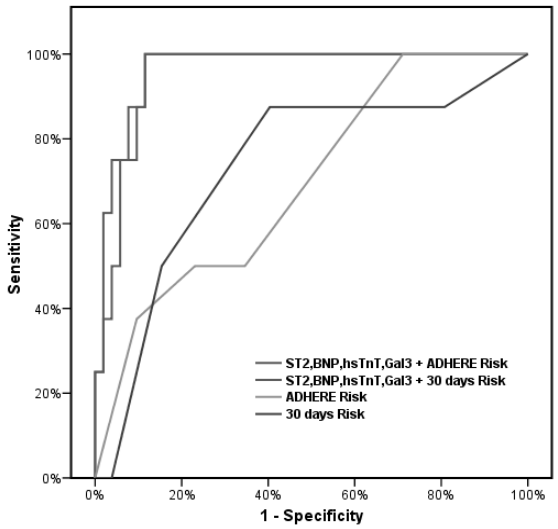

Figure (2):ROC curves for the ADHERE & EFFECT 30 days mortality scores and their combination to the panel of multiple biomarkers

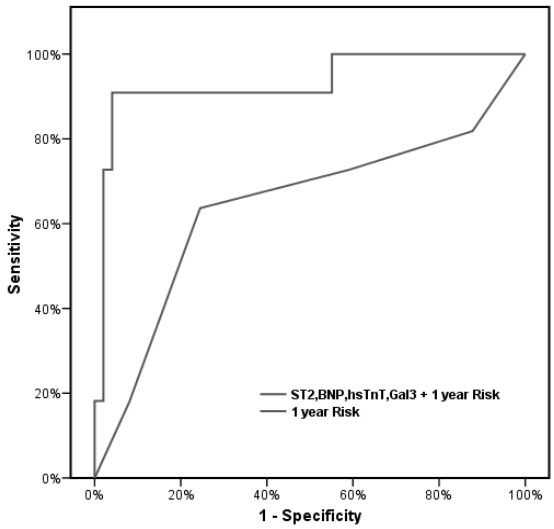

Figure (3):ROC curves for the effect 1 year mortality score and its combination to the panel of multiple biomarkers

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Original Article

Study of the Impact of the Use of a Thiazolidinedione or a DPP-4 Enzyme Inhibitor as an Adjuvant to Standard Immunosuppressive Therapy on the Rate of Recurrent Exacerbations in Behçet's Disease Patients with Uveitis.

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Abstract:

Introduction: Uveitis in Behçet's disease is a potentially blinding condition. It may lead to anterior and posterior synechiae which may lead to secondary glaucoma, cataract, and macular oedema. Posterior segment involvement in Behçet's disease includes posterior uveitis, retinal, macular, and optic disc oedema, and retinal vasculitis. Adequate control of activity of the disease with systemic immune-suppressive therapy in order to salvage vision is of utmost importance, hence the necessity of the combined internal medicine and ophthalmic approach. **Subjects and methods:** The study included 45 subjects, who were divided into three groups, with 15 subjects in each group. After signing the informed consent, each patient was assigned to one of the following three treatment arms: standard immunotherapy (systemic corticosteroids plus azathioprine) alone, standard immunotherapy with Pioglitazone 15 mg per os per day, or standrad immunotherapy with Sitagliptin 100 mg per os per day. **Results:** on comparing the mean improvement in visual acuity at the end of 1 year follow up among the three study groups, the group receiving adjuvant Pioglitazone showed a statistically significant improvement over the group receiving systemic steroids and azathioprine alone, the t = 2.5, level of significance 0.05, degree of freedom 55. Whereas the group receiving adjuvant Sitagliptin showed no statistically significant improvement over the group receiving systemic steroids and azathioprine alone, the t = 0.54, level of significance 0.05, degree of freedom 57. **Conclusion:** A statistically significant improvement in the control of uveitis in Behçet's disease patients was obtained by addition of Pioglitazone to systemic steroids and Azathioprine, but not by addition of Sitagliptin to the same combination.

Keywords: Uveitis, Behçet's disease, Azathioprine, Pioglitazone, Sitagliptin.

Introduction:

Behçet's disease (BD) is a multiorgan disease characterized by an immune-mediated occlusive vasculitis. Major symptoms of BD consist of oral aphthous ulcers, genital ulcerations, skin lesions, and ocular lesions. Due to its poor visual prognosis, eye involvement, which affects approximately 60-80% of patients, is, along with CNS involvement, the most serious manifestation of BD.(1)

So far, the a etiology and pathogenesis of BD remain unclear, with the background of HLA-B51 as the major important predisposing genetic factor.(1)

Uveitis in Behçet's disease is a potentially blinding condition. Common complications of recurrent anterior uveitis in Behçet's disease include anterior and posterior synechiae which may lead to secondary glaucoma, cataract, and macular oedema.(2,3) Posterior segment involvement in Behçet's disease includes posterior uveitis, retinal, macular, and optic disc oedema, and retinal vasculitis.(4) The need for adequate control of activity of the disease with systemic immune-suppressive therapy in order to salvage vision cannot be overemphasized.(5)

Systemic corticosteroids are widely used in the therapy of ocular BD. However, they are not suitable as a monotherapy for longer treatment durations because their doses, necessary to maintain remission of ocular disease, will be too high resulting in unacceptable side effects. Thus, in most cases it will be necessary to add a steroid-sparing immunosuppressive drug as Azathioprine or cyclosporine.[3]

Guidelines for the use of immunosuppressive drugs in patients with ocular inflammatory disorders recommend 1 mg/kg/day of prednisone for severe uveitis. Control of acute inflammation usually takes 2–4 weeks and steroids then need to be slowly tapered for approximately 6 months until their withdrawal.[6]

Azathioprine, cyclosporine, and methotrexate are the immunosuppressive agents that are most frequently used to treat auto-immune uveitis. In a randomized double-blind placebo-controlled trial in Behçet's disease, 2.5 mg/kg/day azathioprine was found to have a significantly preventative effect on new flares of uveitis in patients with previous episodes and patients who had never experienced a flare of uveitis before inclusion in the study.[3]

Thiazolidinediones are a class of drugs originally used for the treatment of type 2 diabetes. They bind avidly to peroxisome proliferator-activated receptor gamma in adipocytes to promote adipogenesis and fatty acid uptake in peripheral fat. By reducing circulating fatty acid concentrations and lipid availability in liver and muscle, these drugs improve the patient's sensitivity to insulin. These compounds have entered clinical practice and there has been a steadily increasing understanding of the multiple biological effects of these drugs. In addition to their antidiabetic effect, thiazolidinediones have got a number of other biological effects including anti-inflammatory effects. Studies have found that these drugs can be useful in controlling the inflammatory process in Behçet's disease.[7]

Sitagliptin is an inhibitor of the enzyme dipeptidyl peptidase-4 (DPP-4). Since DPP-4 mediates pro-inflammatory signals, Sitagliptin exerts an anti inflammatory effect. Sitagliptin induces a reduction in the expression of a series of pro-inflammatory genes and a reduction in plasma concentrations of CRP, TNF-α, and IL-6 in a comprehensive anti-inflammatory effect.[8]

Subjects and Methods:

The subjects who were enrolled in the study were Behçet's disease patients with uveitis, requiring treatment with systemic immunomodulatory therapy to control their intraocular inflammation or to ameliorate their ocular disease course.

The study included 45 subjects, who were divided into three groups, with 15 subjects in each group. After signing the informed consent, each patient was assigned to one of the following three treatment arms:

- Standard immunotherapy (systemic corticosteroids plus azathioprine) alone.
- Standard immunotherapy with Pioglitazone 15 mg per os per day.
- Standrad immunotherapy with Sitagliptin 100 mg per os per day.

Inclusion criteria:

1. The diagnosis of Behçet's disease, according to the International Classification Criteria:
   - Recurrent oral ulceration.

Plus at least 2 of the following criteria:

   - Recurrent genital ulceration.
   - Eye lesions: active uveitis or retinal vasculitis.
   - Skin lesions: erythema nodosum or papulopustular lesions
   - Positive pathergy test.

2. Active uveitis secondary to Behçet's disease in at least one eye.
3. Male and female subjects older than 18 years of age.

Exclusion criteria:

1. Patients treated at any time with chlorambucil or cyclophosphamide.
2. Treatment with intravitreal anti-VEGF agents administered to the study eye within 3 months prior to study screening.

3. Treatment with any injected or implantable corticosteroid-releasing device within the last 3 years.
4. Intraocular surgery or laser photocoagulation in the study eye within the last 6 weeks.
5. Ocular disease that would interfere with ocular evaluations (e.g. corneal scarring, cataract, vitreous hemorrhage, uncontrolled glaucoma, toxoplasma scar, macular scarring).
6. Treatment with any live or live-attenuated vaccine in the last 2 months.
7. Any systemic biologic therapy (e.g. interferon, infliximab, daclizumab, etanercept, or adalimumab) given intravenously or subcutaneously within 3 months prior to screening
8. History of lymphoproliferative disease or any known malignancy within the past 5 years.
9. Heart failure and/or coronary heart disease

The patients were subjected to:

1. History taking.
2. Ophthalmologic examination:
   - Visual acuity (uncorrected and corrected).
   - Slit lamp examination.. to detect anterior chamber cells.
   - Direct ophthalmoscopy.. to detect vitreous haze.
   - Fundus examination using a slitlamp biomicroscope plus a non contact fundus lens.
   - Intraocular pressure measurement using Goldmann applanation tonometer.
3. Systemic evaluation:
   - Systemic manifestations of Behçet's disease eg. oral ulcers, genital ulcers, erythema nodosum.
   - Vital signs: temperature, pulse, blood pressure, respiration.
   - Adverse events of the administered drugs.
   - Laboratory investigations: CBC, urea, creatinine, lipid profile, liver enzymes, high sensitivity CRP.

Follow up was done for a period of 12 months.

The data was statistically analyzed using SPSS program version 18. The following statistical tests were used: descriptive statistics (including mean and SD), t-test, ANOVA test, and multiple regression analysis. Probability or p value of <0.05 was considered statistically significant, while $p>0.05$ was considered statistically non-significant.

Results:

The study included 45 Behçet's disease patients with uveitis, requiring treatment with systemic immune-modulatory therapy to control their intraocular inflammation or to ameliorate their ocular disease course. The subjects were divided into three groups, with 15 subjects in each group. The groups received either

- Standard immunotherapy (systemic corticosteroids plus azathioprine) alone.
- Standard immunotherapy with Pioglitazone 15 mg per os per day.
- Standard immunotherapy with Sitagliptin 100 mg per os per day.

Follow up of the 3 groups has been done for a period of 12 months.

Group I: On ophthalmic examination at the beginning of the study, the studied eyes in group I showed an uncorrected visual acuity ranging from Hand Movement to 0.4 and a corrected visual acuity ranging from Hand Movement to 0.5. Examination using the slit lamp biomicroscope was used to detect anterior chamber cells, which ranged from +2 cells to +4 cells with hypopyon. Direct ophthalmoscopy was used to depict vitreous haze which was present in 17 of the studied eyes. On systemic evaluation, all the 15 patients reported having recurrent attacks of oral ulcers, 9 patients having active oral ulcers at the time of examination. All the fifteen patients reported having genital ulcers, ranging from a single attack to 6 attacks.

The patients were started on systemic prednisolone 60 mg/day and azathioprine 100 mg/day. Several follow up visits were arranged with both ophthalmic and systemic evaluation

performed regularly. At the end of 12 months of follow up, the studied eyes in group one showed an uncorrected visual acuity ranging from Hand Movement to 0.6 and a corrected visual acuity ranging from Hand Movement to 0.7 . The level of anterior chamber cells ranged from +1 cells to +3 cells (Figure II), and vitreous haze was persistent in 4 eyes. Two patients continued to suffer from oral ulcers, but no patients were still experiencing genital ulcers.

Group II: On ophthalmic examination at the beginning of the study, the studied eyes in group II showed an uncorrected visual acuity ranging from Hand Movement to 0.3 and a corrected visual acuity ranging from Hand Movement to 0.5. The slit lamp biomicroscope was used to detect anterior chamber cells, which ranged from +2 cells to +4 cells with hypopyon. The direct ophthalmoscope was used to assess vitreous haze which was present in 18 of the studied eyes. On systemic evaluation, all the fifteen patients stated having multiple attacks of oral ulcers, 11 patients having active oral ulcers at the time of examination. All the fifteen patients reported having genital ulcers, ranging from a single attack to 5 attacks.

The patients were started on systemic prednisolone 60 mg/day, azathioprine 100 mg/day, and Pioglitazone 15 mg/day. Several follow up visits were organized and both ophthalmic and systemic evaluation were done regularly. At the end of 12 months of follow up, the studied eyes in group two showed an uncorrected visual acuity ranging from Counting fingers at 60 cm to 0.7 and a corrected visual acuity ranging Counting fingers at 60 cm to 0.8 (Figure I). The level of anterior chamber cells ranged from zero cells to +1 cells, and vitreous haze was persistent in only one eye. No patients continued to suffer from oral or genital ulcers. The patient with mediastinal vein syndrome experienced complete resolution of the face, neck, and upper limbs swelling during the course of follow up.

Group III: At the start of the study, the studied eyes in group III showed an uncorrected visual acuity ranging from Counting Fingers at 30 cm to 0.4 and a corrected visual acuity ranging from Counting fingers at 30 cm to 0.6. Examination using the slit lamp biomicroscope was used to detect anterior chamber cells, which ranged from +1 cells to +4 cells without hypopyon. Direct ophthalmoscopy was performed to examine for vitreous haze which was present in 15 of the studied eyes. On systemic evaluation, the 15 enrolled patients reported having recurrent attacks of oral ulcers, 7 patients having active oral ulcers at the time of examination. Fourteen patients reported having genital ulcers, ranging from a single attack to 6 attacks.

The patients were started on systemic prednisolone 60 mg/day, azathioprine 100 mg/day, and Sitagliptin 100mg/day. Several follow up visits were arranged with both ophthalmic and systemic evaluation performed regularly. At the end of 12 months of follow up, the studied eyes in group three showed an uncorrected visual acuity ranging from Counting Fingers at 50 cm to 0.6 and a corrected visual acuity ranging Counting Fingers at 50 cm to 0.7. The level of anterior chamber cells ranged from +1 cells to +2 cells, and vitreous haze was persistent in 3 eyes. One patient had an attack of oral ulcers and another attack of genital ulcers during the course of follow up that resolved with continuation of treatment.

Table I: Demographic characteristics and duration of disease of the study groups – before treatment.

	Group I (Steroids + Azathioprine) **(n=15)**	**Group II** (Steroids + Azathioprine + Pioglitazone) **(n=15)**	**Group III** (Steroids + Azathioprine + Sitagliptin) **(n=15)**
Age (mean, SD)	44 +/- 3 yrs, 3 ms	43 +/- 4 yrs,1m	46 +/- 2 yrs, 2 ms
Sex (M/F)	12/3	13/2	13/2
Duration of disease	8- 12 years	8-14 years	7-12 years

Discussion:

In the present study, the results of the group of patients receiving systemic steroids and azathioprine, group I, came supportive of those published by these Nussenblatt[3], as both the systemic manifestations (e.g. oral ulcers and genital ulcers) and the ocular manifestations (e.g. anterior chamber cells and vitreous haze) improved in most of the patients receiving the regimen.

At the end of 12 months of follow up in the present study, the studied eyes showed a mean improvement of visual acuity of 0.1 over the pretreatment levels (SD: 0.02). This improvement was approximate to that reported by Yazici et al,[9,10] who recorded a mean improvement in visual acuity of 0.15 over the pretreatment levels. Nine of the 29 examined eyes had persistent Anterior Chamber cells denoting persistence of anterior uveitis activity, while the remaining 20 eyes were free of cells at the end of the follow up period, with a 72.4% improvement rate, slightly lower than that achieved by Yazici et al[11], who mentioned a 79% improvement rate. The vitreous haze was persistent in 4 of the 17 eyes that had vitreous haze at the beginning of the study, and disappeared from the remaining 13 eyes. The rate of improvement in vitreous haze was 76.5 %, a percentage almost similar to that reported in the study by Kotter et al[12], who mentioned improvement in vitreous haze in about 76% of the eyes. The mean frequency of recurrence of attacks of oral ulcers was 2 attacks/ patient /1 year of follow up under treatment (SD: 0.2), and the mean frequency of recurrence of attacks of genital ulcers was 1 attack/ patient /1 year of follow up under treatment (SD: 0.1).

The patients in the second group, who received Pioglitazone in addition to systemic coticosteroids and Azathioprine showed, at the end of follow up, a mean improvement in visual acuity over the pretreatment levels of 0.2 (SD: 0.05). This improvement is fairly lower than that mentioned by Kim et al[13], who achieved a 0.3 mean improvement in visual acuity over the pretreatment levels. Only 2 of the 28 examined eyes had persistent Anterior Chamber cells denoting persistence of anterior uveitis activity, while the remaining 26 eyes were free of cells at the end of the follow up period, an improvement rate approaching 93%, and somewhat exceeding that reported by Kim et al, who reported 85% improvement in anterior uveitis.

Vitreous haze was persistent in only one of the 18 eyes that showed vitreous haze at the beginning of the study, and disappeared from the remaining 17 eyes, the latter representing 94% of the studied eyes, whereas Kim et al achieved disappearance of vitreous haze in 90% of the affected eyes. The mean frequency of recurrence of attacks of oral ulcers was 0.5 attack/ patient /1 year of follow up under treatment (SD: 0.2), and the mean frequency of recurrence of attacks of genital ulcers was 0.1 attack/ patient / 1 year of follow up under treatment (SD: 0.05).

In the third group, who received Sitagliptin in addition to systemic steroids and Azathioprine, there was a mean improvement in visual acuity over the pretreatment levels of 0.12 (SD: 0.05). This mean improvement is the same as that reported by Acacina et al[14], who reported a mean improvement in visual acuity of 0.12 over the pretreatment visual acuity. Eight of the 30 examined eyes had persistent Anterior Chamber cells denoting persistence of anterior uveitis activity, while the remaining 22 eyes were free of cells at the end of the follow up period, a rate improvement of 70% similar to that achieved by Acacina et al[15,16].

On comparing the mean improvement in visual acuity at the end of 1 year follow up, the group receiving adjuvant Pioglitazone showed

a statistically significant improvement over the group receiving systemic steroids and azathioprine alone, the t = 2.5 , level of significance 0.05, degree of freedom 55. Whereas the group receiving adjuvant Sitagliptin showed no statistically significant improvement over the group receiving systemic steroids and azathioprine alone, the t = 0.54, level of significance 0.05, degree of freedom 57.

Regarding the improvement in anterior chamber cells and vitreous haze, the group receiving adjuvant Pioglitazone obtained a statistically significant improvement over the group receiving systemic steroids and azathioprine alone, confidence interval (20.25-20.64). On the other hand, the group receiving Sitagliptin as an add-on therapy, achieved no statistically significant improvement over the control group, confidence interval (2.17 – 2.65).

In conclusion, addition of Pioglitazone to systemic steroids and Azathioprine in treating Behçet's disease patients with uveitis, achieved a statistically significant improvement in the control of uveitis over systemic steroids and Azathioprine alone. On the other hand, the addition of Sitagliptin to the same combination did not obtain a statistically significant improvement over systemic steroids and Azathioprine alone.

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Original Article

Red Cell Distribution width in Type 2 Diabetic Patients.

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Abstract:

Aim: To study the indices of some elements of the complete blood count in type 2 diabetic patients on treatment in comparison with non-diabetic healthy controls and to find out the effects of glycemic control and different medications on these indices. To the best of our knowledge this study is novel in our environment and will serve as a foundation for other researchers in this field. **Patients and Methods:** A retrospective study included 260 type 2 diabetic patients on treatment and 44 healthy control subjects. All data including gender, age, weight, height and blood pressure were available for all the study population. For diabetic patients, duration of diabetes and all medications were available. Complete blood count, fasting plasma glucose, HbA1c and lipid profile, were available for all participants. **Results:** Red cell distribution width (RDW) was significantly higher in diabetic patients than controls (p= 0.008). It was also higher in patients with uncontrolled glycaemia (HbA1c >7%) than those with good control (HbA1c ≤ 7%) [p= 0.035]. Mean platelet volume (MPV) was comparable in both diabetics and healthy controls (p= 0.238). Red cell distribution width and MPV did not significantly correlate with any of the fasting plasma glucose, HbA1c or duration of diabetes. Both aspirin and clopidogrel did not show a significant effect on mean platelet volume (MPV). Both insulin and oral hypoglycemic agents in our study did not show a significant effect on RDW, MPV or any of the studied indices. Diabetics treated with indapamide or the combined thiazides and angiotensin receptor blockers showed no significant difference in RDW when compared with the controls. **Conclusion:** RDW which is recently considered as an inflammatory marker with a significant predictive value of mortality in diseased and healthy populations, is significantly higher in diabetic patients than healthy subjects, and is particularly higher in uncontrolled glycaemia. None of the studied hypoglycemic agents showed a significant effect on RDW. Patients receiving antihypertensive therapy in the form of indapamide or the combined therapy of thiazides and angiotensin receptor blockers have RDW values comparable to those of the healthy population.

Keywords: Red cell distribution width, mean platelet volume, menopausal, diabetes, inflammation

Introduction:

The prevalence of type 2 diabetes has been increasing rapidly throughout the world[1]. It is estimated that over three hundred million people worldwide will become diabetic by year 2025 [2,3]. It is a global health problem because of its associated high morbidity and mortality. The primary cause of mortality in diabetic patients is cardiovascular diseases[4] while the major cause of morbidity is microvascular complications[5].

The evidence associating red cell distribution width (RDW) with a higher risk of mortality has been expanding since the initial report of its prognostic utility in heart failure patients. Red cell distribution width has also been shown to independently predict overall and cardiovascular mortality in the general population and various high-risk populations[6-10]. It is also a strong predictor of mortality in in many conditions such as obesity, malignancies, and chronic kidney diseases[11]. Being of an independent predictive value for various diseases makes it imperative to be studied in diabetes mellitus.

The red cell distribution width is a quantitative measure of the heterogeneity of the volume of red blood cells (RBCs) with higher values reflecting greater heterogeneity in cell sizes (anisocytosis)[12]. It is originally used together with the mean corpuscular volume (MCV) in clinical practice to differentiate between causes of anemia[13-15].

Inflammation has been proposed as a component of diabetes and its complications[16] Diabetic patients show high levels of chronic subclinical inflammation and oxidative stress, which play key roles in the progression of atherosclerotic diseases[7] Researchers showed RDW to be strongly associated with markers of chronic subclinical inflammation, higher oxidative stress and under-nutrition[13,17]. This may postulate an association between RDW and diabetes.

Platelets; another element of the complete blood count (CBC), play a key role in the development of atherothrombosis, a major contributor of cardiovascular events[18] which represent the major cause of mortality in diabetes[19]. Platelet aggregation and adhesion play a major role in intravascular thrombosis on top of atherosclerosis resulting in cardiovascular and cerebrovascular events. They may also be involved as a causative agent in the development of micro- and macrovascular disease in diabetes, with respect to altered platelet morphology and function[20,21].

Platelet hyperactivity has been reported in diabetics both in vivo *and* in vitro[22,23]. Mean platelet volume (MPV) is an indicator of the average size and was suggested by some authors to be an indicator of the platelet activity[24] and the state of thrombogenesis[20,22]. Antiplatelets have been demonstrated to be very effective at decreasing myocardial infarction, stroke, and death[25].

Leukocytes are known to participate in the inflammatory process accompanying atherosclerosis[26]. They are recruited at the site of endothelial injury, and form foam cells in the atheromatous plaque[27]. Interleukins and tumor necrosis factor-α are released from activated leukocytes and cause endothelial dysfunction. White blood cell (WBC) count is positively associated with increased cardiovascular mortality, mainly from coronary heart disease[28].

We aimed to use the complete blood count in type 2 diabetic patients as a simple and costless technique that is routinely done to investigate the state of various indices of blood elements especially those, which are claimed to have a role in the disease process and its complications such as RDW, MPV, platelet count and WBC count. We also elucidated the effects of various medications on these indices.

Patients and Methods:

We conducted a retrospective study which included 260 diabetic patients (98 females and 162 males) and forty-four non diabetic healthy controls (16 females and 28 males) from Internal Medicine Department, Diabetes and Endocrinology Clinics in a tertiary care hospital in KSA. The study was approved by the Hospital Ethical Committee. Patients were excluded because of anaemia, chronic liver disease, dialysis, thyroid disease, pregnancy, heart failure, acute or chronic infection or blood disease. Patients with known inflammatory conditions such as rheumatoid arthritis, systemic lupus erythematosus, those receiving anticoagulants or had a diagnosis of malignancy were also excluded.

Age of all participants as well as weight, height, and blood pressure were all available. Body mass index was calculated as follows [weight (kg)/height $(m)^2$]. In diabetic patients; duration of diabetes and medications were all noted.

Laboratory Analysis: All patients and healthy controls had complete blood count on venous blood samples taken into tripotassium EDTA (ethylene diamine tetracetic acid), using a Roche Minos cell counter and automatic blood counter (CELL-DYN 3500) within two hours of sample collection for platelet indices, WBC count and RBC indices. Standardization, calibration of instrument and processing of samples were done according to manufacturer's instructions. The blood glucose level was measured by glucose oxidase method and hemoglobin A1c (HbA1c) by calorimetrical method in the autoanalyser. Total cholesterol (T-Ch), low density lipoprotein (LDL), high density lipoprotein (HDL) and triglycerides (TG) in whole serum were measured enzymatically using a Cobas

autoanalyzer. Reference values were as follows: T-Ch: 3-5.2 mmol/L, LDL: < 3.4 mmol/L, HDL: 0.62-1.55mmol/L, TG: 0.34-2.28 mmol/L. WBC count: (4-11) 10^9/µl, platelet count: (140-440) 10^9/µl, MPV: 7-13 fl, RDW: 11-14%, MCV: 76- 96 fl.

Statistical Analysis:

Collected data were verified prior to computerized data entry. The Statistical Package for Social Sciences (SPSS, version 21.0) was used for the statistical analysis of data. Descriptive statistics (e.g., frequency, mean and standard deviation) were applied. Pearson's correlation coefficient and tests of significance (e.g., unpaired t-test) were applied. A significant p-value was considered at 0.05 or less.

Results:

Patient and healthy control characteristics are available in table (I).

Red cell distribution width was significantly higher in diabetic patients than healthy controls (p=0.008) while MCV was significantly smaller (p=0.036). No statistically significant differences were noted between both groups in MPV, platelet count or WBC count. Comparing diabetics with HbA1c≤ 7% (47 patients) with diabetics with HbA1c> 7% (213 patients) showed higher RDW (p=0.035) and smaller MCV (p =0.016) in the group with HbA1c > 7% otherwise no other significant differences were noted. Table (II)

In the patient group no statistically significant correlations were noted between RDW and FPG, A1c (p= 0.22, 0.781), blood pressure or duration of diabetes. RDW was strongly and directly associated with the body mass index (p<0.0001). MCV showed to be inversely associated with BMI (p=.016) and HbA1c (p=0.048). Platelet count inversely correlated with age (p=0.035) while the WBC count was directly associated with the duration of diabetes (p=0.049). MPV showed direct but insignificant correlation with FPG and HbA1c (p=0.057, 0.164 respectively). Table (III)

In the diabetic patients RDW, MCV and MPV did not correlate significantly with any of the components of the lipid profile. Platelet count correlated inversely with triglycerides (r= -0.14, p=0.015) and directly with HDL (r= 0.153, p= 0.008). White cell count inversely correlated with both T-Ch and LDL (r=-0.155, -0.152, p= 0.007, 0.008 respectively). Mean platelet volume was inversely correlating with platelet count (r= -0.368, p <0.001). White blood cell count was directly correlating with platelet count (r=0.16, p= 0.01) and RDW although the second correlation did not reach a statistical significance (r= 0.118, p=0.059).Table (III)

In comparison to controls, patients receiving indapamide had comparable RDW (14.79±1.25 vs 13.69 ± 1.20, p=0.473) and MPV (8.68±0.85 vs 8.85±0.97, p=0.456). The thiazide group of patients had significantly larger MPV (9.57±0.84 vs 8.85±0.97, p= 0.009) and RDW (p=0.014). Combined indapamide and angiotensin converting enzyme inhibitors (ACEI) had higher RDW (p <0.0001) and comparable MPV (p=0.403) meanwhile the combined angiotensin receptor blockers (ARBs) and thiazides had comparable RDW (14.34±1.56 vs 13.69±1.2, p=0.15) and comparable MPV (8.75±0.8 vs 8.85±0.97, p= 0.77) to controls. Calcium channel blockers did not show specific effects. Table (IV)

Diabetics receiving antiplatelets in form of ASA (8.99±0.95 vs 8.85±0.97, p=0.368) or clopidogrel (8.88±0.76 vs 8.85±0.97, p= 0.894) did not show significant difference in MPV when compared with controls. Statin therapy did not show a significant effect on CBC. Table (IV)

Hypoglycemic agents including insulin, metformin, sulfonylurea, pioglitazone and dipeptidyl peptidase inhibitors (DPP4I) did not show significant effects on any of the studied hematological indices. Table (IV)

Diabetic women showed significantly higher RDW and lower MCV when compared to men. This difference was persistent for women whether pre or postmenopausal. No significant difference between women in the two age groups was noticed. Tables (V,VI)

Table I: Criteria of Patients and healthy controls

	Healthy controls (n=44)	Diabetics (n=260)	p-value
Duration of diabetes	-	10.98± 6.92	-
Age	54.39±12.26	56.80 ± 11.95	0.221
BMI	30.55 ± 4.51	31.49 ± 5.14	0.255
SBP	125.68 ± 7.37	129.10 ± 16.33	0.174
DBP	76.00 ± 7.30	72.68 ± 9.04	0.021
WBC	6.87±1.82	7.07±2.08	0.549
MCV	84.49 ± 6.63	82.21 ± 6.66	0.036
RDW	13.69 ± 1.20	14.27 ± 1.36	0.008
Platelet count	259.66 ± 53.23	256.33 ± 65.90	0.751
MPV	8.85 ± 0.97	9.04 ± 0.99	0.238
TG	1.63 ±0.79	1.57 ± 0.80	0.666
T-Ch	4.94 ± 0.71	4.4 ±1.05	<0.001
LDL	2.81± 0.93	2.55 ± 0.92	0.079
HDL	1.1 ± 0.49	1.08 ± 0.31	0.587

BMI: body mass index, SBP: systolic blood pressure, DBP: diastolic blood pressure. WBC: white blood cell, MCV: mean corpuscular volume, RDW: red cell distribution width, MPV: mean platelet volume. Measuring units: WBC count: (4-11) 10^9/µl, platelet count: (140-440) 10^9/µl, MPV: 7-13 fl, RDW: 11-14%, MCV: 76- 96 fl. T-Ch: total cholesterol, LDL: low density lipoprotein, HDL: high density lipoprotein, TG: triglycerides. T-Ch: 3-5.2 mmol/L, LDL: < 3.4 mmol/L, HDL: 0.62-1.55mmol/L, TG: 0.34-2.28 mmol/L.

Table II: The studied CBC indices in patients with A1c≤ 7% vs patients with A1c >7%

	≤7 (n=47)	**> 7 (n=213)**	**P-value**
WBC	6.75 ± 1.74	7.17 ± 2.16	0.095
MCV	83.95 ± 6.93	81.94 ± 6.51	0.016
RDW	13.94 ±1.29	14.29 ±1.36	0.035
Platelet Count	254.75 ± 60.80	257.69 ± 65.65	0.706
MPV	8.95 ± 1.05	9.03 ± 0.965	0.524

WBC: white blood cell, MCV: mean corpuscular volume, RDW: red cell distribution width, MPV: mean platelet volume. Measuring units: WBC count: (4-11) 10^9/µl, platelet count: (140-440) 10^9/µl, MPV: 7-13 fl, RDW: 11-14%, MCV: 76- 96 fl.

Table III: Correlations of CBC indices with various variables

	RDW		MCV		MPV		Platelet Count		WBC count	
	R	P	R	P	R	P	R	P	R	P
Age	0.012	0.853	0.075	0.228	-.061	0.326	-0.131	0.035	0.008	0.9
BMI	0.228	<.0001	-.149	0.016	0.064	0.306	0.097	0.117	0.035	0.572
Duration DM	0.103	0.107	-.051	0.424	0.034	0.6	0.034	0.593	0.125	0.049
SBP	0.109	0.078	-.001	0.984	0.002	0.971	0.032	0.603	0.008	0.898
DBP	-0.094	0.13	.088	0.159	-.067	0.28	-0.024	0.699	0.109	0.08
FPG	-0.076	0.22	-.036	0.564	0.119	0.057	-0.009	0.899	0.044	0.481
A1c	0.017	0.781	-.123	0.048	0.087	0.164	0.005	0.934	0.017	0.786
TG	-0.099	0.086	-0.038	0.504	0.099	0.882	-0.14	0.015	0.001	0.991
T-Ch	-0.01	0.857	-0.026	0.658	-0.054	0.345	0.05	0.39	-0.155	0.007
LDL-c	0.013	0.824	-0.071	0.215	-0.021	0.711	0.028	0.628	-0.152	0.008
HDL-c	0.025	0.667	0.042	0.463	-0.057	0.326	0.153	0.008	-0.094	0.103

BMI: body mass index, SBP: systolic blood pressure, DBP: diastolic blood pressure. WBC: white blood cell, MCV: mean corpuscular volume, RDW: red cell distribution width, MPV: mean platelet volume. T-Ch: total cholesterol, LDL: low density lipoprotein, HDL: high density lipoprotein, TG: triglycerides. Measuring units and reference ranges: WBC count: (4-11) 10^9/µl, platelet count: (140-440) 10^9/µl, MPV: 7-13 fl, RDW: 11-14%, MCV: 76- 96 fl.T-Ch: 3-5.2 mmol/L, LDL: < 3.4 mmol/L, HDL: 0.62-1.55mmol/L, TG: 0.34-2.28 mmol/L.

Table IV: P values of the effects of different medications in diabetics (in comparison with controls)

	RDW	MCV	MPV	Platelet count	WBC count
Indapamide	0.473	0.214	0.456	0.15	0.716
Thiazides	0.014	0.18	0.009	0.686	0.14
ACEI	0.001	0.028	0.557	0.928	0.493
ARBs	0.023	0.315	0.106	0.317	0.009
CCB	0.003	0.12	0.45	0.458	0.379
ACEI+indapamide	<0.0001	0.002	0.403	0.111	0.623
ARBs+thiazides	0.15	0.567	0.77	0.109	0.143
Metformin	0.12	0.064	0.16	0.91	0.471
SU	0.01	0.17	0.46	0.776	0.293
Insulin	0.022	0.019	0.200	0.899	0.39
Pioglitazone	0.021	0.102	0.247	0.94	0.637
DPP4I	0.043	0.278	0.869	0.788	0.067
ASA	0.009	0.159	0.368	0.951	0.33
Clopidogrel	0.021	0.132	0.894	0.667	0.59
Statins	0.006	0.078	0.435	0.789	0.346

ACEI: angiotensin converting enzyme inhibitor, ARBs: angiotensin receptor blockers, CCB: calcium channel blockers, SU: sulphonylurea, DPP4I: dipeptidyl peptidase 4 inhibitors, ASA: acetyl salicylic acid, WBC: white blood cell, MCV: mean corpuscular volume, RDW: red cell distribution width, MPV: mean platelet volume. Measuring units and reference ranges: WBC count: (4-11) 10^9/µl, platelet count: (140-440) 10^9/µl, MPV: 7-13 fl, RDW: 11-14%, MCV: 76- 96 fl.

Table V: Comparison of the studied CBC indices in pre and post-menopausal females

	Pre (M±SD) n=20	Post(M±SD) n=78	p-value
RDW	15.10 ± 1.67	14.60 ± 1.46	0.183
MCV	77.96 ± 7.63	81.31 ± 6.93	0.061
MPV	9.06 ± 1.04	9.12 ± 1.09	0.831
Platelet count	293 ± 75.29	267.54 ± 70.11	0.156
WBC count	6.96 ± 1.64	6.91 ± 1.79	0.921

Pre: premenopausal, Post: postmenopausal, WBC: white blood cell, MCV: mean corpuscular volume, RDW: red cell distribution width, MPV: mean platelet volume. Measuring units and reference ranges: WBC count: (4-11) 10^9/µl, platelet count: (140-440) 10^9/µl, MPV: 7-13 fl, RDW: 11-14%, MCV: 76- 96 fl.

Table VI: CBC indices in pre and post-menopausal females in comparison with males

	Males (n=162) M±SD	Premenopausal females M±SD	P-value	Postmenopausal females M±SD	P-value
RDW	14.01 ± 1.19	15.1 ± 1.67	<0.0001	14.60 ± 1.4562	0.001
MCV	83.18 ± 6.15	77.96 ± 7.63	0.001	81.31 ± 6.93234	0.035
MPV	8.99 ± 0.94	9.06 ± 1.04	0.77	9.12 ± 1.09	0.365
Platelet count	246.27 ± 60.24	293 ± 75.29	0.002	267.54 ± 70.11	0.016
WBC count	7.17 ± 2.26	6.96 ± 1.64	0.686	6.91 ± 1.79	0.347

WBC: white blood cell, MCV: mean corpuscular volume, RDW: red cell distribution width, MPV: mean platelet volume. Measuring units and reference ranges: WBC count: (4-11) 10^9/µl, platelet count: (140-440) 10^9/µl, MPV: 7-13 fl, RDW: 11-14%, MCV: 76- 96 fl.

Table VII: r and p values of the correlations between WBC count and other CBC indices

	r	p-value
RDW	0.118	0.059
MCV	0.036	0.562
MPV	0.038	0.542
Platelet Count	0.16	0.01

WBC: white blood cell, MCV: mean corpuscular volume, RDW: red cell distribution width, MPV: mean platelet volume. Measuring units and reference range s: WBC count: (4-11) 10^9/µl, platelet count: (140-440) 10^9/µl, MPV: 7-13 fl, RDW: 11-14%, MCV: 76- 96 fl.

Discussion:

In the present study, higher RDW in diabetic patients than healthy controls (p = 0.008) indicates the presence of anisocytosis which is related to impairment of erythropoeisis and degradation of erythrocytes by fragmentation or agglutination[29-32]. This occurs in presence of chronic inflammation and increased level of oxidative stress[33].

Hyperglycemia has several effects on RBCs, besides formation of glycated hemoglobin[34], it leads to reduced deformability and changes in mechanical properties of RBCs[35,36], increased adhesion[37] and increased osmotic fragility[38] leading to changes in erythrocyte structure and hemodynamic characteristics[39].

Hyperglycemia reduces RBC life span leading to high variability of the RBC volumes[40,41]. Charles has mentioned reduced average lifespan of RBCs in diabetic patients[42]. This was also demonstrated by Emilia who showed that an extracellular oxidative milieu can be responsible for erythrocyte caspase-3 activation in type 2 diabetes. Activated caspase-3 impairs the maintenance of erythrocyte shape and function, thus contributing to the shortened life span of RBCs[43].

The original studies of Charles[42] showed a modest but consistent increase in erythrocyte half-life after the establishment of tight glycemic control compared with the same patients studied in poor control. In our study, there was a significant difference in RDW, being significantly higher in patients with HBA1c > 7% indicating shorter life span with anisocytosis in uncontrolled diabetes. This suggests that tighter glycemic control might offer a hematologic benefit to diabetic patients undergoing chemotherapy or having a chronic transfusion or erythropoietin requirement.

Our report of higher RDW in diabetics is in contrast to a report by Lutfullah et al[44] who did not find a difference in RDW among diabetics and non diabetics (p=0.53). Moreover, he did not find a significant difference in RDW in patients with HbA1c <7% or > 7%. He also did not find a significant difference in RDW when diabetes duration was longer or shorter than 10 years. In our study we did not observe a correlation between RDW and duration of diabetes (p= 0.107).

Similar to our study, Amparo mentioned a significant strong correlation between RDW and BMI[45]. Obesity is associated with a low-grade inflammatory process in the white adipose tissue[41,46,47]. Some studies elicited the strong association between RDW and markers of chronic subclinical inflammation, so its positive association with obesity is reasonable.

In agreement with our findings, Heba did not observe significant correlations between RDW and HbA1c, SBP, DBP, or duration of diabetes[48]. Contrary to our results, she did not find a significant correlation between RDW and BMI.

In Amparo's study[45], an inverse correlation was observed between RDW and triglycerides, but was gender dependent and was evident only in women ($p<0.05$). In another study in the general population of unselected out patients, Lippi found an inverse association with HDL in both genders and a direct association with hypertriglyceridaemia and Cholesterol/HDL ratio only in women[49]. No significant associations between RDW and lipids were noted in our study.

We did not find significant effects of insulin, metformin, sulfonylurea, pioglitazone or DPP4I on any of the studied hematological indices. To our knowledge there is no previous report about the effects of hypoglycemic agents on RDW or any of the other blood indices mentioned in our study.

In the present study, absence of a significant difference in MPV between diabetics and non-diabetics is in consonance with results obtained by Dada et al in Nigerian diabetics (p=0.593)[50]. On the contrary, several other authors stated a significantly higher MPV in patients with diabetes such as Hekimsoy[20], Demietunc[51], Papanas[52], and Thomas[53]. This also agrees with the findings seen in studies conducted by Zuberi[21] and Jindal[54]. One explanation for the higher MPV in diabetics is that a significant number of these studies was done in diabetic patients post myocardial infarction. This leads to

quicker consumption of smaller platelets that are compensated for by production of younger platelets with larger MPV[55, 56]. Our study was done while the patients were stable with no recent insult.

Absence of a significant correlation between MPV and FPG or HbA1c in our study is consistent with reports by Sharpe[57], and Unubol[58]. Similarly, Ezgi[59] found no association between MPV and FPG, HbA1c, patient age, duration of diabetes, or blood pressure. This is also in agreement with our report and other reports (60, 61). Similar to Thomas we did not find a significant difference in MPV in patients with HbA1c≤7% or >7% and no association with the BMI was found[53]. Peterson, in his study, found no difference in mean platelet survival in uncontrolled diabetics and diabetics with good control[42]. Their reports and ours suggest that other factors rather than hyperglycemia may account for the thrombotic potential of diabetics with time. If vascular damage was only due to increased number of large and reactive platelets, then the rate of damage would have been constant for the duration of disease and independent of diabetic control. This clearly shows that platelet reactivity alone cannot explain the progression of vascular complications in DM since there are other vascular risk factors that may be influenced by the degree of control of diabetes[20,62]. This was supported by the non significant statistical correlation between MPV and duration of diabetes. A direct relation between platelet dysfunction and the development of diabetic complications has yet to be firmly established[20, 22].

Platelet hyper-reactivity and increased baseline activation in patients with diabetes is multifactorial and can not be attributed only to hyperglycemia. It is associated with biochemical factors such as hyperglycemia and hyperlipidemia, insulin resistance, an inflammatory and oxidant state and also with increased expression of glycoprotein receptors and growth factors[63- 66].

On the other hand, positive correlations were demonstrated by Shah[67], Dada[50], Giuseppe[68] and Demirtunc[51] among the diabetics between MPV and FPG and duration of diabetes. They suggested that achieving good glycemic control may limit platelet activation and delay the onset and progression of vascular complications.

Giuseppe[68] demonstrated that MPV is not related to platelet aggregation, the extent of coronary artery disease and carotid intimal medial thickness. Accordingly, he concluded that MPV cannot be considered as a marker of platelet reactivity or a risk factor for coronary artery disease. This necessitates further work up to confirm if MPV can be used as an indicator of platelet function.

Similar to Giuseppe[68], we found an inverse relation between MPV and platelet count. However, in contrast to his report, we did not find a significant association of MPV with age, triglycerides or statin use. Table (4)

Increases in MPV are often associated with decreases in platelet count[69, 70] perhaps as a result of small platelets being consumed in order to maintain a constant platelet functional mass[71]. Also in consistence with our study, a negative linear relationship between MPV and the number of platelets (p=0.006) was observed in Dada's report [50].

Absence of a significant effect of antiplatelets in the form of ASA (aspirin) and clopidogrel on MPV is in agreement with a report by Colkesen[72]. In his study, aspirin treated patients did not show a significant difference in MPV (p=0.9). Shechter et al mentioned individual variability of platelet response to clopidogrel which affects the clinical outcome[73]. In one study, clopidogrel reduced MPV in patients with stable angina after two months of treatment [74]. In another study by Shah, he stated that, when standardized, MPV is a reproducible marker of platelet size and not affected by low-dose aspirin and that MPV is modestly associated with some, but not all, markers of platelet activity[75]. Absence of an effect of aspirin on MPV and the extent of platelet aggregation was mentioned by Giuseppe[76]. Both studies suggest that larger MPV does not imply higher platelet reactivity and may not be considered to monitor platelet reactivity and the efficacy of antiplatelet therapies.

The antiplatelet effects of perindopril and other ACE inhibitors appear to be small. In Gupta's study, perindopril treatment did not affect platelet indices[77]. Other studies of the ACE inhibitor quinapril[78] and the angiotensin

receptor blocker losartan[79,80] similarly have shown little effect on MPV. This is quitely in consistence with our findings as ACE inhibitors and ARBs did not show a significant effect on MPV (p=0.291, 0.106 respectively).

Analysis of the effects of antihypertensive medications in our study elicited that the use of indapamide may be preferable to the use of thiazides because it is associated with comparable RDW and MPV to the controls. On the other hand, the combination therapy of thiazides and ARBs may be preferable to the combination of indapamide and ACE inhibitors for the same reason. Table (4)

In our study, Platelet count inversely correlated with triglycerides and directly with HDL (p=0.015, 0.008). In another study, platelet count was correlating negatively with triglycerides in hypertriglyceridemia (r = -0.489, P < 0.05)[86]. This is in agreement with our study results (r = - 0.1, p= 0.015) which state that the higher the triglycerides, the lower the platelet count.

Both Iolanda and Papatheo[81,82] demonstrated a positive association between WBC count and platelet count. In our study WBC count was positively associated with RDW and platelet count (p= 0.01), although the association with RDW did not reach a statistical significance (p=0.059). No association was found between WBC count and MPV (p=0.542) or MCV (p= 0.562) although the relation between WBC count and MPV was reported by Iolanda[81]. The association of platelet count and RDW with WBC count may underline the role of both platelets and erythrocytes in inflammation.

No association was found between WBC count or platelet count and the FPG, BMI, or lipids in the study conducted by Papatheo [82]. This is in agreement with our report apart from the relation to lipids. Our study revealed a strong negative correlation between WBC count and LDL (r= -0.152, p= 0.008) and total cholesterol (r=-0.155, p=0.007). This seems a paradox which advocates further studies.

In the study conducted by Peter, subjects with higher WBC counts had longer disease duration, higher SBP, DBP, BMI, HbA1c, FPG, LDL cholesterol, TGs and lower HDL [26].

In consistence with Peter's findings, our study results demonstrated a positive correlation between WBC count and duration of diabetes (p= 0.049). However, the association between WBC count and T-ch and LDL was an inverse one. We did not find a significant association with any of the FPG, HbA1c, TG, SBP or DBP and WBC count.

Pre and postmenopausal women had higher RDW, platelet count (p<0.0001, 0.002 respectively) and smaller MCV (p=0.001) than men. Higher platelet count in women can not be explained by the different hormonal profiles or a compensatory mechanism associated with menstrual blood loss, because the difference was persistent regardless of women's age. We did not find a significant difference between pre and post menopausal women in any of the studied indices except for the MCV.

Lippi found higher RDW in non diabetic men than women[49]. Some researchers found no correlation and no statistically significant differences in MPV between both sexes[83, 84]. In another study, platelet count was lower in postmenopausal women compared to young menstruating women. However, MPV values were similar in both groups [85].

Achie, in agreement with our report, did not find a significant difference in RBC indices between pre and post menopausal healthy women[86]. The increase in MCV in menopausal women was elicited by Chalmers, although it was not statistically significant in our study population (p=0.061). Higher MCV indicates a probable risk for developing anaemia especially vitamin B12 and folate deficiency anemia[87].

Our results in pre and menopausal women whether in comparison to each other or to men can be explained by the fact that following menopause the cardioprotective effects of endogenous estrogen is lost[88,89]. Interestingly, sex difference, which normally vanishes after menopause, is rapidly lost in premenopausal T2DM patients, with cardiovascular disease reaching 2- to 5-fold higher rates than

in age matched non-diabetic women[90] and several-fold higher rates of death related to coronary artery disease, with event rates nearly identical to those observed in T2DM men[91]. Diabetes potentiates the effects of major atherosclerotic cardiovascular diseases than in the normal population in a percentage of the diabetic patients, most of them are females[92]. This can explain such differences found even in premenopausal women when compared with men.

Conclusion:

It can be concluded that RDW is higher in type 2 diabetic patients than healthy population. In diabetic patients the glycemic control does affect the RDW. Our study adds to the studies which considered RDW as a marker for subclinical inflammation because of its higher values in diabetics and its positive association with the BMI. The study in our population can be considered as an initial one that necessitates further studies to define the relation between RDW and different diabetic complications and its prognostic value. Further studies are also required to define specific values of the RDW to indicate specific risks.

This study also emphasizes the positive effects of some medications in diabetic patients such as indapamide and the combined therapy of thiazides and ARBs. These positive effects may be of importance in analysis of the beneficial effects of these medications on various morbidities and mortalities in diabetes.

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Original Article

Quality of Life in Type 2 Diabetic Patients Attending Primary Health Care Centers in Kuwait.

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Abstract:

HRQOL is an essential outcome of medical care of chronic diseases, in addition to disease-specific measures. The current study aimed to evaluate the health-related quality of life (HRQOL) of diabetic patients attending primary health care centers in Kuwait, and to explore the socio-demographic and disease-related variables affecting it. The Arabic version of the World Health Organization quality of life questionnaire, short version (WHOQOL-BREF) was administered to 453 type 2 diabetics. The overall mean score was 63.8±12.5 (out of 100), and ranging from 62.5 to 65.2 on the individual subscales. Multiple regression analysis revealed that several factors significantly influenced the total HRQOL. These factors include being non-Kuwaiti, unmarried, illiteracy or having less than secondary education, presence of other co-morbid disorders, presence of diabetes complications, longer duration of diabetes, poor adherence to the prescribed medications and lack of exercise practicing. Nationality, level of education and the degree of adherence to the prescribed medications were the most powerful variable influencing the total HRQOL.

Introduction:

Diabetes is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction, and failure of different organs, especially the eyes, kidneys, nerves, heart, and blood vessels. [(1)] Diabetes is a major lifestyle disorder, the prevalence of which is increasing globally. Its high prevalence constituted chiefly by type 2 diabetes (T2DM), is a global public health threat.[(2)]

In most countries, diabetes has increased. Some 382 million people worldwide, or 8.3% of adults, are estimated to have diabetes. By 2035, some 592 million people, or one adult in 10, will have diabetes.[(3)] In 2013, the International Diabetes Federation (IDF) reported that three of the world's top 10 countries with the highest prevalence of diabetes are in the Middle East and North Africa Region: Saudi Arabia (24.10%), Kuwait (23.0%), and Qatar (22.9%).[(3)]

In Kuwait, the socio-economic development which followed the discovery of oil resources brought about considerable changes in the food habits and lifestyle of the Kuwaiti population. Excessive caloric intake and decreased energy expenditure due to a sedentary lifestyle have led to a rapid increase in obesity, diabetes and other non-communicable chronic diseases in the population.[(4)]

The reported prevalence of T2DM among the Kuwaiti population varied from one source to another. In a study that included 8336 type 2 Kuwaiti diabetic subjects aged 20 years and above registered in two health areas, a total crude prevalence of 7.6% in both health areas was reported.[(5)] In another study that aimed at determining the prevalence rates of NIDDM among a representative sample of the Kuwaiti adult population aged 20 and older, the overall prevalence was found to be

14.8%.[6] A later study was carried out using the WHO Stepwise approach for surveillance of non-communicable disease risk factors. This study represented a national survey for Kuwaiti nationals aged between 20 and 65 years. The prevalence of diabetes was 17.9%.[7]

WHO defines quality of life as the individual perception of their position in life on the context of the culture and value systems in which they live and in relation to their goals, expectations, standards and concerns.[8] QOL has been recognized as an important health outcome in patients suffering from chronic diseases. Health care for such patients focuses mainly on maximizing daily life functions and achieving the highest level of well-being may reduce health care costs.[9] HRQOL is an essential outcome of medical care, in addition to disease-specific measures.[10]

There are a number of studies showing that HRQOL is reduced in T2DM patients compared to the general population and also somewhat lower than in patients with other chronic disease entities.[11,12] The diagnosis of diabetes as with other major chronic illnesses, affects many aspects of an individual's quality of life. The burden of disease management, complex and expensive therapeutic regimens, dietary restrictions, and the need to inject insulin and test blood and urine drastically impair quality of life.[13,14] Once the quality of life has been affected, self- management, the adherence to therapeutic regimen and treatment success are in peril. Therefore, efforts to improve quality of life will lead to better management of the disease for a satisfactory outcome.[15]

A limited number of studies have been conducted in the Middle East to document the HRQOL of patients with DM. More importantly, only a limited number of studies have been conducted in Kuwait to document the QOL of diabetes patients.

The present study aimed to assess HRQOL in T2DM patients in Kuwait, and identify factors that could be associated with poor QOL among them.

Methods:

A comparative cross-sectional survey was carried out. It included 453 type 2 diabetic patients attending specialized diabetes clinics located in the primary health care centers in the state of Kuwait. For conducting the study, a multistage random sample with a proportionate allocation technique was adopted to select one diabetes clinic from each health region (N=5). Five health care centers were randomly selected, one from each health region in Kuwait for conducting the study.T2DM patients recruited from each clinic were proportionate to the number of diabetic patients registered in each clinic.

Data were collected using the following tools:

a. An interview format to collect data about socio-demographic characteristics, diabetic history, history of acute diabetes complications, exercise practicing and level of adherence to diabetes related self- care practices.
b. Measurements of weight, height, waist circumference, hip circumference and blood pressure.
c. Laboratory investigations included FBS, Glycosylated hemoglobin, micro-albuminuria, total cholesterol and triglycerides.
d. Transfer sheet to collect data from patients' electronic records regarding presence of co-morbid conditions other than diabetes and the presence of long-term diabetes complications.

e. Translated Arabic version of WHO-QOL-BREF questionnaire [16] to assess patients' quality of life. It consists of 26 questions each with five possible responses (a five-point Likert Scale). The questionnaire is divided into four domains namely physical health, psychological health, social relationship and environment. There are also two items that were asked separately: question 1 asked about an individual's overall perception of QOL, and question 2 asked about an individual's overall perception of their health.

Statistical analysis:

- Data were fed to the computer using Statistical Package for Social Science (SPSS, version18).
- Many new variables were created through re-categorization of original variables including body mass index (BMI), state of glycemic control, presence of dyslipidemia, degree of adherence to medications, physical exercise practicing and regularity of follow up visits.
- Normal levels for blood lipids were identified as 5.6 mmol/L for total cholesterol, 3.9 mmol/L for TG, 2.6 mmol/L for LDL, and 1.04 mmol/L for HDL. [16]
- Physical activity was considered if it was practiced for 10 minutes at least 3-4 times a week, and classified as follow.[17]
  - No: < 10 minutes per day even if daily
  - Mild: 10 minutes per day at least four or more days per week (walking slowly)
  - Moderate: 10 minutes per day at least four or more days per week (walking rapidly- carrying light things)
  - Vigorous: 10 minutes per day at least four days per week with sweating and increased heart rate (sport, running, worker, carrying heavy things)
- QOL score was calculated by getting each item in the questionnaire code that ranged from 1-5 in a positive direction except three negatively phrased questions (Q3, Q4 and Q26). RECODE Q3, Q4 and Q26: (1=5) (2=4) (3=3) (4=2) (5=1). This transforms negatively framed questions to positively framed questions. The raw score of items within each domain is used to calculate the domain score. To make domain scores comparable with the scores used in the WHOQOL-100, raw scores were converted to transformed scores using the following transformation formula:

Transformed scores=

$$\frac{\text{Actual raw score - lowest possible score}}{\text{Possible raw score range}} \times 100$$

This method converts domain scores to a 0-100 scale to enable comparisons to be made between domains composed of unequal numbers of items. The overall QOL score was calculated as the mean of scores for all domains by summing the mean scores of the four domains and divided by four.[18]

- Simple descriptive statistics as numbers and frequency distribution for categorical variables, and mean with the standard deviation for quantitative variables were used.
- In order to control simultaneously for possible confounding effect of the variables, multiple linear regression was used to identify factors that could be associated with poor QOL. Factors that entered the model were those that showed significant association with the total quality of life percent score in bivariate analysis using student t-test and bivariate correlation. Those include both quantitative and qualitative variables. All the multi-level

categorical explanatory variables included in the regression model were categorized into dichotomous dummy variables (Level of education, marital status, working status). Both weight status and state of glycemic control were excluded to avoid muticolinearity.

- Analysis was performed at 0.05 level of significance.

Results:

Description of the sample:

a. Socio-demographic characteristics (Table I): -

The cross sectional survey included 453 diabetic patients (53.9% were males and 46.1% were females) with a mean age of 54.9± 10.4 years. Nearly two thirds (64.9%) of them were Kuwaiti the others were non-Kuwaiti. Illiterates, those who can read and write and primarily educated patients constituted more than one third (36.6%). 21.2% of them were highly educated (university or higher). Not working and retired patients constituted 46.4% of the studied sample, and the majority of them (84.1%) were ever married. Monthly income for nearly one half (48%) of them was less than 1000 KD.

b. Clinical characteristics (Table II):

More than three quarters of the studied patients were either obese or overweight (80.6%). 64% of them gave a history of one or more co-morbid diseases other than diabetes including: chronic hypertension, cardiac disease, chronic renal disease, chronic liver disease and /or endocrinal disease. Duration since discovery of diabetes ranged from one to 34 years with a mean of 8.4±6.4 years. Only one third (33.3%) were adjusted on insulin therapy to control blood sugar level. Chronic diabetes complications were documented in electronic files of 39.5% of patients including retinopathy, nephropathy, diabetic foot complications or sexual problems. Level of glycosylated hemoglobin HBA1c for 70% of patients was more than 7%denoting poor glycemic control. Micro-albuminuria was diagnosed in only 15.2% of cases, while dyslipidemia was found in 78.1%.

c. Diabetes related self- care behavior. (Table II)

The majority of the studied diabetics (80.8%) attended at least 70% of the appointments in last year. On the other hand, only 58.3% of them reported adequate adherence to the prescribed anti diabetic treatment. Moreover, only 26.5% practiced moderate or heavy exercise.

d. Quality of life of diabetic patients:

Only few studied diabetic patients rated their general QOL as very bad or bad (0.2% and 4.2% respectively). Less than a fifth (18.8%) stated that their life was of moderate quality whereas the majority stated that they lived good (64.0%) or very good (12.8%) QOL in the last four weeks. Only 1.1% of the studied diabetic patients perceived their general health as very bad, and 10.4% as bad. The majority of patients rated that their general health as moderate (22.5%) or good (59.6%). On the other hand, a minority (6.4%) stated that their health was very good.

Table (III) illustrated the total QOL score (out of 100) as well as that for each of the individual domain for studied diabetic patients in terms of minimum, maximum, mean, median and standard deviation. Overall, the total QOL score ranged from 14.3 to 91.0 with a mean score of 63.8±12.5. The mean values for the total quality of life as well as four subdomains were poor as the highest mean subscale scores were for social relationship (65.2±15.3).

e. Factors influencing health related QOL of type 2 diabetic patients in Kuwait.

Results of regression analysis (Table IV) revealed that socio-demographic characteristics, clinical and diabetes-related variables affected the total QOL, and accounted for 29.5% of variance in the total HRQOL of studied diabetic patients (F= 16.778 , P<0.001).

The table shows that being non Kuwaiti, unmarried, lower levels of education, longer duration of diabetes, non-adherence to prescribed medications, non-practicing of physical exercise, presence of other co-morbid diseases or chronic diabetes complications significantly lower the total HRQOL.

Comparing Beta of all correlates showed that nationality, level of education and poor adherence to medications greatly influenced the total QOL of subjects with diabetes.

Table I: Distribution of diabetic patients according to their Socio-demographic characteristics (n=453).

Socio-demographic characteristics	Total(n=453)	
	No	%
Gender		
Male	244	53.9
Female	209	46.1
Age (years)		
<50	159	35.1
50-	145	32.0
≥60	149	32.9
Mean ± SD	54.9±10.4	
Min – Max	37 – 82	
Nationality:		
Kuwaiti	294	64.9
Non-Kuwaiti	159	35.1
Level of education		
1ry / less	166	36.6
Intermediate / 2ry / diploma	191	42.2
University / higher	96	21.2
Working status		
Not working/retired	210	46.4
working	243	53.6
Marital status		
Currently married	381	84.1
Divorced/widow	57	12.6
Single	15	3.3
Monthly income (KD):		
<1000	217	48.0
1000-1499	127	28.1
≥1500	109	23.9

Table II: Distribution of diabetic patients according to their Clinical characteristics and self -care practices (n=453).

Clinical characteristics and self -care practices	Total(n=453)	
	No	%
Weight status		
Average weight	88	19.4
Overweight /obese	365	80.6
History of associated co-morbid conditions		
Yes	290	64.0
No	163	36.0
Duration of diabetes		
Mean ± SD	8.4±6.4	
Min – Max	1.0 – 34.0	
Diabetes treatment		
Insulin	151	33.3
Non-Insulin	302	66.7
History of chronic diabetes complications		
Yes	179	39.5
No	274	60.5
Glycemic control		
Controlled	136	30.0
Uncontrolled	317	70.0
Microalbuminuria		
Present	69	15.2
Absent	384	84.8
Dyslipidemia		
Present	351	78.1
Absent	99	21.9
Exercise practicing		
No/light	333	73.5
Moderate/vigorous	120	26.5
Adherence to treatment@		
Adherent	264	58.3
Non adherent	189	41.7
Follow up visits#		
Regular	366	80.8
Irregular	87	19.2

@ Patient was classified as non-adherent to treatment if answered three or more questions by "yes".
Regular follow up visit (at least 70% of the appointments last year).

Table III: Total and subdomain QOL scores of studied diabetic patients (transformed score out of 100)

QOL Domains	Mean±SD	Median	Min-Max
Physical health	63.1±16.7	63.0	6.0-100
Psychological health	62.5±14.7	69.0	19.0-100
Social relationship	65.2±15.3	69.0	6.0-100
Environment	64.4±13.3	69.0	13.0-94.0
Total score	63.8±12.5	65.8	14.3-91.0

Table IV: Results of regression analysis for variables associated with total health-related quality of life (HRQOL) in patients with diabetes mellitus (DM)

	B#	Beta@	t	Significance
(Constant)	62.269		26.729	.000
Gender	-2.234	-.089	-2.024	.044
Nationality	-5.290	-.202	-4.160	.000
Level of education	5.053	.202	4.626	.000
Working status	-.070	-.003	-.054	.957
Marital status	4.388	.128	3.044	.002
Duration of DM	-.225	-.115	-2.393	.017
Insulin therapy	-.445	-.017	-.371	.711
Other comorbidities	-3.145	-.121	-2.837	.005
Chronic diabetes complications	-3.674	-.144	-3.119	.002
Adherence to medications	4.979	.196	4.743	.000
Exercise practicing	3.567	.126	2.937	.003

partial regression coefficient, @ standardized regression coefficient

gender (0 for males and 1 for females), nationality (0 for Kuwaiti and 1 for non-Kuwaiti), marital status (married=1, single, divorced, widow=0), level of education (illiterate and lower education=0, secondary education or higher=1) , working status (currently working=1, not working or retired=0), presence of other comorbid conditions(yes=1and no=0), presence of chronic diabetes complications (yes=1and no=0), adherence to medications(adherent=1,non-adherent=0), exercise practicing (no or light=0, moderate or heavy=1)

Discussion:

Many studies have generally shown an almost universally negative impact of diabetes on all domains of the QOL. In the present study, out of 100, the average of the physical domain score was 63.1, psychological domain score was 62.5, social domain score was 65.2, environment domain score was 64.4, and the total QOL score was 63.8. These results were comparable with a recent study, it was conducted in Iran, where the physical score was 65.45, the psychological score was 66.52, the social score was 69.37, the environmental score was 62.87, and the total score was 65.54. [19]

The mean score of the present study in physical functioning was comparable with studies that was conducted by Papadopoulos et al.(2007) [20] who studied 229 Greek type 2 diabetics and showed that the mean score of this domain was 64.5, and Clouet et al. study (2001)[21] on 282 French type 2 diabetics who gained his domain mean score as 63.2.

However, it was higher than a study that was conducted in Tehran (2012)[22] by Kazemi-Galougahi et al. who reported a mean score of physical functioning as 57.42, but lower than another one that was conducted by Ribu et al. (2007)[23] who studied 221 Norwegian diabetics and gained mean score of 77.3 for this domain.

Also, the mean score of social functioning in the present study was 65.2 which was lower than that obtained by Papadopoulos et al. who reported it as 74.8 and Clouet et al. who measured it as 68.3 and higher than that reported by Kazemi-Galougahi et al. as 47.81.[20-22]

Several studies are being, and have been, conducted to find out which factors influence the quality of life of patients with T2DM. The results

have been contradictory, especially concerning the effect of the metabolic control of diabetes on quality of life, the treatment regimen and the duration of the disease. In the current study, many factors were proved to affect HRQOL of diabetic patients; namely: lower level of education, low income, poor glycemic control, obesity, comorbidity and long term complication.

Javanbakht et al. found that education level had a linear relationship with QOL. As the educational level increased the quality of life increased. This could be because they would have a better understanding of the disease, its effect on them, and would avail themselves the best treatment they can afford.[24] Kakhki et al. stated that some physical functioning differences were found among levels of education.[25]

Lower BMI has been proved to improve the QOL of participants. Reports in the literature typically concluded that weight loss is associated with positive effects on HRQOL for individuals of varied age, ethnicity and gender over a wide range of BMI and weight changes.[26.27] This goes in accordance with the present study where overweight and morbidly obese patients were significantly more prone to have lower QOL.

Co-morbidity in patients with T2DM is associated with reduced HRQOL and self-care. This has been proved in the present study as patients with one or more comorbid conditions had increased risk of being dissatisfied with their QOL. Also, the risk increased with the increased number of comorbidities. In agreement with this, Wasem et al. suggested that co-morbidity reduces HRQOL in T2DM patients who had a substantial co-morbidity which ranged however from none to more than 6 comorbidities.[29] In agreement with the present study, O'Shea et al. in their study proved that QOL scores decreased with an increasing number of comorbidities.[30]

Diabetes patients are more susceptible to macro or microangiopathic complications. The incidence of diabetic complications has been shown to have a significant impact on QOL in a number of studies.[30.31] Al-hayek et al. study indicated a significantly lower HRQOL among patients with diabetic complication than the patients with diabetes alone. Further, multivariate analysis indicated complication of DM as an independent risk factor for all subscales of SF36 except energy.[32]

Arghese et al. and Saito et al. in two different studies showed that as duration of the disease increases, the health of the patient will gradually worsen depending on his control of diabetes.[31.33]

In Demirci et al. study, it was found that insulin treatment and duration of diabetes were the two most important factors associated with quality of life and that insulin treatment reduced the quality of life.[34] In the present study glycemic control had a significant effect on QOL score. In fact, the relationship between glycaemic control and HRQOL of patients with T2DM is unclear. It has been suggested that reducing glucose to normal levels may enhance well-being, particularly as fatigue is frequent in patients with elevated blood glucose levels.[35] But while some studies have shown that improved A1C is associated with short-term improvement in HRQOL.[36] Others were not able to confirm this.

In contrary to the current study, other studies reported that insulin treatment was one of the important factors associated with HRQOL. Insulin-treated diabetic patients reported reduced impact on HRQOL than tablets/diet-treated patients.[37, 38] Al-Hayek et al. also observed a significantly better HRQOL among insulin treated patients than oral hypoglycemic drugs treated patients. In addition, the combination of insulin and oral hypoglycemic drugs treated patients had better HRQOL than those treated with insulin alone. [32] The current study also shows that practicing moderate or heavy exercise is associated with significant increase in the total quality of life. This was in agreement with Daniele et al. who found an improved HRQOL in physically active patients with diabetes. This might provide, beyond improvements of

metabolic control and body weight, an opportunity to improve HRQOL.[39] However, Wasem et al. were not able to show a direct impact of physical activity on HRQOL. [29]

Being non adherent to medications is closely related to higher risk of uncontrolled hyperglycemia with subsequent increase in the risk of occurrence of acute and chronic diabetes complications, this might explain the greatest impact of adherence to treatment on the quality of life of diabetic patients as revealed by multiple regression in the current study.

Conclusion and recommendations:

The current study demonstrated a relatively poor total HRQOL of diabetic patients and all its subscales. This was significantly related to many sociodemographic and disease related characteristics. Factors influence HRQOL of diabetic patients should be considered while planning management policies for diabetics in Kuwait. Introduction of ongoing health education and support during visits emphasizing the importance of having nutritious diet and engagement in a carefully-planned regular structured exercise as well as adherence to prescribed medications. Intensive education for patients at high risk to have poor QOL, particularly unmarried, with low income and lower level of education and those suffering from comorbidity or diabetic complications.

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Original Article

Chemerin Level, High Sensitivity-CRP, Insulin Resistance & Severity of Coronary Heart Disease in Type 2 Diabetes.

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Abstract:

Background: Chemerin has been recently modified as a novel adipokine having a role in inflammation and atherosclerotic cardiovascular disease. It has also been suggested to have a role in glucose homeostasis and insulin action. The aim of this study was to assess the relationship between serum Chemerin levels, hs-CRP, insulin resistance and the severity of coronary artery disease (CAD) in type 2 diabetic patients. **Methods:** The study included 160 patients; divided equally into 4 groups; CAD having type 2 diabetes, CAD without type 2 diabetes, type 2 diabetes without CAD and a healthy control group. Serum Chemerin levels, hs-CRP, HOMA-IR were assessed for the 4 groups. SYNTAX score was calculated for the CAD groups. **Results:** Our study showed that there was a statistically significant difference in serum Chemerin level between subjects with CAD when compared to subjects without CAD ($p < 0.001$). There was no statistically significant difference in serum Chemerin level between diabetic and non-diabetic subjects ($p = 0.16$). Among our studied groups, there were no statistically significant correlations between serum Chemerin and fasting plasma glucose, HbA1c, fasting serum Insulin and HOMA2-IR. Our results show a significant positive correlation of serum chemerin with waist circumference (p-value=0.024) and waist/hip ratio (p-value=0.044). Patients with CAD (group A and B) were further divided according to their SYNTAX score into 3 groups. There was a statistical significance between groups regarding both Chemerin level ($p <0.001$) and hs-CRP level ($p =0.04$). **Conclusion:** There is a significant correlation between serum Chemerin level with various metabolic risk factors and atherosclerotic cardiovascular disease. It was also significantly correlated to the severity of CAD according to the SYNTAX score. However, the exact role of Chemerin in glucose homeostasis and its relation to diabetes needs further future research.

Keywords: Chemerin, inflammation, atherosclerosis, type 2 diabetes.

Introduction:

The relationship between diabetes (DM) and atherosclerotic coronary artery disease (CAD) has been well established. The presence of diabetes is associated with an increases risk for the development of CAD by two to four folds. Ischemic CAD is a major cause of mortality among diabetic patients leading to about three-quarters of deaths among patients with diabetes.[1]

Macrovascular complications associated with diabetes, especially atherosclerosis are a leading cause of both disability and deaths in diabetic patients. Macrovascular diseases in diabetes are related to both vascular and metabolic abnormalities. Vascular abnormalities involve endothelial dysfunction, abnormalities in vascular smooth muscles, platelets dysfunction. The metabolic abnormalities characterizing diabetes include hyperglycemia, and the presence of advanced glycated end products (AGEs) deposited in various tissues. Insulin resistance shares in provoking molecular mechanisms and elevated levels of free fatty acids that contribute to further vasculopathy. [2]

Abbreviations: HOMA-IR: homeostasis model assessment of insulin resistance, **Hs-CRP:** high sensitive C-reactive protein, **CAD:** Coronary artery disease, **DM:** Diabetes mellitus.

Adipose tissue is considered now an endocrine organ which is actively producing many cytokines and proteins known as adipokines, rather than only storage for excess energy in the form of fat.[3-7] Adipokines exerts several effects on various organs, including liver, brain, β-cells, gonads, muscles and vascular system.[8] The exact role of adipose tissues in the development of CAD in patients with diabetes and patients with metabolic syndrome has been established in many studies.[9-13] Adipose tissue leads to vascular complications by two important mechanisms; either directly through inflammatory response induction and development of atherosclerosis or indirectly through promotion of insulin resistance.[14]

Various adipokines mediate these atherosclerosis-inducing pathways. These adipokines include adiponectin, leptin, and resistin.[9-12,15] Understanding the role of various adipokines may help us to further understand the mechanisms involved in the development of atherosclerosis and insulin resistance leading to better understanding of vascular complications.

A newly discovered chemo-attractant molecule, known as Chemerin, is a 16 kDa protein. It serves as a binding ligand for the G-protein receptor CMKLR1 (ChemR23 or DEZ). [16,17] Chemerin is formed by adipose tissue and secreted in the form of pro-chemerin (18 kDa) which is then transformed by serine protease into an active protein.[17, 18]

When Chemerin is in its active form, it has immune regulatory functions. Chemerin induces inflammation through its effect on the recruitment of inflammatory cells mainly macrophages and dendritic cells.[16, 19] Studies with Psammomysobesus have shown that Chemerin may be associated with adipogenesis. Although Chemerin and CMKLR1 affect all tissues, they show higher expression in the liver, adipose tissue and kidneys. [20]

The expression of Chemerin and CMKLR1 by undifferentiated adipose tissue is at low levels compared the mature adipocytes.[16] On experimental knockdown of chemerin or CMKLR1 expression in pre-adipocytes or mature adipocytes, this has resulted in severe impairment of adipocytes differentiation and reduction of the expression of various genes involved in glucose and lipid metabolism. [16]

In mature 3T3-L1 adipocytes, the effect of Chemerin treatment blocked isoproterenol-stimulated lipolysis by means of a CMKLR1-independent mechanism. On the other hand, Chemerin treatment promoted insulin-stimulated glucose uptake in adipocytes through increased tyrosine phosphorylation of IRS-1. [21]

Clinical studies have reported elevated serum total Chemerin levels in patients with metabolic syndrome compared with healthy controls. In these cases, positive correlations were observed between serum total Chemerin, measures of body fat and a number of markers of metabolic syndrome including various anthropometric measures, blood pressure, fasting glucose levels, fasting insulin levels and serum triglycerides.[20,22,23] One study identified a positive correlation between common genetic variations in the chemerin gene locus (SNPs rs17173608 and rs10278590) and the development of visceral adiposity.[24]

These studies showed that, according to the degree of obesity, serum total Chemerin levels are dynamic and modifiable. Also these observations suggest that chemerin may have an effect on insulin sensitivity and the occurrence of macrovascular complications, especially atherosclerosis. Chemerin is thought to share in the development of atherosclerosis through its effect on inflammatory processes. Chemerin induces leukocyte migration as its receptors also expressed by tissue macrophages. [17-19]

Studies showed a positive correlation between Chemerin levels with hs-CRP levels, which is an established marker of inflammation. CRP, is formed mainly by the liver in response to IL-6.[25] It was shown that CRP levels were higher in patients with metabolic syndrome compared to healthy individuals. Hs-CRP level is considered as an independent risk factor for both type 2 diabetes and cardiovascular disease.[25-27] CRP promotes the expression of adhesion molecules by human endothelial cells thus directly inducing inflammation and atherosclerosis.[28] There are growing evidences of an association between acute inflammatory markers as CRP and the risk of atherosclerotic CAD .The release of various

acute phase reactants as a response to inflammation is an important marker plaque and underlying atherosclerosis and atherosclerotic CAD. [29, 30]

Aim of The Work: The aim of this work was to investigate the link between serum Chemerin and high sensitivity -CRP (hs-CRP) as markers of inflammation and insulin resistance in the different degrees of severity of atherosclerotic CAD in patients with and without type 2 DM.

Subjects & Methods:

This cross-sectional study was conducted on 160 subjects divided into four groups:

- Group A: 40 Type 2 diabetic patients with CAD.
- Group B: 40 CAD patients without type 2 DM.
- Group C: 40 Type 2 diabetic patients without CAD.
- Group D: 40 Healthy control subjects matched for age, sex and socioeconomic status.

Patients of groups A & B were recruited from patients presenting to the Catheter Lab of Alexandria University Hospital. Patients of groups A and B had angiographically documented CAD. Coronary artery disease was excluded in patients of group C by the presence of negative stress test and/or coronary angiography. Subjects of group C were recruited from those undergoing coronary angiography and/or stress test for a justified indication at the Cardiology Department in Alexandria University Hospital and were proven to be free of CAD. Control subjects were subjected to full history taking, clinical examination, ECG and to laboratory investigations as the other groups.

Subjects were included after exclusion of the following criteria: History of acute coronary syndrome in the last 6 months, heart failure, hepatic or renal disease, type 1 diabetes, pregnancy and lactation, any metabolic or endocrinal disease other than diabetes, anaemia, history of previous infection within the last 2 months.

The study was approved by the Medical Ethics Committee of Alexandria University Hospital .Each subject has signed an informed consent to participate in the study.

Blood Sampling:

Blood samples were drawn from all our subjects after overnight fasting of 12 hours. The taken samples were then divided into two parts: the first part was collected for the assay of HbA1c (mmol/mol and %) in disodium salt (Na2)-EDTA containing tubes; the second part of sample was collected for serum preparation used for fasting plasma glucose level, fasting insulin level, lipid profile (including total cholesterol level, serum triglycerides, HDL-cholesterol level, LDL-cholesterol level), ALT, hs-CRP and Chemerin. We used Homeostasis Model Assessment 2 (HOMA2) calculator to estimate insulin resistance (%S) (HOMA-IR) using an updated computer based HOMA2 mode.[31] Human serum Chemerin level was measured using commercially available Chemerin ELISA assays kit. All ELISA procedures were carried as given by the manufacturer's instructions using a Hyprep Automated ELISA system. [20]

Angiographic Analysis :[32]

All patients underwent coronary angiography with the Judkins technique. Coronary angiography was performed with standard femoral approach with a 6-F diagnostic catheter. The SYNTAX score (SS) is a semi-quantitative score which was used before to assess and classify the angiographic findings of subjects included in the SYNTAX trial. SYNTAX score was based on previously existing classifications merged together. The score depends on various variables, including: the number of lesions, dominance, the occurrence of chronic total occlusions, bifurcation, the segment involved per lesions, presence of tortuosity, the presence of long lesions, thrombi, calcifications and a diffuse disease. Then a separate value was calculated for each lesion according to the several variables. On Line calculator was used to calculate the total SYNTAX score by summing up the separate values. www.lr nwr.ru/calculators/syntaxcore.htm. According to SS value, subjects in groups A and B were classified as mild CAD (score of 0-22), moderate CAD (score of 23-32) and severe CAD (score≥33).

Statistical Analysis:

We used for our data collection and analysis the IBM statistical package for social sciences (PASW) previously called (SPSS) statistics version 18.0 (IBM Corp., Chicago, IL, USA). We considered the probability of error at 0.05 to be significant; while the highly significant was that at 0.01 and 0.001.

Results:

Table I shows the anthropometric, biochemical and clinical variables of our study subjects.

The mean HbA1c levels were 8.13±1.69 % in the CAD with diabetes group, 5.86±0.39 % in the CAD non-diabetic group, 8.59±1.96 % in the diabetic patients without CAD, 5.72±0.56 %in the control group. There were statistical differences between group A compared to group B & group D. There was also statistically significant differences between group C compared to groups B & D ($p < 0.05$).

There were no statistically significant differences between groups regarding the mean fasting serum insulin levels which were 14.10±9.14μIU/ml in the CAD with diabetes group, 12.39±8.95 μIU/ml in the CAD non-diabetic group, 11.37±7.51 μIU/ml in the diabetic patients without CAD, 11.55±8.66 μIU/ml in the control group ($p > 0.05$).

The mean HOMA2-IR values were 2.14±1.53 in the CAD with diabetes group, 1.60±0.96 in CAD non-diabetic group, 1.62±1.71 in the group of diabetic patients without CAD and 1.50±1.14 in the control group. There were statistically significant differences between group A compared to the control group ($p < 0.05$).

The mean hs CRP levels were 27.72±57.54 mg/L in the CAD with diabetes group, 22.77±54.55 mg/L in the CAD non-diabetic group, 11.63±10.92 mg/L in the diabetic patients without CAD, 5.58±6.66 mg/L in the control group. There were statistical significant differences between group A compared to group D ($p < 0.05$).

The mean serum Chemerin levels were 447.54±81.17 ng/ml in the CAD with diabetes group, 452.73±80.80 ng/ml in the CAD non-diabetic group, 371.05±84.35 ng/ml in the diabetic patients without CAD, 323.22±67.49 ng/ml in the control group. There were statistical significant differences between group A compared to groups C and D, also between group B compared to groups C and D and significance between group C compared to group D ($p < 0.01$).

There were no statistically significant differences between groups regarding the mean serum ALT values. There was statistically significant difference regarding the presence of albuminuria (ACR>30mg alb/gm Cr) between our study groups. The percentage of subjects having albuminuria was 45% in the CAD diabetic patients, 20% in the CAD patients without diabetes and 27% in diabetic patients without CAD ($p < 0.05$). These findings are shown in Figure 5.

The correlations between serum Chemerin and both age and anthropometric parameters among the studied subjects showed that there was a significant positive correlation of age (p-value=0.015), waist circumference (p-value=0.024) and waist/hip ratio (p-value=0.044) with serum Chemerin. On the other hand there was no significant correlation with weight, body mass index or hip circumference. There was a statistically significant correlation between serum Chemerin and serum hs-CRP ($p = 0.002$), as shown in Figure 1. Among our studied subjects, there was a statistically significant difference in the serum Chemerin level between the hypertensive subjects & those without hypertension ($p = 0.007$.

We then divided our study population into 2 groups, according to the presence or absence of diabetes. Among our studied groups, there was no statistical significant difference in serum Chemerin level between diabetic (group A & group C) and non-diabetic (group B & group D) subjects ($p = 0.16$). Our subjects were also divided into 2 groups, according to the presence or absence of CAD. There was a statistical significant difference in serum Chemerin level between subjects with coronary artery disease (group A & group B) and subjects without coronary artery disease (group C & group D) ($p < 0.001$).

Patients with CAD (group A and group B) were further divided according to their SYNTAX score into 3 groups. There was a statistical significance between groups regarding both Chemerin level ($p<0.001$) and hs-CRP level ($p = 0.04$).

Table I: Descriptive Data of the Studied Subjects (n = 160)

	Minimum	Maximum	Mean	SD
Age (years)	39.0	77.0	55.35	7.65
Weight (Kg)	60.00	110.00	82.59	10.29
Height (cm)	155	187	171.62	6.64
BMI (Kg/cm^2)	20.23	37.11	28.08	3.50
Waist circumference (cm)	67.00	132.00	100.07	8.18
Hip circumference (cm)	70.00	130.00	103.81	7.73
Waist Hip Ratio	0.80	1.10	0.96	0.029
Systolic BP (mmHg)	100	150	127.00	11.54
Diastolic BP (mmHg)	60	110	81.19	9.74
Glucose (mg/dl)	65.00	380.00	120.94	62.49
Total Cholesterol (mg/dl)	66.00	266.00	171.34	39.20
Serum Triglycerides (mg/dl)	38.00	406.00	145.17	67.61
HDL-c (mg/dl)	8.00	88.00	39.18	12.99
LDL-c (mg/dl)	26.50	192.20	103.13	33.56
ALT (IU/L)	7.00	118.00	25.12	15.12
hsCRP (mg/L)	0.40	281.00	16.92	40.74
Insulin (µIU/mL)	1.03	81.90	12.35	10.01
HbA1c (%)	4.10	13.00	7.072	1.86
HOMA2IR	0.10	9.20	1.71	1.38
Chemerin	191.80	723.90	398.63	95.08

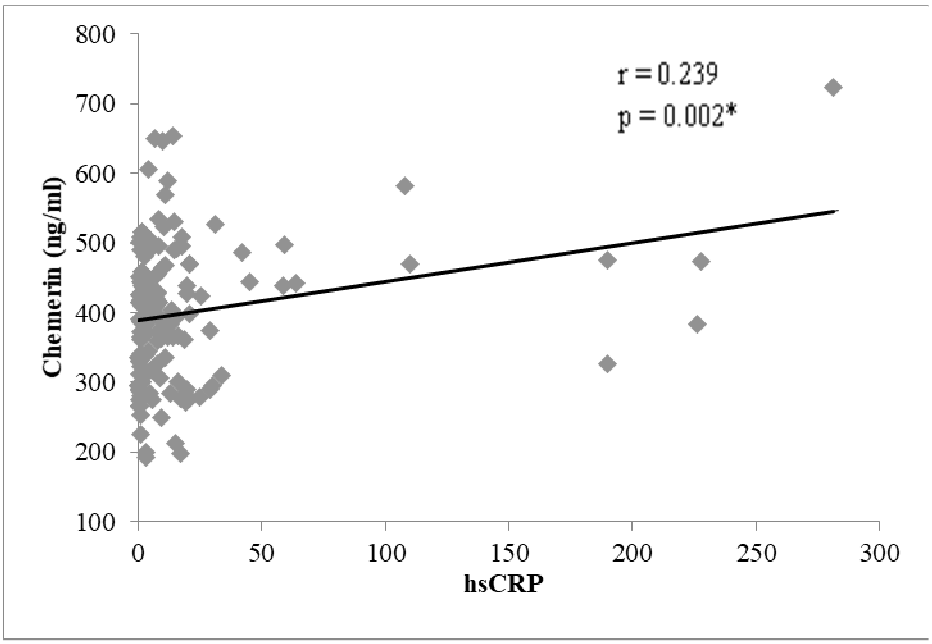

Figure 1: Correlation between Serum Chemerin & hs-CRP in the Studied Subjects

Discussion:

Inflammation is proposed to initiate endothelial dysfunction and cardiovascular complications present in patients with type 2 diabetes. Type 2 diabetes and obesity-related atherosclerotic diseases are considered as a condition of chronic low-grade inflammation, thus understanding the different adipokines and inflammatory markers would help better understanding of various mechanisms related to cardiovascular disorders .[33]

Chemerin affects adipose tissue differentiation, maturation and function through its autocrine and its paracrine actions. Chemerin also shows some important endocrine roles in immunity and metabolism. Chemerin was initially described in 2003 as a novel chemoattractant protein.[34] Chemerin undergoes enzymatic proteolysis and performs its functions through several G protein receptors such as CMKLR1, GPR1, and CCRL2 [35–37]. In 2007, the role of Chemerin as a novel adipokine was discovered [37] and its effects on adipogenesis, metabolism and sustained inflammation were then established.

Several studies have shown that elevated serum chemerin levels correlate positively with body mass index (BMI),waist circumference, waist to hip ratio,[20,38,39,40] and visceral adiposity.[41,42] In our study, Chemerin showed significant positive correlation with waist circumference and waist/hip ratio. On the other hand there was no significant correlation with weight, body mass index or hip circumference.

Elevated Chemerin levels that were shown in obesity, were correlated with adipose tissue infiltration by macrophages and production of well-established inflammatory mediators such as CRP and IL-6.[43,44] The relation between Chemerin levels and adiposity can be summarized in the following three points: (1) The presence of sustained inflammation in adipose tissue is an initial signal for increased Chemerin formation and production, (2) Adipose tissue promotes Chemerin activation and inactivation through introduction of the required proteases, and (3) Adipose tissue regulates the ratio of active to total chemerin acting within adipose tissue thus affecting the inflammatory functions induced by immune cells recruitment.

Among our studied population, there was no statistically significant correlation between serum Chemerin and various glycemic parameters, including fasting plasma glucose, HbA1c, fasting serum Insulin or HOMA2-IR, a finding in accordance with the study conducted by Hu & Feng.[45] Studies showed conflicting results regarding the role of Chemerin in insulin sensitivity and glucose uptake. Some studies have shown both increased [46] and decreased [47] insulin stimulated glucose uptake after Chemerin treatment in adipocyte tissues. The relationship between Chemerin and diabetes is unclear. Some studies showed that high Chemerin levels might be a cause or might be a consequence of abnormalities in the circulating insulin levels and insulin sensitivity. [48, 49]

Given the alterations in Chemerin levels in both obesity and metabolic syndrome states, a limited number of studies have assessed the role of Chemerin in atherosclerotic CAD. On further comparison between subjects who had both CAD and type 2 diabetes to the control healthy group, there was a positive statistically significant correlation showing a possible role of Chemerin in the development of atherosclerotic CAD among our subjects.

Chemerin contributes to endothelial dysfunction and arterial wall abnormalities through induction of vascular markers such as E-selectin and ICAM. [41] Our study showed that Chemerin level was positively correlated to the severity of atherosclerotic CAD when patients were classified according to the SYNTAX score. Some studies showed that the presence of CAD was associated with significantly elevated circulating Chemerin levels; however, it is unclear whether increased Chemerin level can predict CAD severity[50,51] or atherosclerotic plaque morphology or not.[43]

Our study showed that Chemerin was significantly correlated with the presence of hypertension, which is consistent with previous studies that reported that Chemerin is significantly associated with elevated blood pressure after confounding variables control. [20,22] The relation of Chemerin and hypertension can be explained by high levels of Chemerin expressed within the kidneys, which is a key site of BP regulation.[20,52] Also there is a structural relation between chemerin and kininogen. Kininogen is a proteolytic product of the vasoactive peptide bradykinin thus adding to the effect of Chemerin on the blood pressure measured. [17]

The principal limitation of the present study is the relatively small study cohort; therefore the ability to generalize our findings is limited. Also, the limited sample size in our subgroups undermines the statistical power and precision. This is a cross-sectional studies represents a snapshot in time; this limits our ability to determine the temporal sequence of the associations reported and prone to many confounders Our sample included subjects of Egyptian nationality only; it is uncertain whether our findings are applicable to other ethnic groups.

Conclusions:

Serum Chemerin level was significantly correlated with various metabolic risk factors and atherosclerotic cardiovascular disease. It was also significantly correlated to the severity of CAD according to the SYNTAX score. Results of our study suggests an interplay between serum Chemerin level, hs CRP and the occurrence of atherosclerotic cardiovascular disease among patients with type 2 diabetes. However, the exact role of Chemerin in glucose homeostasis and its relation to diabetes needs further future research.

Conflict of Interest: None

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Original Article

Study of Serum and Urinary Cystatin C as A Marker of Nephropathy in Type 1 Diabetic Patients.

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Abstract:

Background: Diabetic nephropathy (DN) is a significant cause of morbidity and mortality in patients with diabetes mellitus (DM). The condition is characterized by persistent albuminuria and may be decline in the glomerular filtration rate (GFR). Serum cystatin C has been proposed as a simple, accurate, and rapid endogenous marker of GFR. **Aim of the study:** To evaluate clinical usefulness of cystatin C levels of serum and urine in predicting DN in patients with type 1 diabetes and to evaluate the association between albuminuria and serum/urine cystatin C. **Subjects and Methods:** The present study included 40 male type 1 diabetic patients 10 of them was normoalbuminuric (group I), 15 patients was microalbuminuric (group II) and 15 patients macroalbuminuric (group III). In addition 10 healthy male individuals served as control group (group IV). Any patients or control with renal disease, hypertension, hepatic disease, thyroid dysfunction and on steroid therapy were excluded. All subjects underwent full history taking and complete clinical examination, and laboratory investigations in the form of urine analysis ,urinary albumin / creatinine ratio (ACR), Serum urea and creatinine, Serum albumin and total protein, Liver enzymes ALT and AST, TSH , Serum and urinary cystatin C, Fasting plasma glucose, glycated hemoglobin (HbA_{1c}), eGFR using MDRD and ultrasound examination of the abdomen and pelvis. **Results:** The cystatin C levels of serum and urine increased with increasing degree of albuminuria, reaching higher levels in macroalbuminuric patients (P <0.001). It was positive correlation between ACR and serum and cystatin C in micro and macroalbuminuric groups and it was negative correlation between serum and urinary cystatin C and eGFR in micro and macroalbuminuric groups **Conclusions:** the serum and urinary cystatin C may serve as a biomarker of diabetic nephropathy in type 1 diabetic patients.

Keywords: Cystatin C, Diabetic Nephropathy, Albuminuria.

Introduction:

The number of people with diabetes is increasing due to population growth, aging, urbanization and the increasing prevalence of obesity and physical inactivity. According to the World Health Organization (WHO), the prevalence of diabetes for all age groups worldwide was estimated to be 2.8% in 2000 and 4.4% in 2030.(1) Diabetic nephropathy is one of the most important long term complications of diabetes mellitus, recognized as a major cause of the end stage renal disease (ESRD). (2) The onset and course of nephropathy can be favorably influenced by appropriate therapy such as tight glycemic control, effective antihypertensive treatment, lipid lowering strategies, and protein restriction. Such treatment can delay the appearance of microalbuminuria, proteinuria, and (ESRD).(3) General recommendation for subjects with DM is to perform kidney function as screening, in type 1 diabetic subjects five years after diagnosis and type 2 diabetes at the diagnosis.(4) One of these marker is microalbuminuria by using urinary excretion rate of albumin by 24 hr collection of urine equal (30-300mg/24hr) or a spot urine albumin to creatinine (ACR) ratio equal (30-300µg/mg). Microalbuminuria has currently emerged as sensitive indicator of early renal damage. (5) Moreover, impaired renal function may be present even in patients with normal urinary albumin excretion rate.(6) Morphological changes in DN known to start earlier than laboratory abnormality. Also some patient with macroalbuminuria have normal renal structure, while some normoalbuminuric diabetic have

well established nephropathic lesions.[7] Also albumin excretion rate is a predictor of renal disease in hypertension and cardiovascular diseases , so it is not a sensitive marker for DN.[8] Measuring glomerular filtration rate (GFR) is the best functional parameter in renal disease using creatinine clearance. This requires 24h urine collection and blood sample and measure creatinine level in blood and urine and volume of urine. There are several factors that may interfere with the accuracy of the test like incomplete collection of urine.[9] Other methods of assessment of GFR are Cockcroft-Gault formula or modified diet in renal disease formula (MDRD).[10] Gold standard procedures for glomerular filtration rate (GFR) measurement, based on the clearance of Cr^{51}- EDTA or iohexol, are impractical in clinical settings and for larger research studies.[11] Thus, we are still in need of identify more earlier marker early markers of DN needed to be identified. Cystatin C is an alternative and more sensitive marker for estimation of renal function in diabetic subjects for early detection, prevention, and treatment strategies for DN.[12] Cystatin C was first discovered in 1961, is a member of cystatin superfamily (inhibitors of cysteine proteinase). It has a molecular weight of 13,000 KD. Cystatin C is non-glycosylated basic protein, which is produced at a constant rate by all nucleated cells released into blood stream with half-life ~2hr, also present in urine, CSF, saliva, semen and colostrum.[13] The serum concentration does not depend on muscle mass, sex, age and not affected by inflammation, fever.[14]Therefore, the plasma concentration of cystatin C is almost exclusively determined by the GFR making cystatin C an excellent indicator of GFR. It also correlates with the appearance of proteinuria in diabetic subjects.[15] Only a few circumstances have been identified that have impact on the production of cystatin C, such as very large dose of glucocorticoids and thyroid dysfunction. [16]Urinary cystatin C is another marker for nephropathy. In healthy kidney is almost freely filtered by the glomerulus and reabsorbed in proximal tubule and with no tubular secretion and it is virtually absent in final urine like other low molecular weight (LMW) proteins, and only appear with tubular damage. [17] Thus, we explored the possibility of the cystatin C levels of serum and urine as markers of early renal impairment in patients with diabetes. We also evaluated the relationship of albuminuria and serum/urine cystatin C.

Subjects and Methods:

The present study included 40 male type1 diabetic patients from the diabetes and metabolism outpatient clinic of Alexandria Main university hospital. Patients were divided into 3 groups according to ACR. Group I, 10 type 1 diabetic patients with normoalbuminuria (ACR less than 30 mg/g), group II, 15 patients with microalbuminuria (ACR 30-300 mg/g) group III , 15 patients with macroalbuminuria (ACR >300mg/g) and 10 healthy male individuals as group IV.

Detailed medical history and clinical assessment were performed. After taking an informed consent from each subject. Measuring blood pressure and anthropometric data were obtained for each subject. They included weight in kilograms, height in centimeter and calculation of body mass index (BMI) (weight in kilograms/ height in meters squared). [18]

All patients with hypertension, hepatic diseases, renal disease other than DN, thyroid dysfunction and patients on steroids therapy were excluded.

Fasting blood samples were taken for measurement of FBG, HBA1c,[19] BUN, Creatinine,[20] ALT, AST,[21] total protein , serum albumin,[22] and TSH. [23]Early morning urine sample for complete urine analysis and ACR. [24]

The cystatin C levels of serum and urine were measured by the latex agglutination test (Modular P800, Roche, Diagnostics, Mannhein, Germany). [25] The eGFR level was calculated using the Modification of Diet in Renal Disease (MDRD) formula

MDRD = 186 × (serum creatinine [mg/dL])-1.154 × age-0.203. [26]

Statistical Analysis:

Data were fed to the computer and analyzed using IBM SPSS software package version 20.0. Quantitative data were described using range (minimum and maximum), mean, standard deviation and median. The

distributions of quantitative variables were tested for normality using Kolmogorov-Smirnov test, Shapiro-Wilk test and D'Agstino test, also Histogram and QQ plot were used for vision test. If it reveals normal data distribution, parametric tests was applied. If the data were abnormally distributed, non-parametric tests were used. For normally distributed data, comparison the studied groups were analyzed using F-test (ANOVA) and Post Hoc test (Scheffe). Correlations between two quantitative variables were assessed using Pearson coefficient. Kruskal Wallis test was used to compare between different groups and pair wise comparison was assessed using Mann-Whitney test. Significance test results are quoted as two-tailed probabilities. Significance of the obtained results was judged at the 5% level.

Results:

All patients and control groups were of male sex and there was statistical significance differences as regard age (P<0.001). As regard the duration of diabetes there was significance difference between diabetic groups (P<0.001) and was longer in macroalbuminuric group 11.40 ± 2.90. All patients and control groups were matched as regard BMI (P=0.807). As regard the patients glycemic control, there was no significant difference between the patients groups for there FPG (P=0.901) also HbA1c% There was no statistically significant difference between the patients groups (P=0.012).

As regard the renal functions, BUN there was statistically significant difference between the patients groups (P=0.002) and also for serum creatinine (P=0.044). For eGFR using MDRD, there was There was statistically significant difference between the patients groups (p<0.001) and was lower in the macroalbuminuric group (79.73 ± 13.39) than in microalbuminuric group (88.0 ± 14.4) and normoalbuminuric group (111.8 ± 12.4) (Table I).

Differences in the cystatin C levels of serum and urine according to albuminuria (TableII, III**):**

There was a statistically significant difference in comparing between patients groups (P<0.001) and the level of serum cystatin C showing stepwise increase with the albuminuria and there was higher in the group of macroalbuminuria (2.79 ± 0.35) than in microalbuminuric group (0.99 ± 0.26) and normoalbuminuric group (0.97 ± 0.17). As regard urinary cystatin C also there was a statistically significant difference between patient groups (P<0.001) and the level of urine cystatin C also showed higher levels in macroalbuminuric group (2.17 ± 0.50) than microalbuminuric group (0.82 ± 0.37) and normoalbuminuric group (0.09 ± 0.08).

Correlation studies:

For serum cystatin C (Table IV)

There was positive correlation between serum cystatin C with ACR only in microalbuminuric group (r=0.958, P<0.001) and in macroalbuminuric groups (r=0.818, P<0.001) (figure 1,2).

Also there was a statistically significant negative correlation between serum cystatin C and eGFR in microalbuminuric group (p=0.020, r= -0.593) and macroalbuminuric group (p=0.029, r= -0.562) (figure3,4).

In microalbuminuric group, there was a statistically significant positive correlation between serum cystatin C and ACR, s.Cr, urinary cystatin C (P<0.001, r=0.985, P=0.001, r=0.774, p<0.001, r=0.858 respectively).

In macroalbuminuric group, there was a statistically significant positive correlation between serum cystatin C and ACR, HbA1c, s.Cr and urinary cystatin c (P<0.001, r=0.818, P<0.001, r=0.823, p=0.026, r=0.572, p<0.001, r=0.922 respectively).

In all studied groups there was no statistically significant correlation between serum cystatin C with age, BMI and BUN.

For urinary cystatin C (table V)

In normoalbuminuric group

There was no statistically significant correlation between urinary cystatin C and age, BMI, ACR, HbA1c, eGFR, BUN and s.Cr (P=0.839, 0.300, 0.681, 0.640, 0.603, 0.195, 0.528 respectively).

In microalbuminuric group

There was no statistically significant correlation between urinary cystatin C and age, BMI, HbA1c, BUN (P=0.293, 0.171, 0.878, 0.380 respectively).

There was a statistically significant positive correlation between urinary cystatin C and ACR , s.Cr (P<0.001, r=0.861, p=0.002, r=0.742 respectively) (Figure 3).

There was a statistically significant negative correlation between urinary cystatin C and eGFR (P=0.037, r=0.502).

In macroalbuminuric group

There was no statistically significant correlation between urinary cystatin C and age, BMI, BUN (P=0.610, 0.784, 0.196 respectively).

There was a statistically significant positive correlation between urinary cystatin C and ACR, HbA1c, s.Cr (P<0.001, r=0.831, P<0.001, r=0.849, p=0.008, r=0.653) (figure4).

There was a statistically significant negative correlation between urinary cystatin C and eGFR (p=0.034, r= -0.562).

In the control group:

There was no statistically significant correlation between urinary cystatin C and age, BMI, ACR, HbA1c, eGFR, BUN, s.Cr (P=0.631, 0.888, 0.264, 0.867, 0.680, 0.530, 0.463 respectively).

Table I: Demographic and laboratory data in patients groups.

	Group I (n=10)	Group II (n=15)	Group III (n=15)	Test of sig.
Age(years)	20.0 ±16.70	23.73 ± 5.85	27.80 ± 5.20	$^{F}p<0.001^{*}$
ACR (mg/g)	14(165.0–21.0)	180(40.0–298.0)	10.50(5.0–21.0)	$^{KW}p<0.001^{*}$
FBS (mg/dl)	210.0 ± 182.80	187.67 ± 35.30	182.40 ± 41.92	$^{F}p=0.901$
HBA1c (%)	8.05 ± 0.49	9.27 ± 1.50	9.98 ± 1.72	$^{F}p=0.012^{*}$
e GFR	111.83 ± 12.44	88.02 ± 14.37	79.73 ± 13.39	$^{F}p<0.001^{*}$
BUN (mg/dl)	18.86 + 2.82	27.32 ± 7.46	22.0 ± 5.01	$^{F}p=0.002^{*}$
Serum creatinine (mg/dl)	0.87 ± 0.16	1.14 ± 0.25	1.01 ± 0.28	$^{F}p=0.044^{*}$
Serum cystatin C (mg/dl)	0.97 ± 0.17	0.99 ± 0.26	2.79 ± 0.35	$^{F}p<0.001^{*}$
Urine cystatin C (mg/dl)	0.08 (0.01–0.31)	0.88 (0.33–1.47)	2.29 (1.19–2.73)	$^{KW}p<0.001^{*}$

Group I : Normo albuminemic **Group II:** Microalbuminemic **Group III:** Macroalbuminemic

Table II: Comparison between the studied groups according serum cystatin C (mg/L):

	Group I (n=10)	Group II (n=15)	Group III (n=15)	Group IV (n=10)	p
Serum cystatin C (mg/L)					
Min. – Max.	0.65 – 1.17	0.66 -1.43	2.04 – 3.23	0.51 – 0.90	
Mean ± SD.	0.97 ± 0.17	0.99 ± 0.26	2.79 ± 0.35	0.66 ± 0.14	<0.001*
Median	0.98	1.05	2.85	0.66	
p_1	0.093	0.034*	<0.001*		
p_2	<0.001*	<0.001*			
p_3	0.997				

Group I: Normo albuminemic **Group II:** Micro albuminemic **Group III:** Macro albuminemic **Group IV:** Control

p: p value for Kruskal Wallis test for comparing between the different studied groups

p_1 : p value for Mann Whitney test for comparing between group IV and each other groups

p_2 : p value for Mann Whitney test for comparing between group III and each other groups

p_2 : p value for Mann Whitney test for comparing between group II and group I

*: Statistically significant at p ≤ 0.05

Table III: Comparison between the studied groups according to urinary cystatin C:

	Group I (n=10)	Group II (n=15)	Group III (n=15)	Control (n=10)	p
Urine cystatin C (mg/dl)					
Min. – Max.	0.01 – 0.31	0.33 – 1.47	1.19 – 2.73	0.01 – 0.09	
Mean ± SD.	0.09 ± 0.08	0.82 ± 0.37	2.17 ± 0.50	0.05 ± 0.03	<0.001*
Median	0.08	0.88	2.29	0.5	
p_1	0.068	<0.001*	<0.001*		
p_2	<0.001*	<0.001*			
p_3	<0.001*				

Table (IV): Correlation between Serum cystatin C with age, ACR, BMI, HbA1C, eGFR, BUN and serum creatinine

	Group I (n=10)		Group II (n=15)		Group III (n=15)		Group IV (n=10)	
	r	p	r	p	r	p	r	P
Age	0.340	0.336	0.527	0.063	0.238	0.394	0.160	0.658
BMI	0.345	0.328	0.612	0.150	-0.299	0.278	0.240	0.505
ACR	0.125	0.731	0.958*	<0.001	0.818*	<0.001	0.505	0.136
HbA 1C	-0.004	0.992	0.181	0.520	0.823*	<0.001	0.101	0.979
eGFR	0.156	0.666	-0.593*	0.020	0.562*	0.029	-0.639	0.547
BUN	0.378	0.281	0.333	0.225	0.365	0.181	0.241	0.503
Serum Creatinine	-0.125	0.730	0.774*	0.001	0.572*	0.026	0.546	0.103
Urinary cystatin C	-0.550	0.099	0.858*	<0.001	0.922*	<0.001	0.159	0.662

r: Pearson coefficient
***:** Statistically significant at $p \leq 0.05$

Table (V): Correlation between urinary cystatin C with age, ACR, BMI, HbA1C, eGFR, BUN and serum creatinine

	Group I (n=10)		Group II (n=15)		Group III (n=15)		Group IV (n=10)	
	r	p	r	p	r	p	r	P
Age	0.074	0.839	0.291	0.293	0.143	0.610	-0.174	0.631
ACR	-0.149	0.681	0.861*	<0.001	0.831*	<0.001	-0.391	0.264
BMI	-0.365	0.300	0.373	0.171	-0.078	0.784	0.051	0.888
HbA 1C	0.169	0.640	0.043	0.878	0.849*	<0.001	0.061	0.867
eGFR	-0.188	0.603	-0.502*	0.037	-0.549*	0.034	0.150	0.680
BUN	-0.447	0.195	0.539	0.380	0.354	0.196	0.625	0.530
Serum Creatinine	0.227	0.528	0.742*	0.002	0.653*	0.008	-0.263	0.463

r: Pearson coefficient
***:** Statistically significant at $p \leq 0.05$

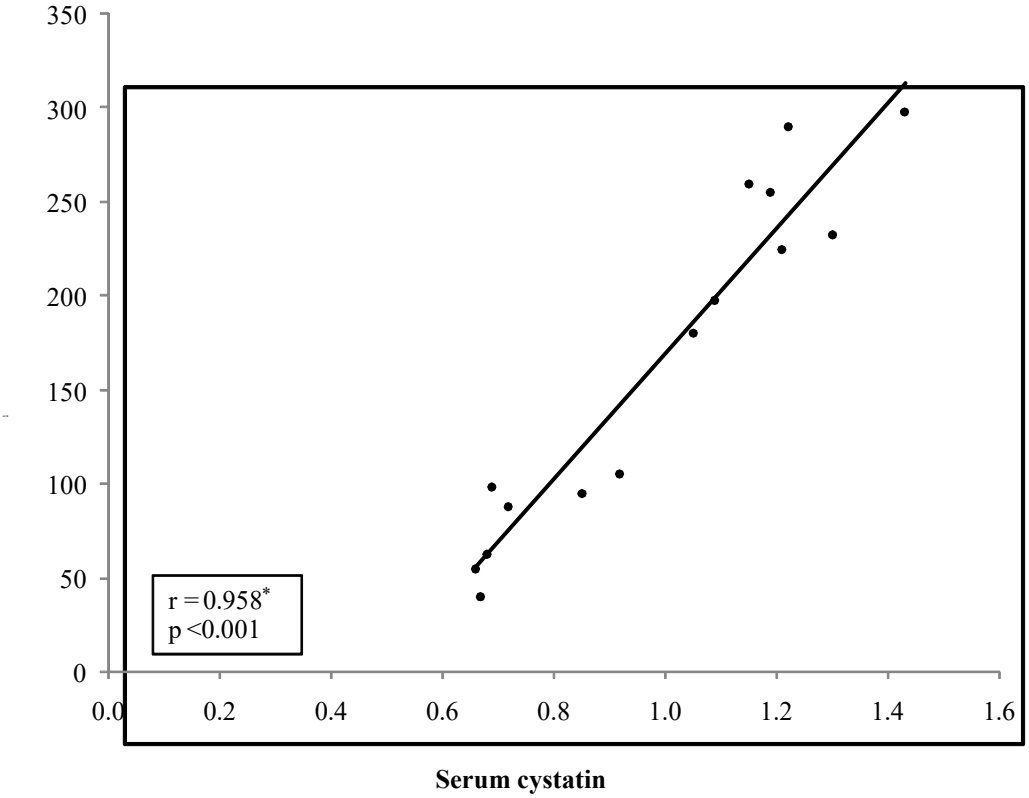

Figure 1: Correlation between Serum cystatin with ACR in group II

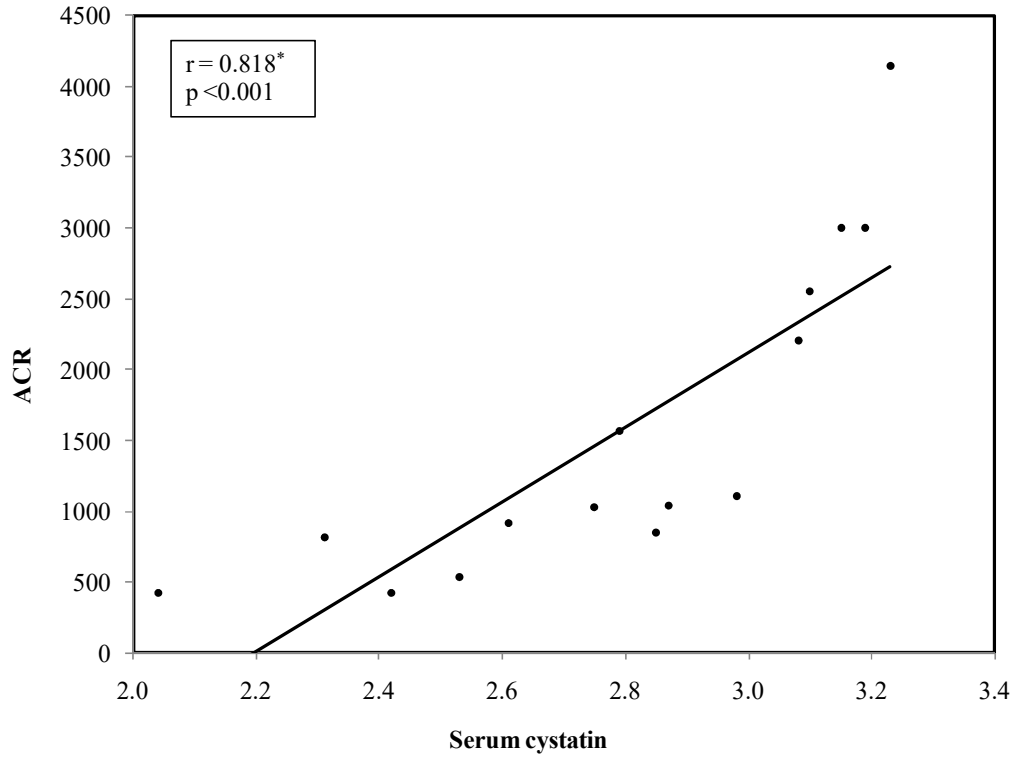

Figure 2: Correlation between urine cystatin with ACR in Group III

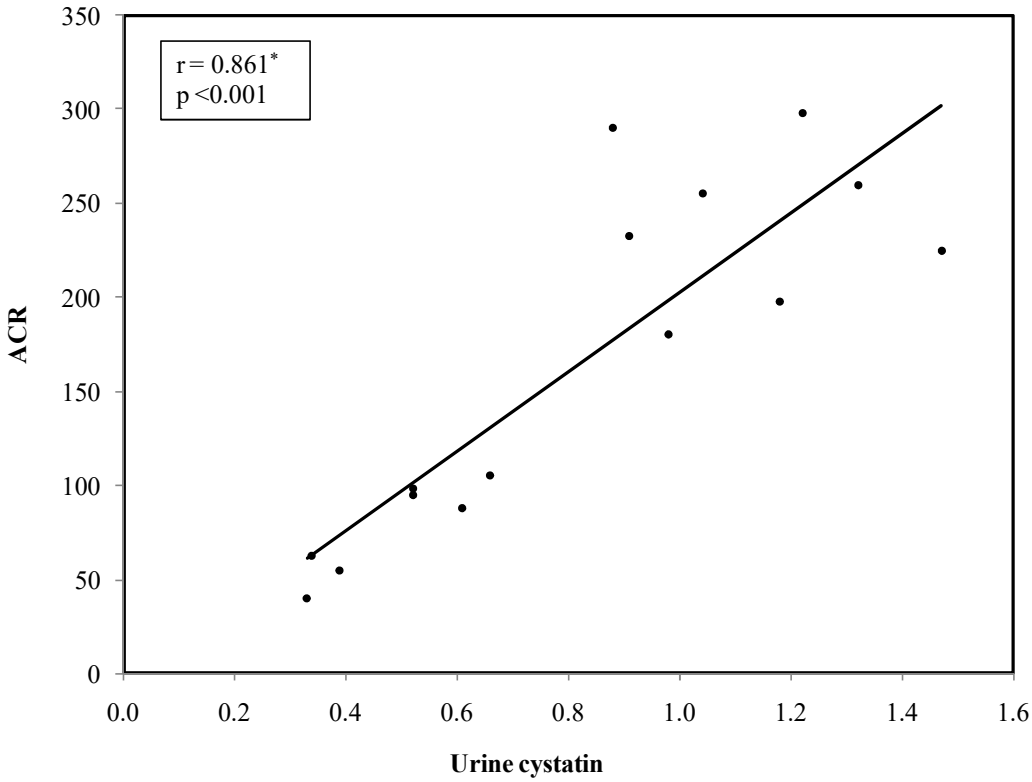

Figure (3): Correlation between urine cystatin with ACR in group II

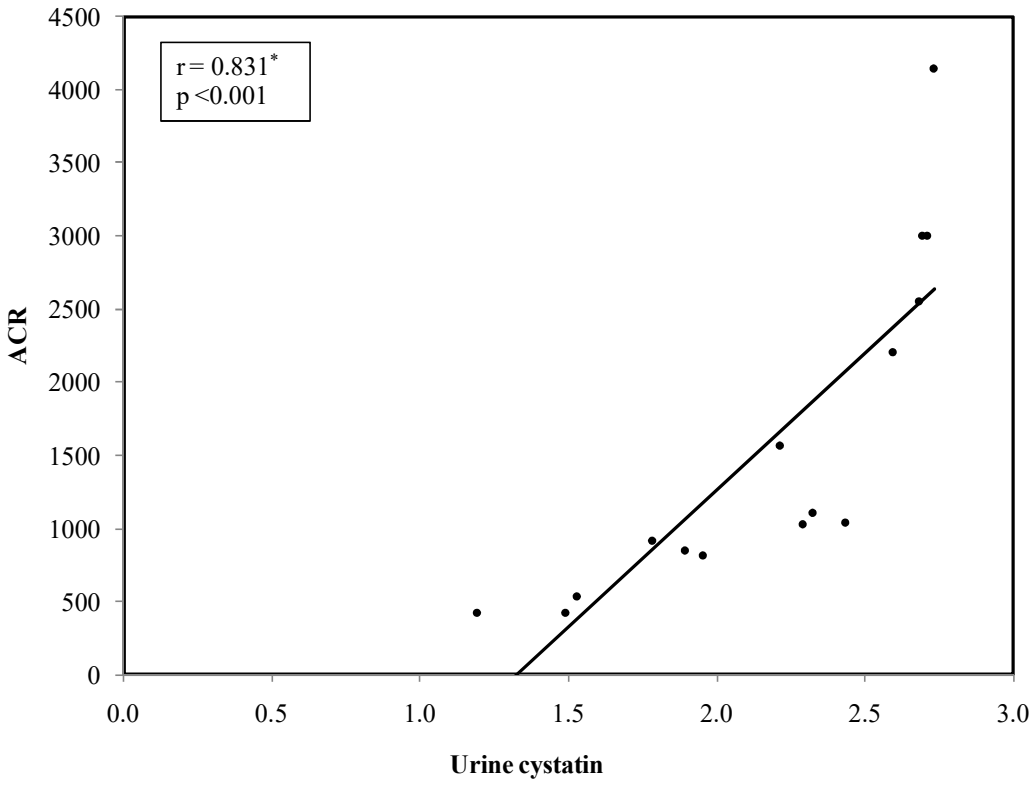

Figure 4 : Correlation between uArine cystatin with ACR in group group III

Discussion:

Data presented in our study dealt with 40 type 1 diabetic patients as cases and 10 healthy individual as controls. There was statistical significant difference between the patients groups and the control groups regarding to age (P < 0.001). The groups of micro and macroalbuminuria were older than the others groups their mean age were (23.7 ± 5.9 and of 27.8 ± 5.2 years respectively). Also the diabetes duration was longer in micro and macroalbuminuric groups (mean 8.53 ± 3.54 years and 11.40 ± 2.90 respectively) than in normoalbuminuric groups (mean 3.69± 1.90 years). This data was confirmed by the fact that DKD takes years to develop in type 1 diabetic patients. It is rare to see DN earlier than 3 years after the diagnosis of diabetes; it is usually seen after 5 to 15 years in patients with type 1 diabetes. (27)

In our study, there was no significant correlation between serum, urinary cystatin C and the age in the control group and the patients groups. This data matched with Filler et al (28) who found that measuring of cystatin C not affected by the age. Also Uchida K et al(29) found that measuring of urinary cystatin C was not affected by the age. In contrast, Christensson et al (30) reported that there was significant positive correlation noticed between serum cystatin C and age.

In our study all patients and healthy controls were of male sex. Regarding to measuring cystatin C, there was conflicting evidence regarding whether cystatin C levels vary by gender or not. Some authors like Croda-Todd et al (31) and Finney H et al (32) found that there was statistically significant differences as regarding measuring cystatin C in adults males and females and was higher in male gender than female. In contrast, Erlandsen JE et al (33) and Uhlmann EJ et al (34) reported that there was no significant difference as regarding measuring cystatin C in both gender.

Regarding BMI, in our study, there was no statistically significant difference between the patients groups and the control group (P=0.807) and there was no statistical significance correlation between serum and urinary cystatin C with BMI. The same result was reported by Galteau et al (35) who have reported a moderate but biologically insignificant correlation between BMI and cystatin C. In contrast, Al Wakeel JS et al (36) and Muntner P et al (37) have reported a significant correlation between serum cystatin C and BMI.

Regarding HbA1c, in our study, there was a statistically significance difference between the patients groups regarding HbA1c (P=0.012) and the mean levels were higher in macroalbuminuric group (mean=9.98± 1.72). There was positive significance correlation between ACR and HbA1c in the macroalbuminuric groups (P=0.002).

In our study there was statistically significance positive correlation between serum and urinary cystatin C with HbA1c only in macroalbuminuric group (P <0.001). The same result was reported by Jeon YK, et al. (17) as cystatin C as a marker of DN is also related to increase level of HbA1c and poor glycemic control. In contrast, Tian et al(38) observed there was non-significant correlation between cystatin C and glucose level indicating that serum cystatin C levels are independent of blood glucose level.

In our study, there was no significance correlation between BUN and serum and urinary cystatin C but there was statistical significance positive correlation between serum Creatinine and serum and urinary cystatin C in the groups of micro and macroalbuminuric groups. The same result was reported by Tian et al(38) there was significant positive correlation between serum cystatin C and each of serum creatinine suggesting that serum cystatin C increased similar to serum creatinine for measuring renal function in diabetic patients.

In our study, there was a significant negative correlation between eGFR with serum cystatin C only in the microalbuminuric (P=0.020) and macroalbuminuric groups (P=0.029) not with normoalbuminuric group (P=0.666). As cystatin C is produced at a constant rate by nucleated cells and released into the blood stream with a half-life of ~2 h and is freely filtered and almost completely taken up and degraded, but not secreted, by proximal tubular cells, so with the occurrence of pathological changes in diabetic

kidney the filtration capacity is decreased with subsequent retention of cystatin C and increased serum level.

Some previous studies on the role of cystatin C in detecting early renal failure in diabetic patients were contradictory. Some authors showed that cystatin C was more effective than creatinine in detecting initial reduction of GFR in T2DM as well as in T1DM. Mussap et al[39], Xia et al[40], and Harmoinen et al[41] showed that serum cystatin C was more sensitive than serum creatinine for estimation of GFR in T2DM patients and Tan et al[42] showed the same in T1DM patients. In contrast to these result, Oddoze et al [43] found that serum creatinine was better than serum cystatin C for the estimation of GFR in microalbuminuric and proteinuric diabetic patients. Oddoze et al selected a heterogeneous group of type 1 and type 2 diabetic patients.

In our study, there was no correlation between urinary cystatin C and eGFR in normoalbuminuric (P=0.603) group but there was negative significance correlation between urinary cystatin C and eGFR in microalbuminuric (P=0.037) and macroalbuminuric (P=0.034) groups.

Sang Soo et al[44] reported that urinary cystatin C was not associated with a decline in eGFR in normoalbuminuric patients. It is necessary to assess tubular damage independent of albuminuria in patients with early development and progression of DN because tubular damage may play a significant role in the normoalbuminuric renal insufficiency. Stefan et al [45] reported that increased urinary cystatin C reflects structural and functional renal tubular impairment independent of GFR.

The mean value of the cystatin C was significantly higher in the macroalbuminuric group compared to normoalbuminuric group, and it also significantly higher in the macroalbuminuric(P<0.001) group compared to microalbuminuric (P<0.001) group, In agreement with these results, Mojiminiyi et al [46] found that cystatin C was significantly higher in patients with DN than in normoalbuminuric diabetic patients, which can be explained in that there is already impaired renal function in DN patients. In addition, Yang et al[47] reported that serum cystatin C concentration increased significantly in patients from normo- to macro- and micro- to macroalbuminuria.

In our study, there was strong positive correlation between serum cystatin C and ACR only in the microalbuminuric group (P<0.001) and macroalbuminuric group (P<0.001) and there was no correlation in the normoalbuminuric group (P=0.731).

The strong correlation between serum cystatin C in the micro and macroalbuminuric groups may be due to decrease eGFR in these groups in comparing to normoalbuminuric group.

In contrast to our study Jeon YK et al[17] found that in normoalbuminuric patients, the cystatin C levels of serum and urine were significantly increased in patients with GFR ≤ 60 mL/min/1.73 m2 than those with GFR > 60 mL/min/1.73 m2. It was thought that this increment was probably due to the tubular phase before glomerular manifestation. He suggested that the cystatin C levels of serum and urine are related to subclinical tubular impairment and can be an earlier measurable markers of renal involvement before onset of albuminuria.

The level of urinary cystatin C was higher in groups of microalbuminuric (P <0.001) macroalbuminuric group (P<0.001) in comparing to normaalbuminuric group and control group. There was strong positive correlation between the urinary cystatin C and ACR in microalbuminuric group (P <0.001) and macroalbuminuric group (P<0.001) and there was no correlation in the normoalbuminuric group (P=0.681).

From these result, urinary cystatin c can use parallel to microalbuminuria to diagnosis of DN and for follow up the progression of the disease. The same result was reported by Kim SS et al [48] who reported that several tubular markers as urinary cystatin C increase more in diabetic patients thanin healthy controls, and this correlated with the severity of albuminuria. Also Sang SK et al[44] reported that urinary cystatin C mainly increased in the macroalbuminuria group

and was not significantly different between the microalbuminuria and normoalbuminuria groups.

In our study, there was no correlation between serum and urinary cystatin C in control group but there was a statistically positive correlation between serum and urinary cystatin C in the micro and macroalbuminuric groups. As in the micro and macralbuminuric group the eGFR were relative lower than the others group so the serum cystatin C increased and also urinary cystatin C secondary to decrease the reabsorption capacity of renal tubules. The same result reported by Jeon YK, et al. [17] In contrast to our result, Sang SK et al [44] reported that urinary cystatin C was a predictor of renal impairment independent of serum cystatin C. Although serum cystatin C itself, an indicator for the eGFR, is very important for predicting renal dysfunction.

Conclusion:

The present study supports the importance of cystatin C measuring in serum and urine as a marker of diabetic nephropathy and its correlation with albuminuria in type 1 diabetic patients.

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Original Article

Role of Serum Retinol Binding Protein-4 and Insulin Resistance as Cardiovascular Risk Factors in Non Diabetic Post-Menopausal Women.

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Abstract:

Background: Retinol binding protein-4 (RBP-4) is a newly discovered adipokine, which is mainly secreted by liver and originally known to be the only specific transport protein for vitamin A in the circulation. Recently, RBP-4 is found to be expressed in adipose tissue and correlated with obesity, insulin resistance (IR) and type 2 diabetes mellitus (T2DM). **Objective:** The aim of the present work was to study the role of serum RBP-4 concentration and IR as cardiovascular risk factors in non diabetic post-menopausal women. **Subjects and Methods:** The present study included 25 apparently healthy premenopausal women (group I), 25 apparently healthy postmenopausal women (group II) and 25 postmenopausal women with cardiovascular diseases (CVD) (group III). Informed consents were obtained from all females. The following were done for all participants: history taking, complete physical examination, laboratory tests including lipid profile, fasting (FG)and postprandial plasma glucose(PPG) level, fasting plasma insulin , assessment of IR by homeostasis model assessment (HOMA score) and determination of plasma RBP-4. **Results:** Data showed that, the mean fasting plasma insulin levels and mean HOMA-IR in postmenopausal women with CVD were statistically significantly higher than those without CVD ($p_2 < 0.05$). There was a significantly higher mean level of RBP-4 in postmenopausal without CVD than in the premenopausal women ($p_1 < 0.05$). In addition, the mean value of RBP-4 levels of postmenopausal women with CVD was statistically significantly higher than those without CVD ($p_2 < 0.05$). Plasma RBP-4 was positively correlated with the age, serum total cholesterol (TC), serum triglycerides (TG), serum TG/HDL-C ratio, plasma (FG), plasma (PPG), plasma insulin and (HOMA-IR) in postmenopausal women with CVD. **Conclusions:** RBP-4 is significantly elevated in postmenopausal women with CVD as compared to postmenopausal women without CVD. RBP-4 is positively correlated with lipids and HOMA-IR in postmenopausal women with CVD. It affects glucose and lipid homeostasis and contributes to the onset of IR which may play a role in the development of CVD. RBP-4 might serve as a novel biomarker of CVD.

Keywords: Retinol binding protein-4, insulin resistance, cardiovascular disease, Postmenopausal women.

Introduction:

Cardiovascular disease is a class of diseases that involve the heart or blood vessels (arteries and veins), principally cardiac disease, vascular diseases of the brain and kidney, and peripheral arterial disease. The causes of cardiovascular disease are diverse but atherosclerosis and/or hypertension are the most common.(1) Cardiovascular diseases (CVD) are a principal cause of death worldwide and are linked to obesity and metabolic syndrome. Several adipokines secreted by the increased adipose tissue mass, together with the infiltrating macrophages, have been identified as key components of the 'adipo-cardiovascular axis' and are main contributors to the pathogenesis of atherosclerosis and other cardiovascular diseases. Among these, RBP4 has been identified as an adipokine associated with obesity, type 2 diabetes (T2DM) and metabolic syndrome. (1)

Retinol binding protein-4 (RBP-4) is a newly discovered adipokine, which is mainly secreted by the liver and excreted by the kidneys. It is the sole carrier of retinol (vitamin A) in blood, and serves to transport it from liver stores to the peripheral tissues. Circulating RBP4 levels have been shown to rise and positively correlated with body mass index (BMI) (2,3) and

to be associated with insulin resistance (IR).[2-5] Growing evidence suggests that RBP4 plays a role in lipid metabolism to an even greater extent than insulin resistance. In fact, many human studies have found a strong relationship between RBP4 and triglycerides; some have found associations with insulin resistance [3, 6] and others have failed to do so. [7-9]

Menopause is defined as the permanent cessation of menses as a consequence of the loss of ovarian follicular function or of surgical removal of ovaries. During this period, many psychological, physiological, and pathological modifications occur; in particular cardiovascular disease (CVD). It is well known that there is a high prevalence of cardiovascular risk factors and metabolic syndrome (MS) in postmenopausal women. Postmenopausal status is believed to be a risk factor for IR in women. IR has a causal role in the development of T2DM. Even in the absence of hyperglycemia or diabetes, IR contributes to an increased risk of CVD. [1, 10]

The aim of the present work was to study the role of serum RBP-4 and IR as CVD risk factors in non-diabetic post-menopausal women.

Subjects and Methods:

The present study included 25 apparently healthy premenopausal women (group I), 25 apparently healthy postmenopausal women (group II) (both groups were chosen from the staff members of MRI), and 25 postmenopausal women with CVD (group III) (recruited from the Internal Medicine Department, Cardiology unit MRI). All the subjects were matched as regards body mass index. Informed consents were obtained from all females. Exclusion criteria: patients with impaired kidney function, liver cirrhosis, diabetes mellitus and hypertension.

The following were done for all participants: history taking with special stress on family history of diabetes mellitus and any drug intake, complete physical examination including body mass index (BMI) which was calculated as body weight (kg) divided by body height squared (m^2), Waist to hip ratio (WHR) and blood pressure measurement and laboratory investigation including determination of serum total cholesterol (TC), high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol, Triglycerides (TG), calculated TG/HDL-C ratio, fasting and postprandial plasma glucose level, and fasting plasma insulin by chemiluminescence method.[11] Assessment of IR was done using homeostasis model assessment HOMA score (HOMA-IR = fasting plasma insulin (mU/ml) × fasting plasma glucose (mmol/l)/22.5.[12] Plasma creatinine was determined[11] and the estimated glomerular filtration rate (eGFR) was calculated using Cockcroft Gault equation:

eGFR = (140-age) × body weight (kg) × 0.85 if female / 72 × serum creatinine (mg/dl). [13] Liver enzymes including serum aspartate aminotransferase (AST) and serum alanine aminotransferase (ALT)[11] were determined. Plasma RBP-4 was estimated by ELISA method using commercially available kit.[14] Special studies included; echocardiography and ECG for suspected cases with ischemic heart diseases (IHD), or Doppler on carotid arteries and CT brain for suspected cases with cerebrovascular strokes (CVS).

Statistical analysis:

Statistical analysis was performed with the SPSS version 19 software package. Statistical significance was defined as a 2-sided P-value of ≤0.05.The comparison between the studied groups was done using Mann-Whitney and t-test. Correlation analysis was done using Pearson coefficient and Spearman coefficient tests.

Results:

Out of the 25 postmenopausal women with CVD, 16 cases were diagnosed as IHD and 9 cases were diagnosed as CVS. As regard BMI, there was no statistical significant difference between the three studied groups (p_1&p_2 > 0.05). Table I

Data showed that, there were statistical significant differences in the mean TC and mean TG between group I&II (p_1 < 0.05). In

contrast, there were no statistical significant difference between group I&II as regard mean HDL-C, mean LDL-C levels and TG/HDL-C ratio ($p_1 > 0.05$). It was also observed that the mean TC, mean LDL-C, mean TG levels and TG/HDL-C ratio were statistically significantly higher in group III than group II ($p_2 < 0.05$), while there was no statistical significant difference between the two groups in the mean HDL-C ($p_2 > 0.05$).(Table II)

There were no statistical significant difference in the mean plasma FG, mean plasma post prandial glucose, mean insulin levels and mean HOMA-IR between group I&II ($p_1 > 0.05$). On the other hand, the mean plasma insulin levels and mean HOMA-IR in group III were statistically significantly higher than in group II ($p_2 < 0.05$). (Table III)

There was a statistical significant difference in the mean serum RBP-4 between groupI&II ($p_1 < 0.05$). In addition, the mean serum RBP-4 was statistically significantly higher in group III than in group II ($p_2 < 0.05$). (Table IV)

Correlation analysis of the plasma RBP-4 levels with clinical and biochemical parameters in premenopausal women and postmenopausal women with and without CVD revealed that; plasma RBP-4 was positively correlated with serum total cholesterol (TC) ($r = 0.409$, $p = 0.042$) and post prandial glucose ($r = 0.412$, $p = 0.041$) in postmenopausal women without CVD. Also it positively correlated with the age ($r = 0.540$, $p = 0.005$), serum total cholesterol (TC) ($r = 0.442$, $p = 0.027$), serum triglycerides (TG) ($r = 0.654$, $p < 0.001$),serum triglycerides / high density lipoprotein-cholesterol ratio ($r = 0.627$, $p = 0.001$), fasting plasma glucose (FG) ($r = 0.475$, $p = 0.017$), plasma post prandial glucose ($r = 0.404$, $p = 0.045$), plasma insulin ($r_s = 0.431$, $p = 0.032$), and homeostasis model assessment insulin resistance level (HOMA-IR) ($r_s = 0.455$, $p = 0.022$) in postmenopausal women with CVD. While there were no correlation between the plasma RBP-4 levels with clinical and biochemical parameters in premenopausal women. (Table V)

Table I: The age (year) and body mass index (kg/m^2), of the three studied groups

	Premenopausal women (gp1) (n = 25)	**Postmenopausal women without cardiovascular disease(gpII) (n = 25)**	**Postmenopausal women with cardiovascular diseases(gpIII) (n = 25)**
Age (year)			
Range	27.0 – 52.0	47.0 – 59.0	52.0 – 66.0
Mean ± SD	37.60 ± 7.65	52.56 ± 3.28	59.52 ± 4.72
p_1		$p_1 < 0.001^*$	
p_2			$p_2 < 0.001^\#$
BMI (kg/m^2)			
Range	20.69 – 36.79	25.30 – 35.38	24.03 – 40.31
Mean ± SD	29.13 ± 4.48	30.03 ± 2.79	32.48 ± 3.44
p_1		$p_1 = 0.684$	
p_2			$p_2 = 0.064$

p_1 : p value compared postmenopausal women without CVD to premenopausal women.

p_2 : p value compared postmenopausal women with and without CVD.

* : Significant difference between postmenopausal without CVD and premenopausal groups.

\# : Significant difference between postmenopausal groups.

Significance was considered at the level of $p < 0.05$.

Table II: Serum total cholesterol (TC), High density lipoprotein-cholesterol (HDL-C), triglyceride (TG) and TG/HDL-C ratio of the three studied groups.

	Premenopausal women (gpI) (n = 25)	**Postmenopausal women without cardiovascular diseases(gpII) (n = 25)**	**Postmenopausal women with cardiovascular diseases (gpIII) (n = 25)**
TC (mg/dl)			
Range	132.0 – 229.0	124.0 – 276.0	188.0 – 321.0
Mean ± SD	181.96 ± 26.36	204.20 ± 30.42	227.80 ± 28.97
p_1		$p_1 = 0.028^*$	
p_2			$p_2 = 0.018^\#$
HDL-C (mg/dl)			
Range	34.0 – 74.0	31.0 – 77.0	31.0 – 85.0
Mean ± SD	52.36 ± 9.92	57.56 ± 11.39	50.58 ± 11.74
p_1		$p_1 = 0.256$	
p_2			$p_2 = 0.089$
LDL-C (mg/dl)			
Range	66.6 – 156.8	51.0 – 192.8	97.2 – 213.6
Mean ± SD	114.36 ± 24.81	122.82 ± 30.31	143.18 ± 30.05
p_1		$p_1 = 0.579$	
p_2			$p_2 = 0.047^\#$
TG (mg/dl)			
Range	48.0 – 128.0	60.0 – 291.0	85.0 – 287.0
Mean ± SD	76.24 ± 23.77	119.12 ± 53.76	170.20 ± 46.89
p_1		$p_1 = 0.004^*$	
p_2			$p_2 < 0.001^\#$
TG/HDL-C Ratio			
Range	0.65 – 3.15	0.78 – 8.08	1.29 – 8.39
Mean ± SD	1.54 ± 0.69	2.35 ± 1.78	3.58 ± 1.46
p_1		$p_1 = 0.133$	
p_2			$p_2 = 0.010^\#$

p_1 : p value compared postmenopausal women without CVD to premenopausal women.

p_2 : p value compared postmenopausal women with and without CVD.

* : Significant difference between postmenopausal without CVD and premenopausal groups.

\# : Significant difference between postmenopausal groups.

Significance was considered at the level of $p < 0.05$.

Table III: Fasting plasma glucose (mg/dl), post prandial glucose (mg/dl),fasting insulin (µIU/ml) and homeostasis model assessment- insulin resistance in the three studied groups.

	Premenopausal women (gpI) (n = 25)	**Postmenopausal women without cardiovascular diseases (gpII) (n = 25)**	**Postmenopausal women with cardiovascular diseases (gpIII) (n = 25)**
FG (mg/dl)			
Range	61.0 – 101.0	77.0 – 99.0	78.0 – 118.0
Mean ± SD	84.92 ± 9.69	91.32 ± 6.01	98.0 ± 12.58
p_1		$p_1 = 0.076$	
p_2			$p_2 = 0.061$
Post prandial glucose (mg/dl)			
Range	70.0 – 117.0	78.0 – 139.0	77.0 – 131.0
Mean ± SD	97.72 ± 10.33	104.28 ± 16.65	105.28 ± 12.56
p_1		$p_1 = 0.232$	
p_2			$p_2 = 0.966$
Insulin (µIU/ml)			
Range	2.10 – 9.40	2.05 – 18.80	3.0 – 22.60
Mean ± SD	4.52 ± 2.21	5.54 ± 4.04	7.54 ± 4.40
p_1		^{MW} $p_1 = 0.587$	
p_2			^{MW} $p_2 = 0.012^{\#}$
HOMA-IR			
Range	0.49 – 2.02	0.42 – 4.55	0.58 – 6.42
Mean ± SD	0.94 ± 0.49	1.27 ± 0.97	1.90 ± 1.33
p_1		^{MW} $p_1 = 0.265$	
p_2			^{MW} $p_2 = 0.007^{\#}$

p_1 : p value compared postmenopausal women without CVD to premenopausal women.
p_2 : p value compared postmenopausal women with and without CVD.
* : Significant difference between postmenopausal without CVD and premenopausal groups.
\# : Significant difference between postmenopausal groups.
Significance was considered at the level of $p < 0.05$. MW *:* Mann-Whitney test.

Table IV: Serum retinol binding protein–4 (mg/L) in the three studied groups

	Premenopausal women (gpI) (n = 25)	**Postmenopausal women without cardiovascular diseases(gpII) (n = 25)**	**Postmenopausal women with cardiovascular diseases (gpIII) (n = 25)**
RBP-4 (mg/L)			
Range	29.61 – 68.71	53.33 – 91.79	62.05 – 120.00
Mean ± SD	54.28 ± 10.36	70.06 ± 11.81	90.09 ± 13.02
p_1		$p_1 < 0.001^{*}$	
p_2			$p_2 < 0.001^{\#}$

p_1 : p value compared postmenopausal women without CVD to premenopausal women.
p_2 : p value compared postmenopausal women with and without CVD.
* : Significant difference between postmenopausal without CVD and premenopausal groups.
\# : Significant difference between postmenopausal groups.
Significance was considered at the level of $p < 0.05$.

Table V: Correlation of plasma RBP-4 levels with clinical and biochemical parameters in the three studied groups.

	Group I		Group II		Group III	
	Coeff.	**P**	**Coeff.**	**P**	**Coeff.**	**P**
Age	r = -0.072	0.733	r = -0.143	0.496	r = 0.540**	***0.005#***
BMI	r = -0.077	0.715	r = 0.007	0.974	r = 0.117	0.576
TC	r = -0.052	0.806	r = 0.409*	***0.042#***	r = 0.442*	***0.027#***
HDL-C	r = -0.340	0.096	r = -0.107	0.610	r = -0.211	0.312
LDL-C	r = 0.043	0.839	r = 0.380	0.061	r = 0.305	0.139
TG	r = 0.200	0.338	r = 0.200	0.339	r = 0.654**	***<0.001#***
TG/HDL-C ratio	r = 0.271	0.189	r = 0.212	0.309	r =0.627**	***0.001#***
FG	r = -0.001	0.995	r = 0.174	0.406	r = 0.475*	***0.017#***
Post prandial glucose	r = -0.240	0.249	r = 0.412*	***0.041#***	r = 0.404*	***0.045#***
Insulin	r_s = -0.144	0.588	r_s = 0.162	0.440	r_s = 0.431*	***0.032#***
HOMA-IR	r_s = -0.127	0.544	r_s = 0.157	0.453	r_s = 0.455*	***0.022#***

r :Pearson coefficient

rs :Spearman coefficient

** :Correlation is significant at the 0.01 level (2-tailed).

* :Correlation is significant at the 0.05 level (2-tailed).

:Statistically significant at $p \leq 0.05$

Discussion:

RBP4 has long-been known to be released by the liver ,but recently, it has been shown that approximately 15% of circulating RBP4 levels results from adipose tissue secretion.[15] RBP4 down-regulates GLUT4 ,[2] the insulin-activated glucose transporter responsible for translocation of glucose into both muscle and fat cells , and has also recently been shown to induce expression and secretion of pro-inflammatory cytokines in primary human macrophages known to induce insulin resistance.[16] In humans, circulating RBP4 levels were found to be highly negatively correlated with levels of insulin sensitivity, and to be increased with obesity and in those with type 2 diabetes.[3,17,18] However, discrepant results from other studies have questioned these associations, failing to demonstrate associations with either obesity or indicators of glucose homeostasis.[4,19,20]

The aim of the present work was to study the role of serum RBP-4 concentration and IR as CVD risk factors in non diabetic post-menopausal women. The present study included 25 apparently healthy premenopausal women (group I), 25 apparently healthy postmenopausal women (group II) and 25 postmenopausal women with CVD (group III).

The present data revealed that RBP-4 concentration in postmenopausal women without CVD was higher than those in premenopausal women. This result agreed with that reported by Suh JB[21] suggesting that menopausal status might be a major determinant of plasma RBP-4 concentrations. As women reach menopause, estrogen decreases. As such, fat amount or body fat percentage change; and visceral fat increase. As a result, lipid metabolism becomes dysregulated. This change of lipid metabolism may affect plasma RBP-4 concentrations that come from adipocytes. [22]

Accumulating data strongly support the association of RBP4 circulating levels with traditional cardiovascular risk factors (e.g. dyslipidemia, hypertension, albuminuria) and non-traditional cardiovascular risk factors (e.g. cytokines) mainly through the impairment of glucose and lipid metabolism and adipose tissue dysfunction, despite that opposite findings put RBP4 changes in dispute.[23] Although, the involvement of RBP4 in the development of subclinical atherosclerosis has been proven,[24] its prognostic value in carotid or coronary atherosclerosis progression is still obscure. [25]

The present work showed that RBP-4 concentration in postmenopausal women with CVD was higher than those without CVD. In agreement with our work, Pala et al [26] suggested that, for the first time, a major effect of visfatin, adipocyte fatty acid binding protein (aFABP), and RBP4 in the development of cardiovascular disease in their work. Also Lambadiari et al [27] concluded that patients with CAD showed elevated RBP4 serum levels. Notably, increased RBP4 concentration seemed to independently correlate with CAD severity. And regarding the underlying mechanisms, they observed the independent correlation of RBP4 with insulin resistance indices and established markers of inflammation, like CRP. RBP4 levels independently predicted early endothelial dysfunction, linking adipose tissue, inflammation and subclinical atherosclerosis in non diabetic individuals. [28]

Insulin resistance is the main pathologic mechanism that links the constellation of clinical, metabolic and anthropometric traits with increased risk for cardiovascular disease and type II diabetes mellitus. These traits include hyperinsulinemia, impaired glucose intolerance, endothelial dysfunction, dyslipidemia, hypertension, and generalized and upper body fat redistribution. This cluster is often referred to as insulin resistance syndrome. The progression of insulin resistance to diabetes mellitus parallels the progression of endothelial dysfunction to atherosclerosis leading to cardiovascular disease and its complications. In addition, insulin resistance assessed by HOMA has been shown to be independently predictive of CVD in several studies and a one unit increase in insulin resistance is associated with a 5.4% increase in CVD risk.[29&30] The San Antonio Heart Study (SAHS)[30] demonstrated that insulin resistance assessed by HOMA was significantly and independently associated with risk of CVD outcomes among Mexican-American and non-Hispanic white men and women. Logistic regression analysis in SAHS indicated that the risk of a cardiovascular event increased across quintiles of HOMA-assessed insulin resistance after the adjustment for age, sex and ethnicity. [30]

In agreement with the previous reports,[29&30] our data also revealed that the mean plasma insulin levels and mean HOMA-IR in postmenopausal women with CVD were statistically significantly higher than in postmenopausal women without CVD.

Also fasting glucose positively correlated with serum RBP-4 concentrations in postmenopausal women with CVD. The association between plasma RBP-4 concentrations and fasting glucose may be explained by the mechanism through which RBP-4 develops IR in liver. Retinol binding protein-4 induces the expression of the gluconeogenic enzyme PEPCK in the liver.[2] The present results could be due to this mechanism.

In addition, RBP-4 correlate positively with postprandial glucose in the postmenopausal women with CVD. This could be explained by findings of Broch et al [19] who concluded that RBP-4 may impair β cell function in human subjects. As RBP-4 circulates in plasma form a complex with transthyretin, which constitutes a functional component in pancreatic β cell stimulus secretion coupling. Thus it is possible that increased serum RBP-4 prevents transthyretin from exerting its β cell stimulus secretion effects.

Results from the present study also found positive association between RBP-4 and HOMA-IR. A mechanism whereby RBP-4 modulates insulin sensitivity in muscle and liver has been suggested. In skeletal muscle, RBP-4 reduces insulin sensitivity by inhibiting both insulin receptor substrate-1 phosphorylation and phosphatidylinositol 3-kinase activation, while increasing hepatic glucose production by increasing PEPCK expression.[2] Another contributing factor for IR is that RBP-4 down-regulates GLUT4,[2,15] the insulin activated glucose transporter responsible for translocation of glucose into both muscle and fat cells,[15] and has also recently been shown to induce expression and secretion of pro-inflammatory cytokines in primary human macrophages known to induce IR. [16]

Insulin resistance is well established as one of the risk factors for CVD suggesting that RBP-4 might serve as an alternative biomarker of CVD.[31] This need confirmation of some future prospective studies.

Dyslipidemia is a major cause of CVD, which in turn, is the most common cause of female morbidity and mortality.[32] The incidence of CVD increases after menopause due to changes in the plasma lipid and lipoprotein

levels that occur following menopausal transition.[33,34]

In this study it is evident that postmenopausal women had significantly higher concentrations of total cholesterol with respect to premenopausal women ($p<0.001$). These findings are similar to other studies. [35-37] Gorodeski GI and his colleague [38] reported that TC was 19% higher in postmenopausal women compared to premenopausal women, and 1% increase in TC is associated with at least 2% increase in the incidence of CVD. [39] They attributed the elevated concentrations of TC to estrogen deficiency in postmenopausal women.

In our study, postmenopausal women with CVD were having high levels of LDL; this finding is in agreement with other studies. [35,40] Circulating estrogen is a regulator of lipoprotein lipase (LPL), which catalyzes the hydrolysis of very low density lipoprotein (VLDL) to form intermediate density lipoprotein (IDL) and later LDL. After menopause due to estrogen deficiency, there will be increased plasma LPL activity causing increased level of LDL and also leads to down-regulation of LDL receptors.[37,41] The higher the small dense LDL proportion which characterizes the atherogenic shift, the higher is the LDL oxidation,[37] and these particles are associated with a threefold increase in CVD risk. [42]

In the present study when compared to premenopausal women, postmenopausal women showed high TG. This finding is in accordance with that reported by Razay et al. [39] who stated that TG in postmenopausal women was higher by 31% when compared to premenopausal women. The elevated levels of TG observed in postmenopausal women could be interpreted in view of Razay and colleagues,[39] they stated that in the postmenopausal women, the increased fat accumulation, resulting in increased release of free fatty acids into the circulation and excessive free fatty acids provide substrate for hepatic triglyceride and triglyceride rich lipoprotein production.[43]

Furthermore, the ratio between TG and HDL-C was also estimated in post -menopausal women. It was found that TG/HDL-C ratio (atherogenic index) in postmenopausal women with CVD was higher than in the postmenopausal women without CVD. Since increased TG/HDL-C ratio is considered as independent risk factor for CVD,[44, 45] therefore our result may throw light on the possibility of using this ratio in predicting CVD which is more common among postmenopausal women. The importance of TG/HDL-C ratio in CVD risk assessment has been established by previous study. [46]

In the present study, serum RBP-4 correlate positively with TC, TG and TG/HDL-C ratio in postmenopausal women with CVD. Our results are in line with previous findings.[3,47,48] It was suggested that RBP-4 in postmenopausal women with CVD might have a direct role in the progression of lipogenesis by increasing the expression of the gene encoding fatty acid synthesis (FASN) in adipose tissue. [49]

Conclusion:

RBP-4 is significantly elevated in postmenopausal women with CVD as compared to postmenopausal women without CVD. RBP-4 is positively correlated with lipids and HOMA-IR in postmenopausal women with CVD. It affects glucose and lipid homeostasis and contributes to the onset of IR which may play a role in the development of CVD. RBP-4 might serve as a novel biomarker of CVD.

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Original Article

Studying the Effect of Premature Progesterone Rise on Cleavage Stage and Blastocyst Stage Embryos in Cases of Intra Cytoplasmic Sperm Injection.

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Abstract:

Background: This randomized controlled study was performed to investigate the clinical significance of premature elevated progesterone levels in women with good ovarian reserve treated by long agonist (GnRH-a) cycles and ICSI, and its effect on cleavage stage embryos (day 3 embryo transfer) and blastocyst stage embryos (day 5 embryo transfer),and to determine if there are certain cutoff levels for progesterone and progesterone/estradiol ratio on day of hCG (human chorionic gonadotrophin) that would be defined as detrimental for pregnancy. Premature elevated progesterone levels refer to arise in serum progesterone levels on the day of hCG for final oocyte maturation above a threshold level which is arbitrarily defined. **Materials and Methods:** This study will be carried out on 200 infertile females who are candidate for intra- cytoplasmic sperm injection (ICSI) who have ≥ 5 grade A embryos. women recruited from the outpatient clinic at El Shatby Maternity University-Hospital, all cases age < 37 years old, antral Follicular count ≥ 8, Basal FSH < 9 IU/ml and Basal E_2 < 50 pg/ml. Used drugs A GnRH agonist for pituitary down regulation .Gonadotrophins for follicular growth and Human chorionic gonadotrophins for follicular maturation. Cases randomly allocated into 2 equal groups 100 female each, according to the day of embryo transfer (ET), 100 females subjected to embryo transfer on day 3, 100 females will be subjected to embryo transfer on day 5, Serum progesterone (P) and estradiol (E_2) levels will be measured on day of hCG administration, follow up of cases by biochemical pregnancy rate and clinical pregnancy rate. **Aim of the work:** The aim of work studying the effect of premature progesterone rise on cleavage stage and blastocyst stage embryos in cases of intra cytoplasmic sperm injection and the effect of high serum progesterone on embryos quality and blastocyst formation.

Keywords: ICSI, GnRH agonist, progesterone, blastocyst, cleavage embryos, clinical pregnancy rate.

Introduction:

Premature luteinization (PL) remains one of the most controversial topics in reproductive endocrinology. PL is usually defined as subtle premature increases in serum Progesterone (P) concentrations on or before the day of hCG administration.(1)

Gonadotrophin-releasing hormone agonists (GnRH a) and antagonists have been used for pituitary down-regulation during controlled ovarian stimulation (COS) to prevent a premature luteinizing hormone (LH) surge.(2) Despite the use of GnRH analogues, a subtle pre-ovulatory rise in the serum progesterone (P) concentration before the administration of hCG for final oocyte maturation still occurred in 5–30% of COS cycles, this phenomenon has been called premature luteinization (PL).(3)

Assessment of association between progesterone levels (P) and progesterone/ estradiol ratio (P/E2) on the day of human chorionic gonadotrophin (hCG) administration and pregnancy achievement in intra-cytoplasmic sperm injection (ICSI) cycles has been the focus of interest for many years. Several investigators have suggested that (P/E2) ratio more accurately reflects (PL) than a single hormone level, as (P) levels have been found to correlate positively with the number of mature follicles and (E2) levels on hCG day.(4,5)

Several hypotheses have been proposed to explain the possible pathophysiology of PL, such as precocious elevation of follicular LH levels, serum accumulation of hCG or LH from hMG, and increased sensitivity of granulosa cell LH receptors to gonadotrophin.(6) It has been proposed that the term PL is inappropriate

because premature serum P rise occurs when the serum LH concentration is low. Therefore, excess serum progesterone is unlikely to be produced by the luteinization process and is more probably due to accumulation from a large number of follicles.[7]

As early as 1991, concerns were raised that in ovarian stimulation for IVF a preovulatory modest increase in serum P levels is associated with lower pregnancy rates and higher pregnancy loss. Two mechanisms have been proposed: either a poor oocyte quality plus a reduction in their fertilizability or a detrimental effect on endometrial receptivity (ER).[8-12]

Ovarian stimulation, whether using an agonist or antagonist for pituitary suppression, induces more histologically advanced endometrium than in natural cycles. This advancement is about 1–2 days in agonist cycles.[13-15] Exposure of the endometrium to high hormone levels during ovarian stimulation significantly increased PRB (progesterone receptor B) receptor expression at the time of day 3 ET (embryo transfer) in comparison to natural cycle. So, in good responders, it appears that the benefits associated with high steroid levels may be offset by the negative impact of COS on the endometrium or other target tissues, particularly if the embryos are placed in the uterus soon after the steroid hormones peak for oocyte retrieval.[16]

Human embryos obtained through in vitro techniques are routinely transferred to the uterus on day 2 or 3 when they are at the 4-8 cell stage. The implantation rates of these embryos are low.[17] Therefore, the standard treatment is to transfer more than one embryo into the uterus to obtain a resemble pregnancy rate. This results in a high order of multiple pregnancies and twins with increased pregnancy complications such as abortion and premature deliveries.[18]

It has been suggested that transferring embryos at the blastocyst stage instead of selection at an earlier stage without knowing their developmental capability might enhance the implantation rate by a better embryo selection, thereby, reducing the need to transfer more embryo.[19]

Participants:

Participants in this study will be carried out on 200 infertile females who are candidate for intra cytoplasmic sperm injection (ICSI), who have ≥ 5 grade A- embryos The Participants were selected according to inclusion and exclusion criteria. Inclusion criteria were: Women aged < 37 years old, antral Follicular count ≥ 8, Basal FSH < 9 IU/ml and Basal E_2 < 50 pg/ml and exclusion criteria: Poor responders. And women who have <5 grade A embryos.

Intervention:

After full history taking, complete general examination, trans-vaginal sonography, hormonal profile analysis(serum FSH, LH, estradiol & prolactin levels) and an informed consent, 200 Cases who have ≥5 grade A embryos will be randomly allocated into 2 equal groups 100 female each, according to the day of embryo transfer (ET). 100 females subjected to embryo transfer on day 3 and 100 females will be subjected to embryo transfer on day 5. All The included patients will be subjected to the following steps in order: Pituitary down-regulation by wit GnRH agonist starting from Day 21 of the previous cycle, controlled ovarian stimulation (COS) by gonadotrophins will be started once Pituitary down-regulation is confirmed (serum E2 < 50 pg/ml) and ovarian response will be monitored by trans-vaginal ultrasound and serum E_2 level. hCG will be administered when at least 3 follicles ≥ 18 ml in size and serum E_2 level >150pg/ml per follicle. Serum progesterone (P) and estradiol (E_2) levels will be measured on day of hCG administration. Ultrasound guided oocyte retrieval 36 hours after hCG administration. ICSI procedure will be carried out. Embryo transfer will be done randomly in 200 females who have ≥ 5 grade A embryos: 100 females will be subjected to embryo transfer on day 3(Cleavage stage) and 100 females will be subjected to embryo transfer on day 5 (Blastocyst stage). Luteal support will be maintained by progesterone vaginal pessaries. Outcome measures by: Biochemical pregnancy rate by β- hCG measured 2 weeks after E.T and clinical pregnancy rate (positive cardiac pulsations by trans-vaginal U.S).

Results:

The results of the study have showed that by using ROC curve there was a reduction in pregnancy rate with progressively greater concentrations of progesterone> 1.5 ng/ml and progesterone /estradiol ratio of > 0.6 in day of hCG in group 1 (day 3 embryo transfer) while Progesterone rise at day of hCG had no detrimental effect on pregnancy rate in group 2(day 5 embryo transfer) so using roc curve there was no detrimental cutoff point for progesterone and p/E2 ratio.

The two studied groups were compared regarding the demographic data. Age in group I ranged 21.0-37.0 with mean value 30.20±4.22 and in group II ranged 21.0 - 39.0 with mean value 30.66±3.86. Duration of infertility in group I ranged 2.0 - 10.0 with mean value 4.70±1.81 and in group II ranged 2.0-8.0 with mean value 4.57±168. BMI in group I ranged 18.0-31.0 with mean value 23.76±3.44 and in group II ranged 18.5-31.0 with mean value 24.44±3.33. Cause of infertility was higher in tubal disease in group I (35%) and in group II ovulatory factor was higher (37%). There was no statistical significant difference between the two studied groups regarding the demographic data (P > 0.05), and regarding basal hormonal level.

The two studied groups were compared regarding final E2 and final P4. Final E2 in group I ranged 1834.2 - 3828.0 with mean value 3091.23±875.98 and in group II ranged 1450.0-3471.0 with mean value 2436.29±611.68. Final P4 in group I ranged 0.6-2.3 with mean value 1.32±0.44 and in group II ranged 0.7-2.2 with mean value 1.08±0.43. P/E ratio in group I ranged 0.327-0.601 with mean value 0.427±0.212 and in group II ranged 0.483-0.634 with mean value 0.443±0.123. There was statistical significant difference between the two studied groups regarding to final E2 and final P4 (P < 0.05), while there was no statistical significant difference regarding to P/E ration (P > 0.05) table (I). In comparison between the two studied groups regarding the biochemical pregnancy rate. Positive biochemical pregnancy in group I was 41(41%) and in group II was 54(45%) while negative biochemical pregnancy in group I was 59(59%) and in group II was 46(46%). There was statistical significant difference between the two studied groups regarding the biochemical pregnancy rate (P < 0.05). table (II). Comparison between the two studied groups regarding the clinical pregnancy rate. Positive clinical pregnancy in group I was 36(36%) and in group II was 55(55%) while negative clinical pregnancy in group I was 64(64%) and in group II was 45(465%). There was statistical significant difference between the two studied groups regarding the clinical pregnancy rate (P < 0.05). Table (III), in comparison between the two studied groups regarding the P level. Normal P level in group I was 77(77%) and in group II was 71(71%) while rise P level in group I was 23(23%) and in group II was 29(29%). There was no statistical significant difference between the two studied groups regarding the P level (P > 0.05). Table (IV).

Table I: Comparison between the two studied groups regarding final E2 and final P4.

	Group I	Group II	P
Final E2 (pg/ml)			
Range	1834.2-3828.0	1450.0-3471.0	0.031*
Mean	3091.23	2436.29	
S.D.	875.98	611.68	
Final P4 (ng/ml)			
Range	0.6 - 2.3	0.7-2.2	0.041*
Mean	1.32	1.08	
S.D.	0.44	0.43	
P/E ratio			
Range	0.327 - 0.601	0.483-0.634	
Mean	0.427	0.443	0.154
S.D.	0.212	0.123	

Table II: Comparison between the two studied groups regarding the biochemical pregnancy rate.

Biochemical pregnancy	Group I		Group II	
	No.	%	No.	%
Positive	41	41.0	54	54.0
X^2	1.98			
p	0.046*			

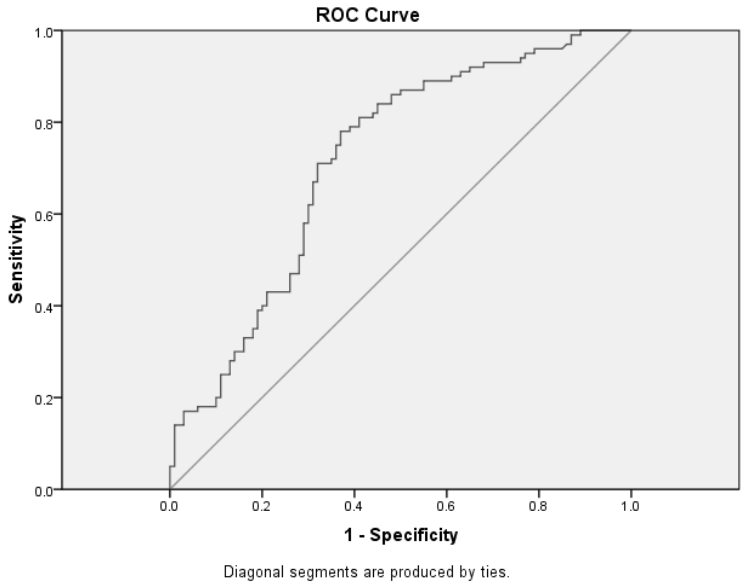
Table III: Comparison between the two studied groups regarding the clinical pregnancy rate.

Clinical pregnancy	Group I		Group II	
	No.	%	No.	%
Positive	36	36.0	55	55.0
X^2	3.65			
p	0.012*			

Table IV: Comparison between the two studied groups regarding the P level.

P level	Group I		Group II	
	No.	%	No.	%
Normal	77	77.0	71	71.0
Rise	23	23.0	29	29.0
X^2	0.936			
p	0.21			

ROC Curve for P.



Area Under the Curve

Test Result Variable(s):E2

Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
			Lower Bound	Upper Bound
1.5	.036	.000	.645	.788

The test result variable(s): E2 has at least one tie between the positive actual state group and the negative actual state group. Statistics may be biased.

a. Under the nonparametric assumption

b. Null hypothesis: true area = 0.5

Coordinates of the Curve

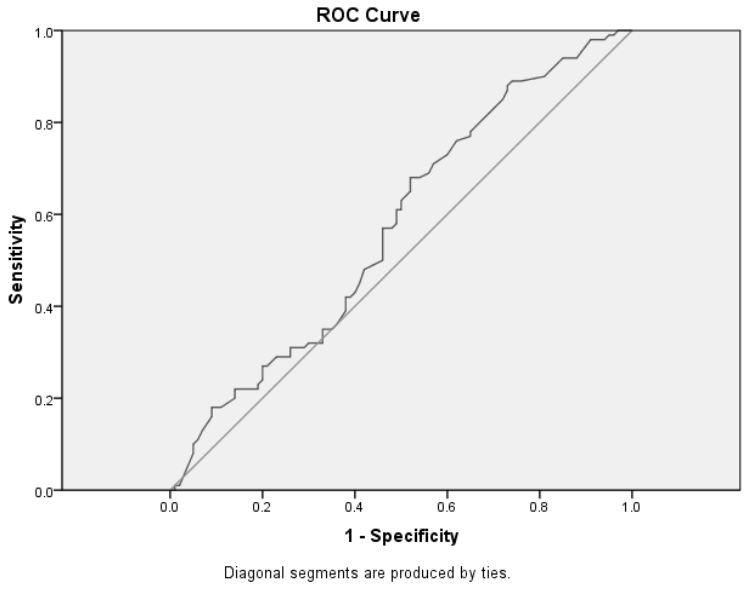
Test Result Variable(s):E2

Positive if Greater Than or Equal To[a]	Sensitivity	1 - Specificity
1887.0100	.950	.770

The test result variable(s): E2 has at least one tie between the positive actual state group and the negative actual state group.

a. The smallest cutoff value is the minimum observed test value minus 1, and the largest cutoff value is the maximum observed test value plus 1. All the other cutoff values are the averages of two consecutive ordered observed test values.

ROC Curve for E2



Area Under the Curve

Test Result Variable(s):P

Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
			Lower Bound	Upper Bound
.573	.041	.074	.494	.653

The test result variable(s): P has at least one tie between the positive actual state group and the negative actual state group. Statistics may be biased.

a. Under the nonparametric assumption

b. Null hypothesis: true area = 0.5

Coordinates of the Curve

Test Result Variable(s):P

Positive if Greater Than or Equal To[a]	Sensitivity	1 - Specificity
.8650	.830	.700

The test result variable(s): P has at least one tie between the positive actual state group and the negative actual state group.

a. The smallest cutoff value is the minimum observed test value minus 1, and the largest cutoff value is the maximum observed test value plus 1. All the other cutoff values are the averages of two consecutive ordered observed test values.

Discussion:

Our results indicate that in long GnRH-a cycles, premature elevation of progesterone on the day of hCG could adversely affect the clinical outcome of ICSI-ET among women with normal ovarian reserve undergoing day 3 cleavage stage embryo transfer, But there is no effect on the clinical outcome of ICSI-ET among women with normal ovarian reserve undergoing day5 blastocyst transfer.

The cause of premature elevation of progesterone in GnRH agonist cycles remains unknown. Many researchers in the past have adopted the term 'premature luteinization' for patients with progesterone elevation on the day of hCG administration for final oocyte maturation.[20] This suggests that the excessive amount of progesterone is produced by granulosa cells that have started the process of luteinization. However, the fact that a significantly higher mean number of cumulus oocyte complexes (COCs), accompanied by a higher mean E2 level on the day of hCG administration, were present in the group of patients with elevated progesterone, when only the agonist studies were pooled, suggests an alternative explanation. It is likely, at least regarding the patients treated with GnRH agonists, that the elevated progesterone might be attributed to an excess number of follicles, each one producing a normal, for the late follicular phase, amount of progesterone. In this way, excess of proliferating granulosa cells leads to an increased progesterone production, independent of LH exposure. Thus, at least for the studies using GnRH agonists to inhibit LH surge, the use of the term premature luteinization' in the presence of normal LH levels might not be appropriate.[21] So, we used the term of premature elevated progesterone levels instead of premature luteinization.

In the late follicular phase of COH, the elevation of P is the result of the total amount of P secreted by maturing follicles[22]. Progesterone levels have been found to correlate positively with the number of mature follicles and with E2 levels on the day of hCG. [23] Younis et al.[24] defined P: E2 >1 on the day of hCG as PL, which was suggested to be an important variable that could result in a manifestation of low ovarian reserve and low clinical PR. Recently, increased serum P: E2 ratio (1.2) on the day of hCG, as an indicator of PL, was reported to have poor predictive value for ICSI outcomes in infertile women with normal ovarian reserve treated with a long GnRH-a protocol.[25] However, in that study group1 (cases of day 3 embryo transfer) there was only 23 progesterone rise patient and 77 non progesterone rise patient and clinical pregnancy rate was (36cases-46.8%) in non-progesterone rise patients but was (5 cases -21.7%) in progesterone rise patients. The numbers of oocytes retrieved, fertilized oocytes and the number of embryo transferred. No. of retrieved oocyte in normal P level ranged 6.0 - 25.0 with mean value 13.644.54 and in rise P level ranged 11.0-31.0 with mean value 18.835.34. No.of fertilized oocyte in normal P level ranged 6.0 - 21.0 with mean value 12.264 and in rise P level ranged 11.0-31.0 with mean value 18.835.34. No. of fertilized oocyte in normal P level ranged 6.0 - 21.0 with mean value 12.264.40 and in rise P level ranged 5.0-20.0 with mean value 8.883.16. No. of embryo transferred in normal P level ranged 2.0 – 6.0 with mean value 4.601.36 and in rise P level ranged 3.0-6.0 with mean value 5.170.98. There was statistical significant difference regarding number of retrained oocytes and number of fertilized oocytes ($P < 0.05$), while there was no statistical significant difference regarding number of embryo transferred ($P > 0.05$).

Many studies have described an adverse relationship between elevated circulating P and

the occurrence of pregnancy.[26] Although the precise mechanism is unclear, the effect can be attributed to a deleterious effect on either the endometrium or the oocyte. Ovarian stimulation is known to advance endometrial maturation and P itself may hasten closure of the window of implantation.[27] Premature luteinization of follicles also may have detrimental effects on oocyte quality as reflected in reduced rates of normal fertilization, cleavage, embryo morphology and implantation.[36] In our study, fertilization rate, the total number of embryos, number of embryos transferred and numbers of good quality embryos transferred were not significantly different between groups, making the proposed theory of impaired oocyte-embryo quality less likely. The diminished implantation rate in the high P group further supported reduced endometrial receptivity.

Recently, Azem et al. demonstrated no deleterious effect of elevated P on embryo quality. However, high serum P adversely affects implantation and pregnancy rates. In cycles with elevated serum P, higher estradiol levels were noted, more oocytes were retrieved and manipulated, and more embryos were available for transfer.

Previously, Kolibianakis et al.[28] showed that advanced endometrium was present in all patients on the day of oocyte retrieval. The P receptors in IVF cycles were upregulated on the day of hCG. Thereby, even moderate increases of P, although still within normal limits, result in an amplification of P action, leading to down-regulation of the ER (endometrial receptivity), so-called luteinization.[29] By performing transfer on day 3, the embryo was exposed to an advanced endometrium and to P-induced uterine growth factor 'out of phase', leading to the establishment of an asynchronous embryo– endometrium cross-dialogue.[30]

In contrast, on day 5 the premature progesterone rise had no effect on pregnancy outcome in the blastocyst transfer group (group 2) the clinical pregnancy rate in non-progesterone rise group was 39cases from 71cases (54.9%) and was 16 cases from 29 cases (55.2%) in progesterone rise group so there was no statistical significant difference at day 5 ($P > 0.05$).

Ovarian stimulation, whether using an agonist or antagonist for pituitary suppression, induces more histologically advanced endometrium than in natural cycles. This advancement is about 1–2 days in agonist cycles.[31] In a recent study, Detti et al,2011 reported that exposure of the endometrium to high hormone levels during ovarian stimulation significantly increased PRB receptor expression at the time of day 3 ET in comparison to natural cycle.[32] So, in good responders, it appears that the benefits associated with high steroid levels may be offset by the negative impact of COS on the endometrium or other target tissues, particularly if the embryos are placed in the uterus soon after the steroid hormones peak for oocyte retrieval. We found that P level > 1.5 ng/ml and P/E2 ratio > 0.6, on the day of hCG were detrimental for CPR among women with normal ovarian reserve undergoing cleavage stage ET (group 1). These results were relatively in line with a recent study including 4,037 patients in which P levels >1.5ng/mL was associated with lower CPR in women undergoing cleavage stage ET after IVF/ICSI cycles.[33]

Importantly, such a hypothetical effect on the endometrium may, to some extent, dissipate day 3 and day 5. Moreover, the absence of endometrial advanced maturation has been demonstrated in mid-luteal phase of stimulated cycles.[34] So, blastocyst transfer not only eliminates the transfer of embryos not destined to reach the blastocyst stage, but also has the advantage of interacting with a less out of phase endometrium.[35] In accordance, it has been reported that the endometrium is optimally receptive about 5–6 days after retrieval, or about 6.5–7.5 days after hCG

administration.[41] pinopode development was reported to be dependent on P secretion, and it was shown that elevated pre-ovulatory serum P levels were associated with accelerated pinopode development. Importantly, observations of pinopode development were used to assess the window of endometrial receptivity and the duration of this window was reported to be < 48 hours in an individual cycle. The start of this brief phase was reported to vary between patients.[36] So, there is concern that high progesterone will accelerate pinopode appearance and prematurely close the implantation window.

However, the specificity of this histological finding for the window of implantation has recently been questioned, because pinopode formation could be detected at nearly all time points following progesterone exposure. [37] In the study of Papanikolaou et al, the P cut-off level of >1.5 ng/ml did not adversely affect outcome of BET (blastocyst embryo transfer), we wondered whether there could be higher detrimental cut offs. Using ROC, neither P nor P/E2 ratio had detrimental cut-offs among BETs. [38]

So, in cases of high progesterone, estradiol and or P/E2 ratio at the end of the ovarian stimulation, targeting ET on day 5 could be a good strategy for better synchronization with an endometrium already affected by COS, rather than prematurely exposing the embryo to such environment. This actually might explain one of scenarios responsible for higher pregnancy of blastocyst in comparison to cleavage stage ET.

Conclusions:

These preliminary findings suggest that premature progesterone rise at day of hCG adversely affect clinical pregnancy rate in cases of day 3 embryo transfer and has no effect on clinical pregnancy rate in cases of day 5 embryo transfer in long agonist ICSI cases.

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Original Article

Vitamin D Deficiency in Patients with Systemic Lupus Erythematosus and its Relation to Disease Activity.

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Abstract:

Introduction: Deficiency of vitamin D is associated with many autoimmune diseases including type 1 diabetes mellitus, multiple sclerosis, rheumatoid arthritis, and systemic lupus erythematosus (SLE). Our study aims to determine the status of vitamin D in SLE patients and its relation to disease activity. **Methods:** 32 SLE patients were recruited in our study. They were collected from Riyadh National Hospital between February, 2012 to November, 2013. They were compared to 20 healthy control subjects. Serum levels of 25(OH)D, anti-dsDNA, C_3 & C_4 were evaluated using enzyme- linked immunosorbent assay (ELISA) technique. SLE disease activity was assessed by systemic Lupus Erythematosus Disease Activity Index (SLEDAI) score. **Results:** 32 SLE patients were enrolled in our study. Serum levels of 25(OH)D in our patients (21.7±16.2 ng/ml) is significantly lower than healthy subjects (39.3±4.2 ng/ml)(p=0.000). Deficiency of 25(OH)D levels is significantly related to SLE disease activity assessed by SLEDAI score (P=0.04), significant direct relationship was present between 25(OH)D and both C_3(r=0.27 & p=0.013) & C_4 (r=0.24& p=0.006) in SLE patients and a significant negative correlation existed between 25(OH)D and anti-ds DNA (- 0.36 , P <0.000). **Conclusion:** Vitamin D deficiency is frequent among SLE patients and its deficiency is related to disease activity.

Keywords: Vitamin D, SLE, disease activity.

Introduction:

Vitamin D is a secosteroid mainly synthesized in the skin from exposure to sunlight [(1)]. The classic function of vitamin D is to regulate calcium homeostasis and bone formation or resorbtion. Non-classic function of vitamin D is known to involve in the immune system. It is noted since the identification of vitamin D receptor in peripheral lymphocytes, macrophage, and thymus tissue[(2,3)], and expression of 1α-OHase in variety of normal human tissue[(4)]. Lifestyle factors have led to an increased prevalence of vitamin D deficiency, while improved availability and reliability of 25-hydroxyvitamin D (25(OH)D) testing have led to better awareness of widespread deficiency.

Apart from effects on bone metabolism, vitamin D deficiency has been related to an increased risk of cardiovascular disease and a higher risk of all-cause mortality in the general population[(5)]. Also, an increased risk of autoimmune diseases, including type 1 diabetes mellitus, multiple sclerosis, rheumatoid arthritis, and systemic lupus erythematosus (SLE), in relation to low vitamin D levels and/or intake, has been reported[(6,7)].

Systemic lupus erythematosus (SLE) is an autoimmune disorder which appears in a group of individuals and which is related to several factors, including environmental and host genetics that contribute to the development of the disease[(8)]. Patients with SLE develop an immune response against numerous, mostly intracellular self-antigens. This results in formation of immune complexes that get deposited in vascular beds in most organs of the body. Immune complex deposition causes local inflammation and tissue damage that probably amplify the autoimmune response[(9)]. This has serious consequences on the outcome of the disease.

Serum level of vitamin D in SLE patients is reported lower than the normal population[(10,11,12-15)]. This is mainly due to the long-term use of sunscreen by patients with SLE because of photosensitivity, corticosteroid therapy, and lack of dietary intake[(10, 16)]. It is also reported that SLE patients produce

anti-vitamin D antibodies[17]. The low level of vitamin D causes impaired immunological response that is thought to increase disease activity in SLE [11]. Our study is to investigate the status of vitamin D in our lupus patients and its relation to the disease activity.

Subjects & Methods:

32 patients diagnosed as systemic Lupus Erythematosus based on the revised American College of Rheumatology(ACR)

Classification criteria[18]. These SLE patients were collected from the outpatient and inpatient departments of the internal medicine and rheumatology of Riyadh National Hospital between February ,2012 to November,2013 . Thorough clinical examination and laboratory investigations were performed. 20 age & sex matched healthy subjects were used as control subjects. The Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) score[19] was calculated.

All recruited patients & control subjects signed informed consents. The study was approved by the local Ethics & Research Committee. The following variables were recorded for each recruited patient: age, sex, BMI, disease duration, activity status, current medications, clinical manifestations & smoking status.

Serum 25(OH)D levels were measured by enzyme- linked immunosorbent assay(ELISA) (K2110, immuno diagnostic [Dutch Company], Holland), which measures vitamin D levels, both the D2 and D3 forms. It measures both the D2 and D3 derivatives of 25(OH)D. Vitamin D deficiency was defined as serum 25(OH)D level <50 nmol/L; severe if 25(OH)D <25 nmol/L, and normal when 25(OH)D >75 nmol/L. Anti-dsDNA was measured by ELISA technique(Quanta Lite,™ dsDNA Kit, INOVA Diagnostic Inc, CA, USA). Serum C_3 & C_4 , urine protein, red cell casts , serum creatinine , GFR , AST, ALT& CPK were examined.

Statistical Analysis:

SPSS (Statistical Package for Social sciences) software was used. The variables were expressed as numbers and percentages. Quantitative data were expressed as mean ± standard deviations. We used Chi square for group differences. Person tests were used for correlation analysis. ANOVA and Student t- test were used for continuous variables. F-test was used to compare between two groups. A P-value is considered significant if < 0.05.

Results:

Clinical manifestations of SLE patients:

32 SLE patients were recruited in our study. The clinical & demographic characters are shown in table 1. 30 (93.8%) patients were females & 2 (6.2%) were males. Their mean age was 34.5 ± 12.6 years. The BMI (body mass index) of the studied group was 22.1 ± 3.6 (km/m^2). The duration of illness was 19.5 ± 20.4 months. The patients were compared to 20 healthy control subjects, all of them were females , with mean age of 33.4 ± 9.2 years & BMI of 23.6 ± 3.4 (kg/m^2). The clinical manifestations of SLE patients were 18 (56.3%) patients with arthritis, 20 (62.5%) patients with anemia, 15 (46.9%) patients with skin manifestations, 18 (56.3%) patients with nephritis, 7 (21.9%) patients with vasculitis, 6 (18.8%) patients with serositis & 3 (9.4%) patients with neuropsychiatric manifestations.

As regards the medications used by the patients at the time of entry of the study, 28 (87.5%) patients used glucocorticoids, 10 (31.2%) patients used hydroxycloriquine, 4 (12.5%) patients used azathioprine, 5 (15.6%) patients used methotrexate & 7 (21.8%) patients used mycophenolate mofetil. 13 (40.6%) patients were active& 19 (59.4%) patients were inactive disease at the time of entry of the study.

Vitamin D in the studied group:

Mean level of 25(OH)D in SLE patients is 21.7±16.2 ng/ml and in healthy control group was 39.3±4.2 ng/ml. There was a significant difference in 25(OH)D level between the SLE patients and the healthy control group (p=0.000).The mean 25(OH)D of the male SLE patients was 24.5±16.7 ng/ml and the female SLE patients was 21.5±14.9 ng/ml. There were no significant differences in 25(OH)D levels between the SLE male and female patients (P=0.8). 28 (87.5%) SLE patients have 25(OH)D deficiency (<50 ng/ml) & 19 (59.4%) SLE patients have severe 25(OH)D (less than 25 ng/ml.).

Vitamin D & SLE disease activity in SLE patients:

The mean SLEDAI among the 32 SLE patients was 16.2 ± 8.43 .The mean 25 (OH) D of the active SLE patients (19.1±14.2 ng/ml) is significantly lower than the inactive group (24.5 ± 16.3 ng/ml) , (P=0.04) , so there was a significant inverse correlation between lupus activity and 25 (OH) D level.

Vitamin D & anti-ds DNA & C3 & C4 in SLE patients:

A significant negative correlation existed between 25 (OH) D and anti-ds DNA (-0.36, P <0.000). A significant direct relationship was present between 25 (OH) D and both C_3 (r=0.27 & p=0.013) & C_4 (r=0.24& p=0.006) in SLE patients.

Table I: Clinical and demographic characteristics of the studied group

Characteristics	SLE patients	Healthy control group
Age (years) (mean±SD)	34.5 ± 12.6 years	33.4 ± 9.2 years
Female, no (%)	30 (93.8%)	20 (100%)
Male , no (%)	2 (6.2%)	-
Duration of the isease (months) (mean± SD)	19.5±20.4	-
Body mass index (kg/m^2)	22.1±3.6 (km/m^2).	23.6±3.4 (kg/m^2).
Active SLE patients , no(%)	13 (40.6%) patients	
Inactive SLE patients , no(%)	19 (59.4%)	
SLEDAI score(mean± SD)	15.3±8.9	-
Smoking (no , %)	10 (31.3%)	5 (25%)
Medications:		-
Glucocorticoids	28 (87.5%)	-
Hydroxycloriquine	10 (31.2%)	-
Azathioprine	4 (12.5%)	-
Methotrexate	5 (15.6%)	-
Mycophenolate mofetil	7 (21.8%)	-

Table II: Clinical manifestations of the SLE patients

Clinical manifestations	Number	%
Arthritis	18	56.3
Anemia	20	62.5
Skin	15	46.9
Nephritis	18	56.3
Vasculitis	7	21.9
Serositis	6	18.8
Neuropsychiatric	3	9.4

Table III: Laboratory parameters of SLE patients

Laboratory parameters	Mean ± SD
Hemoglobin (gm / dl)	10.3 ± 2.2
ESR (mm / h)	71.3± 28.9
CPK (mg / dl)	79.7± 199.8
AST (mg / dl)	37.8± 34.2
ALT (mg / dl)	40. 4± 33.8
Creatinine (mg / dl)	0.89 ± 1.77
Anti ds-DNA (IU/ ml)	532.3± 157.7
C_3 (mg/L)	0.33± 0.32
C_4 (mg/L)	0.12± 0.13
25 (OH) D(ng/ml)	21.7±16.2
SLEDAI	16.2±8.43

Discussion:

We had an observational study in vitamin D in SLE patients of different ethnicities. We detected high prevalence of vitamin D deficiency among the SLE patients, as vitamin D level in our SLE patients (21.7±16.2 ng/ml) is lower than the normal range and lower when compared to the healthy control group (39.3±4.2 ng/ml) . Our finding was consistent with many other studies [20,21,22,23].

Serum level of vitamin D is influenced by several factors such as physical factor (clothing, use of sunscreen or sunglasses, altitude, season, time outdoors, etc.) and biological factors such as pigmentation, use of medication, thick body fat, fat mal-absorption and age [24, 25]. While it has been pointed out that vitamin D deficiency may increase the risk of autoimmune disease, SLE patients were suspected of having a variety of risk of deficiency of vitamin D. Low levels of vitamin D in patients with SLE may be caused by low nutritional vitamin D intake , avoidance of sun as sunlight flares SLE (by the use of sunscreens, the lack of outdoor activities, use clothes that are closed), corticosteroid treatment and the presence of auto-antibodies against vitamin D, vitamin D binding protein and to its receptor (VDR) [17].

Other risk factors have been associated with this deficiency, namely: lupus nephritis due to decreased 1-alpha-hydroxylase activity on 25(OH)D, leading to decreased synthesis of 1,25(OH)2D; use of drugs like hydroxychloroquine, and anticonvulsants that enhance 25(OH)D metabolism[17,26,27]. Another important factor is female gender as SLE and other autoimmune diseases are more common in females than males, also vitamin D deficiency is more prevalent among females.

Our study demonstrated a reciprocal relationship between 25(OH)D level and SLE activity measured by SLEDAI score. This finding was consistent with another study done in China that showed a reciprocal relationship between 25(OH)D level and disease activity measured by SLEDAI score independent of age, disease duration, vitamin D supplementation and immunosuppressive therapy[28]. Another study done in Brazil showed also inverse relation between 25(OH)D and SLE activity[29]. Mok et al. found vitamin D deficiency is a markerof SLE disease activity with comparable specificity to anti-C1q[21].

Among 378 patients with SLE from several European and middle east cohorts, serum 25(OH)D was found to be inversely related to disease activity ($p = 0.018$)[30]. Improving vitamin D status among patients with SLE may benefit other common manifestations as well, such as fatigue[31] and cognitive dysfunction[32].

The conflicting results of the few studies could be due to the diverse study populations, methodological variations and the power of several studies[31,33,34] was probably too low to achieve statistical significance. While the results of these observational studies are helpful for generating hypotheses concerning the effects of vitamin D on the clinical course of SLE, it is unwise to make causal inferences from these studies.

SLE disease activity predicts the subsequent lupus induced organ damage[36]. However, most of the studies failed to prove a causal relation between vitamin D deficiency and SLE induced organ dysfunction[30,31,33,35,36]. This observation actually has no clear explanation.

Anti-dsDNA antibodies and C_3 & C_4 are used as serological markers of SLE activity. Our study showed a negative correlation between serum 25(OH)D & anti-ds DNA . This finding is consistent with many other studies [21,37,38].

Another Indian study by Mandal et al. involved 129 SLE patients showed similar negative correlations between 25(OH) D and anti-ds DNA in these patients[39], however, other studies did not show similar correlations[15,35].

Our study showed a positive correlation between serum 25(OH)D and serum levels of C_3 & C_4 of in our SLE patients. This was consistent with many other studies[36,38].

Another Saudi study involved 95 SLE patients showed similar findings as regards the negative correlation between 25(OH)D and anti-ds DNA and positive correlation between 25(OH)D and C_4 but not C_3 [40].

Szodoray et al in their study on 177 SLE patients found similar findings to the latest study[39], which showed that a positive correlation existed between 25(OH)D and C4 levels but not with C3 levels. In addition, they found that low levels of C3 and C4, were strong predictors for 25(OH)D deficiency in lupus patients. This could be explained by the fact that the classical pathway is the dominant pathway in complement activation in SLE patients, so the level of C4 is always low while C3 may be either normal or lower than normal[41].

One of the important functions of vitamin D3 is maintenance of homeostasis of B cells [42]. Low levels of vitamin D3 contribute to hyperactivity of B cells and enhanced production of auto-antibodies [43].

Furthermore, vitamin D3 is known to modulate various immunological pathways[44] and thus could have a defining role in the development, progression and pathogenesis of SLE. Vitamin D3 also inhibits differentiation of dendritic cells (DCs) and T-helper cells (CD4+)[45], enhances T regulatory cell proliferation and suppresses release of inflammatory mediators [46], which collectively help in control of autoimmune disorder.

Our study has some limitations like the limited number of the studied group of patients which may affect the results, lack of the other studies of different languages, lack of racial effect and vitamin D therapy on the outcome.

Vitamin D is important determinant factor in SLE disease activity as well as other clinical manifestations of the disease and cardiovascular risk factor as lipid and aortic stiffness. It is essential for bone metabolism. Despite vitamin D supplementation is not yet established, many authors recommend it for the SLE patients on daily or weekly basis to maintain adequate levels of 25(OH)D depending on the starting level[30].

Conclusion:

In conclusion of our study, Vitamin deficiency is common in SLE patients. Deficiency of vitamin D is related to the disease activity. Low vitamin D levels is related to increased levels of anti-ds DNA and decreased levels of C3 and C4 as important factors in disease activity. Further research studies are indicated for the role of vitamin D supplementation in controlling disease activity through maintenance of homeostasis of B cells and preventing their hyperactivity and production of auto-antibodies.

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Original Article

C-Terminal Telopeptide of Type Ii Collagen (Ctx-Ii): Correlation with Ultrasonographic, Conventional Imaging and Clinical Findings of Knee Osteoarthritis.

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Abstract:

Background: Osteoarthritis (OA) is the most common form of arthritis, which is a multifactorial disease of the joints characterized by gradual loss of articular cartilage. Type II collagen is the most abundant protein component of hyaline cartilage and c-terminal telopeptide of type II collagen (CTX-II) is released during the degradation of type II collagen, which is a critical event in the pathology of OA. **Aim of the Work**: is to evaluate CTX-II as a marker of OA and correlate its level with ultrasound, conventional imaging and clinical findings. **Subjects and Methods**: 50 patients with knee OA and 20 healthy persons of matched age and sex as a control group , were subjected to detailed history taking and through physical examination including joint examination, laboratory investigations, serum CTX-II level and in some cases synovial CTX-II level, plain radiography of both knees and musculoskeletal ultrasound (MSK US) of both knees . Data was collected and analyzed using computer based program (SPSS). **Results:** There was statistically significant increase in serum CTX-II level in OA patients than the control group (p=0.001), serum CTX-II was significantly positive correlated with ESR (r = +0.44, p = 0.001), CRP (r = +0.541, p = 0.001), OA grade (r = +0.348, p = 0.013) and Lequesne index (r = +0.378, p = 0.007). No correlation was found between CTX-II and VAS, WOMAC and cartilage thickness. In patients with ultrasound evidence of effusion, CTX-II level was significantly higher than in patients without effusion. **Conclusion:** CTX-II may be a useful biomarker for osteoarthritis either in early diagnosis or in assessment of the severity of the disease. Analysis of CTX-II in serum samples provided a sensitive method to detect increased degradation of collagen type II in patients with knee osteoarthritis.

Keywords: c-telopeptide, type II collagen, osteoarthritis, knee ultrasound.

Introduction:

Musculoskeletal diseases are now the second greatest cause of disability in all regions of the world, with osteoarthritis (OA) showing the greatest increase in the last 20 years.(1) Quality of life studies suggest that the impact of OA is comparable to that of cardiac, neurological and pulmonary diseases in terms of effect on daily functioning and health –related quality of life.(2)

Osteoarthritis (OA) is the most frequent chronic musculoskeletal disease and the leading cause of disability in elderly persons. Traditionally OA has been considered as a disease of articular cartilage, but the disease is manifest in all joint structures; cartilage, subchondral bone, synovium, capsule and ligaments.(3,4)

OA has been always classified as non-inflammatory arthritis; yet there is increasing evidence for inflammation occurring with cytokine and metalloproteinase release into the joint.(5,6) Inflammation and angiogenesis are now recognized as contributing to the symptoms and progression of OA.

Osteoarthritis may be classified as primary or secondary according to its cause or major predisposing factor; Primary osteoarthritis is the most common type. Major factors that affect the degree of risk for developing osteoarthritis include age, gender, joint location, obesity, genetic predisposition, joint malalignment and trauma.(7,8)

Molecular markers are molecules, or fragments of connective tissue matrices which are released into biological fluids during tissue biosynthesis and turnover and which can be measured by immunoassays. (9)

Type II collagen molecule has been investigated as a potential source of biomarkers in osteoarthritis. This is because type II collagen the most abundant protein component of cartilage and it is relatively specific for hyaline cartilage. Furthermore, damage to the type II collagen meshwork is a critical event in the pathology of osteoarthritis. (10)

The CTX-II epitope is a part of the non-helical carboxyterminal crosslinked telopeptide and consists of six amino-acids attached to a cross-link (X). It is released during the degradation of type II collagen. It is mainly concentrated in calcified articular cartilage at the junction with sub-chondral bone. (11,12)

Musculoskeletal ultrasound (MSK US) has become an established imaging technique for diagnosis and follow up of patients with rheumatic diseases. It has considerable advantages over other imaging modalities including non –invasiveness, quick to perform, relatively low cost, ability to scan multiple joints, lack of ionizing radiation , repeatability and high patient acceptability .It can also be used for guidance of aspiration , biopsy and injection of treatment. (13)

Ultrasound is useful for detecting joint effusions, including a minimal effusion undetectable upon clinical examination, synovitis and osteophytes.(14) Popliteal cysts can also be visualized by ultrasound, and potential complications including compression of adjacent vascular structures can be diagnosed.

Aim of the Work:

Is to evaluate CTX-II as a biochemical marker and to investigate its association with ultra-sonographic assessment as well as conventional imaging and clinical findings in knee OA .

Subjects and Methods:

The present study included 70 persons divided into 2 groups as follows:

Group 1: 50 adult patients with knee osteoarthritis**,** diagnosed according to the American College of Rheumatology (ACR) clinical and radiographic criteria of knee OA. (15)

Group 2: 20 adult healthy persons age and sex matched as a control group.

Exclusion criteria: patients with secondary osteoarthritis either due to inflammatory joint diseases (e.g; rheumatoid arthritis) or traumatic causes were excluded from the study.

Following informed consent all participants were subjected to the following:

1- Complete history taking

2- Clinical examination: general examination with estimation of body mass index (BMI), as well as detailed joint examination.

3- Osteoarthritis clinical assessment:

- The Western Ontario and McMaster Universities (WOMAC) questionnaire (16)
- Visual analogue scale
- Lequesne functional index will be assessed (16)

4- Routine laboratory investigations :

- Complete blood count
- Blood urea , serum creatinine
- Liver enzymes ,prothrombin time
- Erythrocyte sedimentation rate
- C-reactive protein

5- Specific laboratory investigation :

- Serum CTX-II by Enzyme linked Immunosorbent Assay (ELISA)
- Synovial CTX-II by ELISA, whenever possible in group (1) only .

6- Plain x-ray of both knees using the Kellgren-Lawrence scale for grading of osteoarthritis(17).

- Grade 1 (doubtful OA): doubtful narrowing of joint space and possible osteophytic lipping.
- Grade 2 (minimal OA): definite osteophyte and possible narrowing of joint space.
- Grade 3 (moderate OA): moderate multiple osteophyte, definite narrowing of joint space, some sclerosis and possible deformity of bone contour.
- Grade 4 (severe OA): large osteophytes, marked narrowing of joint space, severe sclerosis and definite deformity of bone contour.

7- Musculoskeletal ultrasound (MSK US) of knee joint for assessment of inflammatory and structural changes in both knees including cartilage thickness, effusion, synovitis.

8- Ultrasound guided aspiration of knee joint for synovial fluid sample whenever possible.

Statistical analysis:

Data were fed to the computer and analyzed using IBM SPSS software package version 20.0.(18) Qualitative data were described using number and percent. Quantitative data

were described using range (minimum and maximum), mean, standard deviation and median. Comparison between different groups regarding categorical variables was tested using Chi-square test. Correlations between two quantitative variables were assessed using Spearman coefficient.

Results:

Table (I) shows demographic characteristic of the studied population, there was no statistically significant difference between the studied groups as regards their age, sex and BMI**.** The mean disease duration among patients was 5.7 years.

Clinical findings in group 1 were as follows: 27 patients had swelling (54%), 23 patients had tenderness (46%) ,20 patients had effusion (40%) , 35 patients had crepitus (70%) and 15 patients had deformities (30%)

There was statistically significant difference between the 2 groups as regards their VAS (P< 0.001) and there was significant decrease in the functional level in patients in comparison to the control group (p < 0.001) also there was significant difference between 2 groups as regard their Lequsene index (p < 0.001) Table (II)

There was statistically significant difference between group 1 and group 2 as regards ESR, CRP and CTX-II. The readings of serum CTX-II among cases of knee osteoarthritis were higher in comparison to that of control group and this difference was of high statistical significance (p < 0.001). Table (III)

Table (IV) shows Correlation between Serum CTX-II with different parameters in group I. There was positive significant correlation between CTX-II level with ESR (r = +0.44, p = 0.001), CRP (r = +0.541, p <0.001), osteoarthritis grading (r =+0.348, p =0.013) (figure 1), Lequsene functional index (r = +0.378, p =0.007) (figure 2). No correlation was found between CTX-II level and age, BMI, WOMAC, VAS, cartilage thickness.

Table (V) shows the serum level of CTX-II in different grades of osteoarthritis among group 1. The mean CTX-II level in grade 1 was 176 pg/ml, in grade 2 was 235 pg/ml, in grade 3 was 262 pg/ml and in grade 4 was 367 pg/ml.

Synovial fluid samples were collected from 15 patients (30%). the level of synovial CTX-II ranged from 200-400 pg/ml with a mean of 238.67 and standard deviation 70 and median 200 pg/ml

Ultrasound findings were in the form of effusion, synovitis, baker cyst and measuring the femoral condyle cartilage thickness. In group 1, 30 patients (60%) had effusion, 26 patients (52%) had low grade synovitis, 10 patients (20%) had baker cyst detected by ultrasound. Table (VI) shows Comparison between the two studied groups according to ultrasound findings.

Table I: Comparison between the two studied groups according to demographic data

	Group 1 (n = 50)		**Group 2 (n = 20)**		**Test of sig.**	**P**
	No.	**%**	**No.**	**%**		
Sex						
Male	20	40.0	7	35.0	χ^2= 0.151	0.698
Female	30	60.0	13	65.0		
Age						
Min. – Max.	45.0 – 65.0		40.0 – 63.0		t= 0.462	0.645
Mean ± SD.	54.02 ± 5.24		54.70 ± 6.33			
Median	53.0		55.0			
BMI						
Min. – Max.	26.0 – 31.50		24.50 – 30.2		t= 1.226	0.113
Mean ± SD.	29.40 ± 1.26		28.40 ± 1.84			
Median	29.50		28.0			
Disease duration (years)						
Min. – Max.	1.0 – 12.0		-		-	-
Mean ± SD.	5.70 ± 3.32		-			
Median	5.0		-			

χ^2: Chi square test t: Student t-test

*: Statistically significant at p ≤ 0.05

Table II: Comparison between the two studied groups according to Womac, Vas mm, Lequsene index

	Group 1 (n = 50)	Group 2 (n = 20)	Z	P
WOMAC				
Min. – Max.	20.0 – 65.0	1.0 – 13.0		
Mean ± SD.	41.80 ± 14.45	6.80 ± 3.53	6.521*	<0.001*
Median	42.50	6.50		
VAS (mm)				
Min. – Max.	20.0 – 65.0	1.0 – 10.0		
Mean ± SD.	43.30 ± 14.62	5.35 ± 2.76	6.523*	<0.001*
Median	42.50	5.50		
Lequesne				
Min. – Max.	8.0 – 17.0	0.0 – 2.0		
Mean ± SD.	11.48 ± 2.43	0.50 ± 0.61	6.549*	<0.001*
Median	11.50	0.0		

Z: Z for Mann Whitney test
*: Statistically significant at p ≤ 0.05

Table III: Comparison between the two studied groups according to ESR , CRP, CTX-II

	Group 1 (n = 50)	Group 2 (n = 20)	Z	P
ESR				
Min. – Max.	10.0 – 26.0	6.0 – 16.0		
Mean ± SD.	18.40 ± 3.66	11.10 ± 2.8	8.015 *	<0.001*
Median	18.0	11.0		
CRP				
Min. – Max.	1.0 – 7.0	0.0 – 2.0		
Mean ± SD.	3.68 ± 1.41	1.20 ± 0,52	6.124*	<0.001*
Median	3.5	1.0		
Serum CTX-II				
Min. – Max.	120.0 – 600.0	110.0 – 200.0		
Mean ± SD.	249.8 ± 125.5	150.5 ± 38.8	4.069*	<0.001*
Median	200.0	130.0		

Z: Z for Mann Whitney test
*: Statistically significant at p ≤ 0.05

Table IV: Correlation between Serum CTX-II with different parameters in group I (n = 50)

	Serum CTX-II	
	r_s	P
Age	-0.225	0.116
ESR	0.440*	0.001
CRP	0.541*	<0.001
OA	0.348*	0.013
WOMAC	0.099	0.496
VAS	0.155	0.284
Lequesne	0.378*	0.007
BMI	0.217	0.130
Synovial ctx	0.430	0.109
Cartilage thickness	-0.230	0.109

r_s: Spearman coefficient
*: Statistically significant at p ≤ 0.05

Table V: Level of serum CTX-II in different grades of osteoarthritis

	OA grading				$^{KW}\chi^2$	P
	Grade 1 (n =10)	Grade 2 (n =18)	Grade 3 (n =15)	Grade 4 (n=7)		
Serum CTX-II						
Min. – Max.	120.0 – 220.0	140.0 – 500.0	130.0 – 500.0	150.0 – 600.0		
Mean ± SD.	176.0 ± 33.4	235.0 ± 92.9	262.0 ± 127.4	367.1 ± 196.3	6.887	0.076
Median	190.0	200.0	200.0	320.0		

$KW\chi^2$: Chi square for Kruskal Wallis test

Table VI: Comparison between the two studied groups according to ultrasound findings

	Group 1 (n = 50)		Group 2 (n = 20)		χ^2	P
	No.	%	No.	%		
Effusion						
Absent	20	40.0	20	100.0	21.000*	<0.001*
Present	30	60.0	0	0.0		
Synovitis						
Absent	24	48.0	20	100.0	16.545*	<0.001*
Present	26	52.0	0	0.0		
Baker cyst						
Absent	40	80.0	20	100.0	4.667	^{FE}p=0.053
Present	10	20.0	0	0.0		
Cartilage thickness						
Min. – Max.	1.0 – 5.0		5.50 – 6.50		Z= 6.650*	<0.001*
Mean ± SD.	2.90 ± 1.04		6.03 ± 0.26			
Median	3.0		6.0			

χ^2: Chi square test FE: Fisher Exact test
Z: Z for Mann Whitney test *: Statistically significant at p ≤ 0.05

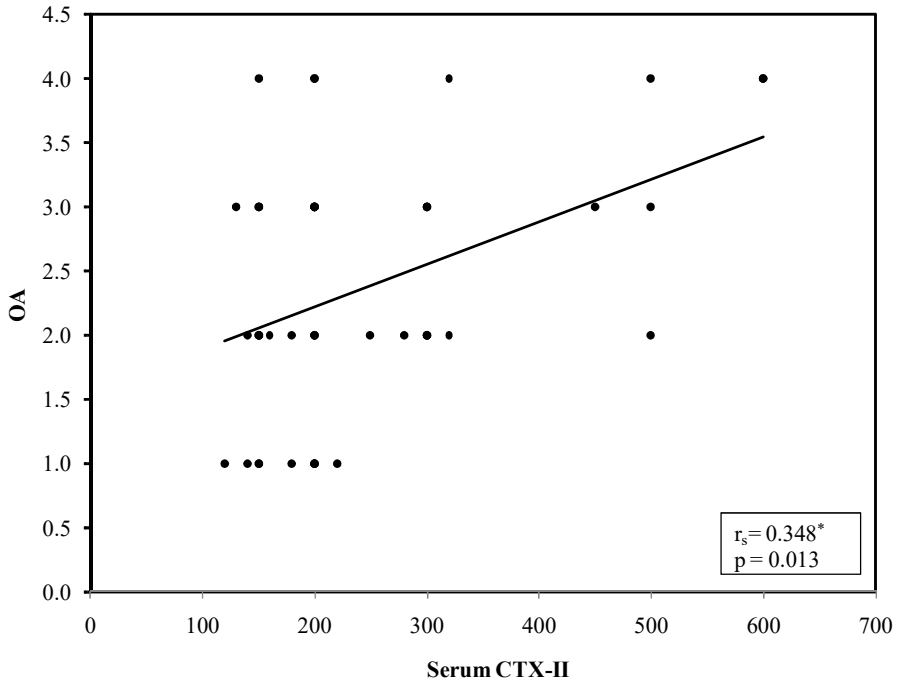

Figure (1): Correlation between Serum CTX-II with OA grading in group I

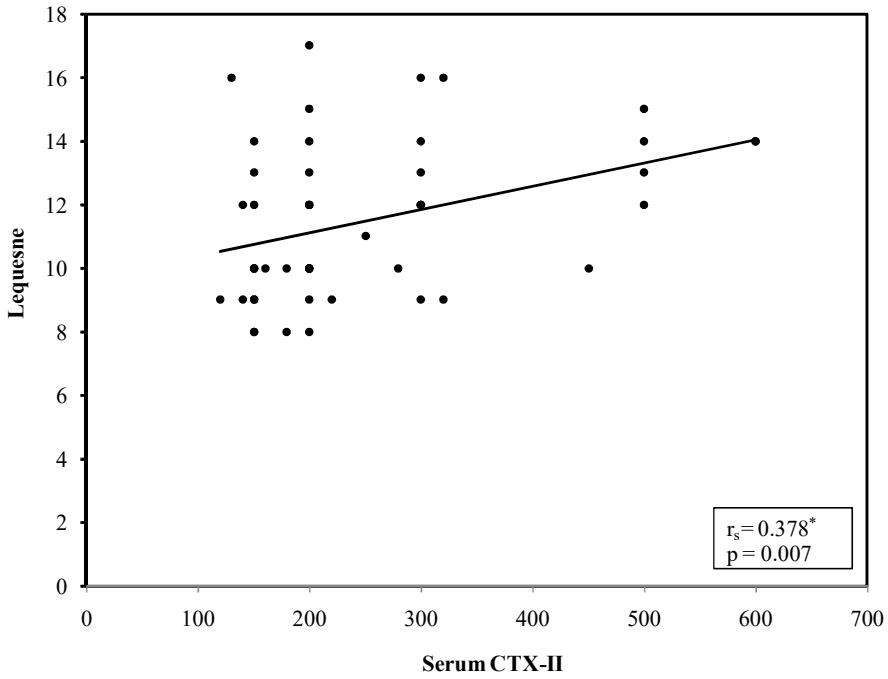

Figure (2): Correlation between Serum CTX-II with lequesne in group I

Discussion:

Osteoarthritis (OA) is a group of chronic painful, disabling conditions affecting the synovial joints. It results from articular cartilage failure induced by a complex interplay of genetic, metabolic and biochemical factors with secondary components of inflammation. The process involves interactive degradation and repair processes of cartilage, bone and synovium. Osteoarthritis is the most common form of arthritis. [19]

OA is a complex, active degradative and repair process of cartilage and subchondral bone with a synovial inflammation. Several factors are involved in this process such as mechanical stress, biochemical and genetic factors. Chondrocytes respond to injuries by producing degradative enzymes and by developing inappropriate repair responses. A lot of pro-degradative agents such as proteinases and proinflammatory cytokines have been extensively studied and might compromise macromolecular synthesis resulting in the development of cartilage breakdown.[20]

In current work , The median BMI of group 1 was 29.5 kg/m^2 ranged from 26 to 31.5 , while that of group 2 was 28 ranged from 24.5 to 30.2. There was no statistically significant difference between the studied groups as regards their BMI. This is in agreement with Averyl et al[21] who found that obesity was related with tibiofemoral OA .

In our study 20 patients (40%) had clinical effusion, 23 patients (46%) had tenderness. this is in agreement with D`Agostino et al[22] who enrolled 600 patients with knee OA in their study and found that 204 patients (34%) had clinical effusion and 52% of them had tender knees.

In the current study there was statistically significant difference between the patients and control group as regards their VAS (P < 0.001). Similar results were obtained by Barthel HR et al. Barthel correlations confirmed significant associations (P < 0.001) between VAS pain intensity and OA.[23] also there was statistically significant difference between group 1 and group 2 as regards WOMAC (p<0.001), and this result agrees with Salaffi F, Leardini G, et al.[24]

There was statistically significant difference between group 1 and group 2 as regards to ESR and CRP (P < 0.001). This went into accordance with the results reported by Sermin et al. [25]

Cartilage damage is one of the main pathological changes in OA. Knee OA is characterized by depressed cartilage synthesis

and increased cartilage degradation. However, this can be monitored by measurement of cartilage- derived synthesis and degradation products of matrix molecules released into synovial fluid and serum .[26]

To the best of our knowledge, all the researches for CTX-II in osteoarthritis in humans were in the urine samples, so the current work is a novel one in detecting the serum level of CTX-II among patients with osteoarthritis.

In the present study the readings of serum CTX-II among the cases of knee osteoarthritis were higher in comparison to that of control group and this difference was of high statistical significane ($p < 0.001$).

Kalai E et al,[27] studied the level of urinary CTX-II among patients with knee osteoarthritis and found that urinary CTX-II levels were significantly higher in knee osteoarthritis patients compared with controls (323.98 vs 218.04 microg/ mol creatinine).

In our study there was positive significant correlation between serum CTX-II level and OA grading with highest levels were in grade 4.this is in agreement with Jordan KM et al [28] who reported that urinary CTX-II was found to be higher in patients with severe radiologic osteoarthritis (Kellgren and Lawrence score¼2, 3, or 4) than in patients with few (Kellgren and Lawrence score¼1) or no radiologic signs of OA.

Synovial fluid CTX-II levels directly represent articular cartilage status in OA. Our present study showed that Synovial fluid samples were collected from 15 patients (30%). the level of synovial CTX- II ranged from 200-400 pg/ml with a mean of 238.67 and standard deviation 70. similar results were obtained by Yuanhui et al [29] they found that synovial CTX- II were significantly higher in OA patients than those in control subjects SF CTX-II (ng/ml) 32.80 ± 14.46 in control group and 66.00 ± 65.11 in OA.

Conclusion:

CTX-II may be a useful biomarker for osteoarthritis either in early diagnosis or in assessment of the severity of the disease. Analysis of CTX-II in serum samples provided a sensitive method to detect increased degradation of collagen type II in patients with knee osteoarthritis also ultrasound assessment of knee joint is better in detecting effusion, Baker cyst and synovitis than clinical examination.

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Original Article

Relationship between the Circulating Levels of Angiopoietin-2 and Progression of Chronic Kidney Disease.

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Abstract:

Introduction: Chronic kidney disease (CKD) is associated with high overall and cardiovascular (CV) mortality that increases as kidney functions decrease. Angiopoietins have been recognized mostly for their involvement in endothelial dysfunction, and inflammation, and it was incriminated in the pathogenesis of atherosclerosis, all these processes are highly prevalent in patients with CKD. High serum angiopoietin-2 (Ang-2) was found in acute kidney injury (AKI) and was a strong independent predictor of mortality. Also, Ang-2 was showed to be markedly elevated in patients on maintenance dialysis and was closely associated with the extent of coronary artery disease (CAD) and peripheral arterial disease (PAD). **Aim:** This study was done to investigate the relationship between the circulating levels of angiopoietin-2 and renal function throughout all stages of CKD and to assess correlations between serum Ang-2 and known CV risk factors. **Subjects and methods:** Serum levels of angiopoietin-2 was measured in 75 CKD patients divided into 5 equal groups representing the five stages of CKD according to K/DOQI guidelines and were compared with angiopoietin-2 levels in 15 nonsmokers healthy control matching the patients group as regard, age, sex, and body mass index (BMI). All participants in the study were subjected to full history taking, thorough clinical examination and routine laboratory investigations. Informed written consent was obtained from every subject. **Results:** The circulating Ang-2 was significantly higher in CKD patients compared to the healthy controls, when we further assessed Ang-2 levels according to CKD stages, Ang-2 levels steadily increased across the following groups: healthy controls, CKD 1, CKD 2, CKD 3, CKD 4, and CKD 5, respectively. However, the Ang-2 levels in patients with CKD stages 1 and 2 were not statistically different from the Ang-2 levels in healthy control but at higher stages a significant increase in circulating Ang-2 compared to healthy controls was found. Next, we performed correlation analyses for Ang-2 and several CV risk factors. A significant direct correlation between Ang-2 and serum phosphorus level was detected. All other tested CV risk factors including patients, age, intact parathyroid hormone (iPTH), mean arterial blood pressure (MAP), serum calcium, cholesterol and triglycerides were not correlated with serum Ang-2 level. Furthermore, there was no detectable influence of erythropoietin or vitamin D treatment on circulating Ang-2 levels. **Conclusions:** circulating Ang-2, a putative marker and potential mediator of accelerated atherosclerosis, is inversely related to GFR and increases with progression of CKD. The finding of the study may serve as a basis for more research to elucidate the mechanisms of release, action of Ang-2 and its interplay with other mediators in patients with CKD.

Keywords: Chronic kidney disease, Endothelial dysfunction, Angiopoietin.

Introduction:

Approximately 50% of individuals with end stage renal disease (ESRD) die from CV cause, a CV mortality that is 15 to 30 times higher than the age adjusted CV mortality in the general population.[1] This disparity is present across all ages, but it is most marked in the younger age group (25 to 34 years old), where the CV mortality is 500 fold greater in ESRD patients compared with age matched controls with normal renal function.[2] Studies showed that 40% of patients who have started dialysis treatments have evidence of CAD, and 85% of these patients have abnormal left ventricular structure and function.[3] In fact, the majority of patients with stage 3 to 4 CKD die of CV causes rather than progress to ESRD.

A growing number of studies have demonstrated that the relationship between renal dysfunction and increased CV morbidity and mortality extends across the spectrum of renal dysfunction to encompass the mildest degrees of renal impairment. [4]

Both traditional and non-traditional risk factors have been implicated in the development of CVD in CKD.[5] Traditional risk factors are those defined in the Framingham heart study and used to predict CAD outcomes in the general population. It includes DM, Hypertension, smoking, dyslipidemia, physical inactivity, and left ventricular hypertrophy. [6] Non-traditional risk factors are uremia related factors that increase in prevalence or severity as kidney function declines and may contribute to the excess risk of CVD seen in CKD. Non-traditional risk factors include, anemia, albumiuria, abnormal calcium and phosphorus metabolism, sympathetic over activity, homocysteine, lipoprotein a, and chronic inflammation. [7]

Endothelial dysfunction and accelerated atherosclerosis are almost universal in CKD; impairment of kidney function is responsible for creation of atherogenic milieu. Retention of toxic substances and/or metabolic changes leads to increased oxidative stress and subclinical low grade inflammatory state. These changes result in endothelial dysfunction. [8]

Reduced nitric oxide synthesis due to the accumulation of the endogenous inhibitor of NOS, asymmetric dimethylarginine (ADMA) has been accused of accelerating progression of endothelial dysfunction in CKD.[9]High levels of ADMA were reported in CKD and were associated with higher intima-media thickness and CV events.[10]

Angiopoietins are protein growth factors that promote angiogenesis, there are four identified angiopoietins: Ang-1, Ang-2, Ang-3, and Ang-4. Of them, Ang-1 and Ang-2 are the most studied. These ligands bind to the transmembrane receptor Tie 2 and possibly Tie1, members of a family of receptor tyrosine kinases expressed primarily in vascular endothelium. [11] The angiopoietins have been recognized mostly for their involvement in endothelial activation, angiogenesis and inflammation, the major processes which lie at the core of atherogenesis,[12] and so; the angiopoietins/Tie2 system has been identified as a potential new player in the pathogenesis of CKD associated atherosclerosis.[13]

Ang-2 was discovered by sequence homology to Ang-1, it is secreted by endothelial cells as it is stored in endothelial Weibel-Palade bodies from where it can be rapidly released upon stimulation. A broad range of factors has been reported to modulate Ang-2 expression, including hypoxia, vascular endothelial growth factor (VEGF), angiotensin II, and leptin.[14]In contrast to the widespread expression of Ang-1, Ang-2 expression occurs in areas of endothelial activation and angiogenesis, for example, in ovaries and tumor vessel endothelia, where it coincides with vessel destabilization during angiogenesis.[14,15]

Ang-1 and Ang-2 have opposing effects on receptor activation, while Ang-1 stimulates Tie2; Ang-2 is capable of antagonizing this effect. Genetic evidence verified that Ang-2 can counteract Ang-1 activity as Ang-2 over expression in transgenic mice leads to embryonic death with a phenotype similar to Ang-1 or Tie2 deletion. [14] Signaling through Tie2 appears to depend on the balance between Ang-1 and Ang-2. Ang-1 has powerful vascular protective effects; it suppresses plasma leakage, inhibits vascular inflammation, and prevents endothelial death. In studies in which Ang-1 is directly administered or overexpressed, it leads to marked improvements in vascular integrity in both growing and adult mice. There is now strong evidence to support the concept that Ang-1 provides a paracrine constitutive tonic signal to promote quiescence of the

endothelium, and this is modified by the more actively regulated autocrine antagonistic signals from Ang-2.[16]

Ang-2 has been shown to be a key regulator of vascular inflammation. Ang2 is required for TNF-α induced monocyte adhesion to cultured human vascular endothelial cells and expression of ICAM-1 and VCAM-1. Mice deficient in Ang-2 have a markedly attenuated inflammatory response to staphylococcus aureus, and other stimuli and administration of Ang-2 reverses this.[13]

High serum Ang-2 was found in acute AKI and was a strong independent predictor of mortality. [17]David S et al. showed that circulating Ang-2 is also markedly elevated in patients on maintenance dialysis and that Ang-2 is closely associated with the extent of CAD and PAD. [18]

Patients and methods:

This study was conducted on 90 subjects; they were subdivided into two groups: group A: included 75 patients from Alexandria Main University Hospital, they subdivided into five equal groups representing the five stages of CKD according to the K/DOQI guidelines (group AI, AII, AIII, AIV, and AV) and group B: included 15 apparently healthy nonsmoking subjects. They were matched with respect to age, gender, and body mass index (BMI) to group A. Smokers, patients having diabetes mellitus, CAD, PAD, systemic lupus erythematosus, vasculitis, and patients positive for hepatitis B surface antigen (HBsAg) or hepatitis C virus antibodies (HCV Abs) were excluded from the study. The study was conducted in accordance with ethical guidelines of the declaration of Helsinki and an informed consent was obtained from every subject.

All subjects were interviewed and subjected to thorough history taking, complete physical examination, and routine laboratory investigation, in the form of CBC, blood urea and serum creatinine and estimated GFR using MDRD formula, liver functions tests, fasting and postprandial blood glucose, serum cholesterol and triglyceride, and serum calcium, phosphorus and intact parathyroid hormone

Determination of serum levels of angiopoietin-2 was done using commercially available enzyme linked immunosorbent assay (ELISA) kit purchased from R&D systems (Quantikine, R&D Systems Inc., Minneapolis, USA; catalog number (Dang20)) following the manufacturer's instructions.

Ultrasound examination of abdomen and pelvis was done to assess kidney shape and size and any abnormalities consistent with kidney disease and to exclude presence of liver cirrhosis or fibrosis.

ECG and assessment of ankle: brachial pressure index (ABPI): was done for all participants in the study to detect signs of myocardial ischemia and to exclude PAD respectively.

Results:

The mean of age were 39.87±10.46, 37.00±13.55, 38.20±9.99, 41.67±13.25, 40.20±12.65 and 38.20±13.07 years for Group AI, II, III, IV, V and group B respectively, there were no statistical significant differences between the studied groups regarding age. (P=0.919).

There were no statistical significant differences between the studied groups regarding sex. (P=0.197).

The mean of BMI was 27.13±1.74, 26.97±0.61, 26.22±1.21, 26.31±1.90, 25.39±2.12 and 26.15±1.86 kg/m^2 for Group AI, II, III, IV, V and group B respectively, there were no statistical significant differences between the studied groups regarding BMI. (P=0.068).

Angiopoietin-2 ranged between 290-660 pg/ml and 361-3222 pg/ml with the mean of

447.67111.63 pg/ml and 1510.9676.275 pg/ml for group B and group A respectively, patients group had values statistically higher than control group. (P=0.0001).

Angiopoietin-2 ranged between 361-616, 490-867, 423-3184, 373-2542, 809-3222 and 290-660 pg/ml with the mean of 494.4771.85, 543.5361.57, 1722.33910.46, 1838.67744.33, 1939.07988.74 and 447.67111.63 pg/ml for group AI, II, III, IV, V and group B respectively, there were statistical significant differences between the studied groups regarding angiopoietin-2 (P=0.001). Group AIII, AIV and AV had values statistically higher than group B

Table I showed the relation between angiopoietin-2 levels and drugs received, it illustrated that: In those who were receiving vitamin D, serum angiopoietin-2 ranged between 361 and 3200 pg/ml with a mean of 1468.2 701.3 pg/ml and in those who were not receiving vitamin D, it ranged between 380 and 3222 pg/ml with a mean of 1605.3 608.3 pg/ml, with no statistically significant difference between them and in those who were receiving erythropoietin, serum angiopoietin-2 ranged between 366 and 3222 pg/ml with a mean of 1506.2 586.9 pg/ml and in those who were not receiving erythropoietin, it ranged between 361 and 3205 pg/ml with a mean of 1525.6 652.3 pg/ml, with no statistically significant difference between them.

Correlation analysis between serum levels of angiopoietin-2 and different CV risk factors, demonstrate that serum angiopoietin-2 levels have a statistically significant positive correlation with serum creatinine levels (r =0.54, p =0.011) and with serum phosphorus levels (r=0.490, p=0.006) while, they showed a statistically significant negative correlation with estimated GFR levels (r=-0.632, p=0.002) as shown in figures III, IV, V respectively.

There was statistically insignificant positive correlation between Ang-2 and age, mean arterial pressure, intact parathyroid hormone, calcium phosphorus product, serum cholesterol a triglyceride.(r = .112, P = .377), (r =.091, P = .496), (r = .131, P = .655), (r = .141, P = .624), (r =.030, P = .876), and (r = .012,P =.949) respectively.

There was statistically insignificant negative correlation between Ang-2 and serum calcium(r = -.168, P = .357).

Table I: The relation between angiopoietin-2 levels and drugs received.

	With treatment	Without treatment	p
Vit. D intake			
n	10	35	0.109(NS)
Range	361-3200	380 – 3222	
Mean	1468.2	1605.3	
S.D.	701.3	608.3	
Erythropoietin			
n	11	34	0.526(NS)
Range	366 – 3222	361 – 3205	
Mean	1506.2	1525.6	
S.D.	586.9	652.3	

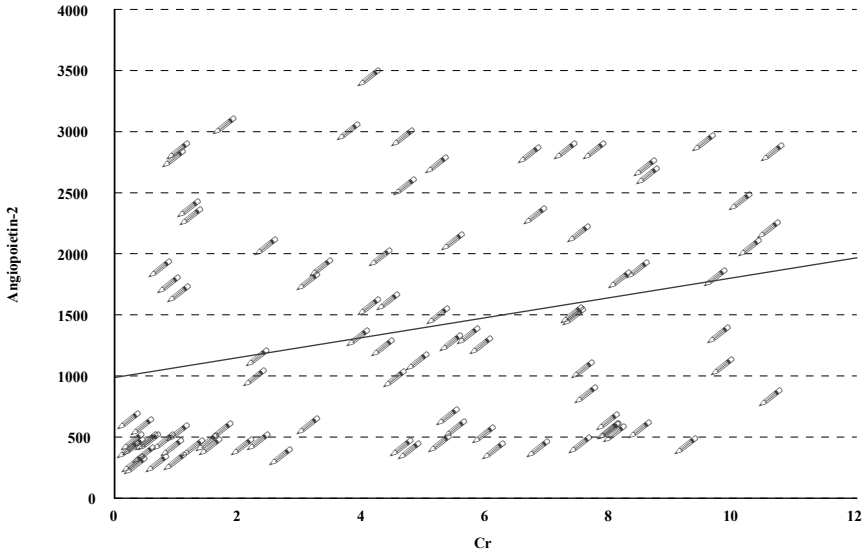

Figure (1): Correlation between serum Ang-2(pg/ml) and serum creatinine mg/dl.

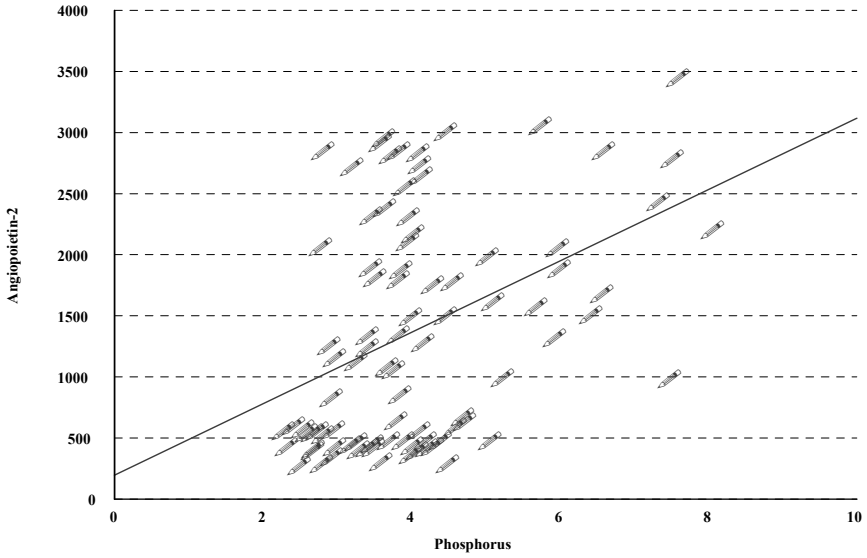

Figure (2): Correlation between circulating angiopoietin-2(pg/ml) levels and serum phosphorus (mg/dl).

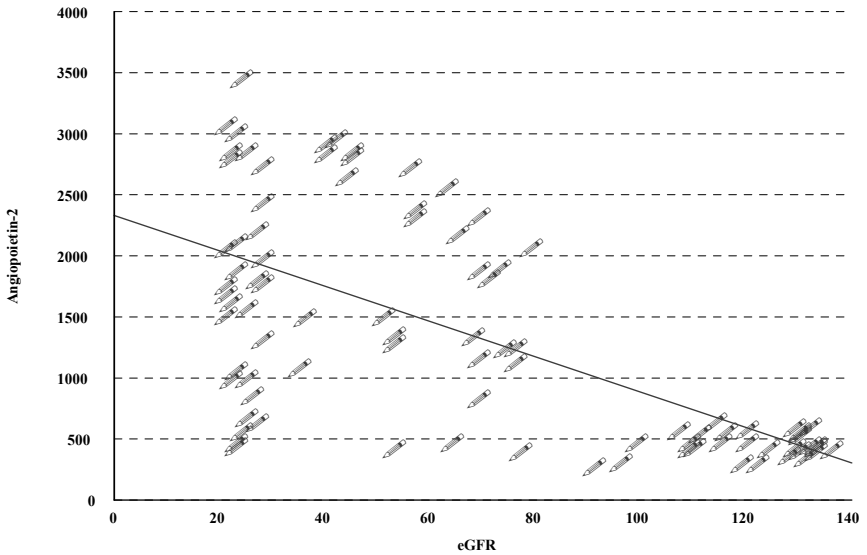

Figure (3):- Correlation between circulating angiopoietin-2 (pg/ml)levels and eGFR (ml/min).

Discussion:

Endothelial dysfunction and accelerated atherosclerosis are almost universal in CKD. Impairment of kidney function is responsible for creation of atherogenic milieu. Retention of toxic substances and/or metabolic changes leads to increased oxidative stress and subclinical low grade inflammatory state, these changes result in endothelial dysfunction. [8]

Angiopoietins have been recognized mostly for their involvement in endothelial activation, angiogenesis and inflammation, the major processes which lie at the core of atherogenesis,[12] and so the angiopoietins/Tie2 system has been identified as a potential new player in the pathogenesis of CKD associated atherosclerosis. [13]

Elevated levels of Ang-2 have been found in arteriosclerosis, [19] acute coronary syndrome,[20] , PAD [21] as well as acute and chronic CHF.[22] Patients with hypertension had higher concentrations of plasma Ang-2 that were predictive of MI.[23] In a study by Freestone B et al. they demonstrated that in patients with atrial fibrillation, the levels of plasma Ang-2 correlate with vWF, this finding points to the link between endothelial damage/dysfunction and Ang-2.[24] Increased concentrations of plasma Ang-2 were detected in patients with diabetes. Patients who received intensified diabetes management had a reduction of plasma Ang-2 if CVD was absent but not in the presence of CVD. [25] Atherosclerotic plaques with high microvessel density show higher concentrations of Ang-2 than Ang-1 and plaque Ang-2 levels are associated with higher concentrations of matrix metalloproteases (MMPs) which promote extracellular matrix degradation,[26] a phenotype associated with unstable and rupture prone plaques. [27]

In the present study, serum levels of Ang-2 were significantly higher in patients with CKD compared to healthy controls. Indeed, Ang-2 levels steadily increase with the progression of CKD. The Ang-2 levels in patients with low CKD stages (i.e. 1 and 2) were not statistically different from the Ang-2 levels in healthy controls. However, at higher stages (i.e. CKD 3–5), there was a significant increase in circulating Ang-2 compared to healthy controls. There was a strong negative correlation between the eGFR and the circulating Ang-2 levels and a strong positive correlation between serum creatinine and the circulating Ang-2 levels.

These findings are supported by previous results by Futrakul N et al. who found that the circulating levels of Ang-2 were increased in patients with mildly and moderately impaired renal function. [28]

David S et al. studied the angiopoietins levels in patients treated by means of dialysis and kidney transplantation, the association of altered angiopoietins levels with atherosclerosis, and the changes in altered levels after renal transplantation. They found that, circulating Ang-2 level was increased in patients treated with dialysis and its level correlated significantly with scores of CAD and PAD. Indeed, Ang-2 levels have been normalized 3 months after kidney transplantation. [18]

In a succeeding study by David S et al. they showed that, serum Ang-2 levels steadily increase with progression of CKD as shown by significant positive and negative correlations with serum creatinine and GFR respectively. They further added power to the relation between CKD and Ang-2 by investigating the effect of sudden loss of GFR after unilateral nephrectomy for kidney donation on the serum Ang-2 levels. They detected a close inverse correlation between the mean changes (0–72 h) in Ang-2 level and the decrease in GFR. [29]

In another study conducted by David S et al. who investigated the impact of Ang-2 level on the outcome in CKD, they prospectively studied 128 CKD patients [43 CKD stage 4, 85 CKD stage 5 (57 hemodialysis,

28 peritoneal dialysis)] over a follow-up period of 4 years. They found that, Ang-2 values were significantly higher in CKD patients than in controls. Furthermore, Ang-2 was significantly higher in dialysis than in stage 4 CKD patients and correlated with markers of vascular disease [cholesterol, high sensitivity CRP (hsCRP), osteoprotegerin (OPG)]. Moreover, Ang-2 was an independent predictor of mortality in both unadjusted and models adjusted for age and vascular calcification. (30)

The relation between Ang-2 and AKI was studied by Kümpers P et al.; they measured circulating Ang-2 by ELISA in 117 critically ill patients with AKI at inception of renal replacement therapy (RRT) in the ICU. Mortality, length of stay and renal recovery were prospectively assessed during a study period of 28 days. They found that, the circulating Ang-2 levels were significantly higher in AKI patients with RIFLE category-Injury or Failure, compared to patients with RIFLE category-Risk. Elevated levels of circulating Ang-2 correlated with impaired oxygenation, low mean arterial pressure and vasopressor dose. Ang-2 concentrations were significantly higher in non-survivors than in survivors at day 0 and day 14 after initiation of RRT. There was a strong independent prognostic impact of elevated Ang-2 on patients 28 day survival. (17)

El-Banawy HS et al. showed that the Ang-2 levels were significantly increased in SLE patients than controls, and it was significantly higher in patients with lupus nephritis than in patients without. Ang-2 was significantly positively correlated with proteinuria and histological activity index, and was negatively correlated with C3, eGFR and FMD. (31)

Similarly, Kümpers P et al. showed that, Ang-2 levels were increased and Ang-1 levels were decreased in patients with active SLE compared to healthy controls and that, this tendency still present in inactive SLE, although less pronounced. Ang-2 concentrations correlated well with SLE disease activity index (SLEDAI) score, proteinuria, double-stranded DNA (dsDNA) titre and soluble vascular cell adhesion molecule-1 (sVCAM-1). In this study, renal involvement was the only independent predictor for elevated Ang-2 level and serum Ang-2 was identified as a strong predictor for disease activity. Additionally, Protein expression of Ang-2 was upregulated in glomeruli of patients with lupus nephritis. (32)

There are three theoretical possibilities for how Ang-2 levels increased in parallel to loss of renal function. First, this may occur due to reduced renal excretion of Ang-2 either due to reduced GFR or diminished tubular secretion with gradual accumulation of Ang-2 in the circulation but there are several observations that argue against this theory, purified recombinant Ang-2 protein exhibits predominant single bands of a molecular mass of 62 kDa. Furthermore, in vivo Ang-2 exists mainly as a multimeric protein; so, its glomerular excretion is rather unlikely.(33) Indeed, Ang-2 is neither detectable in urine from apparently healthy subjects (34) nor cleared by dialysis. (17,35)

A second possibility is that, the damaged glomerular endothelium may over secrete Ang-2 into the circulation. This theory is supported by the finding that, in normal mature glomeruli, Ang-2 levels are low or undetectable(36,37) but reported to be upregulated in certain disease models, including diabetic nephropathy(38) and glomerulonephritis.(37) Although such a scenario is more likely to occur in AKI, it would not also explain Ang-2 level raising after the unilateral nephrectomy without inducing kidney disease in the remaining healthy kidney described by David S et al.(29)

A third possibility is that, CKD and the associated uraemic environment might trigger the release of Ang-2 from distant systemic endothelium via circulating uraemic toxins.

This theory may be supported by the decrease in serum Ang-2 level observed after correction of uremic environment by kidney transplantation in a study done by David S et al. [18]

David S et al. found that in their cohort of CKD patients, that ADMA correlated significantly with circulating Ang-2. Also ADMA remained a significant predictor of Ang-2 after adjusting for GFR, serum phosphate and homocysteine in a multiple linear regression model.[29] ADMA is the most potent endogenous inhibitor of NO which starts to increase at the earlier CKD stages.[39] ADMA has been accused of accelerating progression of endothelial dysfunction in CKD.[9] High levels of ADMA were associated with higher intima-media thickness and CV events in CKD.[10] So, it is conceivable to assume that, the elevated Ang-2 levels in CKD patients might reflect excess exocytosis from Weibel palade body in which it is stored. This may be due to decreased NO bioavailability in the presence of high ADMA levels, as NO inhibit Ang-2 exocytosis from WPB.

In the present study, there was significant positive correlation between serum Ang-2 and serum phosphorus level, one of the non-traditional CV risk factors known to increase with loss of renal function due to secondary hyperparathyroidism, reduced GFR or hypocalcemia, this correlation was also supported by David S et al. [29]

In the present study, there was no significant correlation between Ang-2 level and any of the other tested CV risk factors including patients' age, iPTH, MAP, serum calcium, cholesterol or triglycerides. This may be explained by the effect of drugs that control or nearly control patients' blood pressure, elevate serum calcium to near normal values and malnutrition that may accompany CKD with associated negative impact on serum lipid. Furthermore, there was no detectable influence of erythropoietin or vitamin D treatment on circulating Ang-2 levels.

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Original Article

Nutritional Assessment of Patients with End Stage Renal Disease Undergoing Hemodialysis in Alexandria, Egypt.

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Abstract:

Background: Malnutrition (MN) is so prevalent in hemodialysis patients (HD patients) worldwide that it adversely affects their prognosis; being associated with an increased morbidity and mortality in these patients. However, recent data regarding the nutritional status among HD patients in Egypt is lacking. The purpose of this study was to evaluate the nutritional status of these patients at the dialysis unit of ElMoasat University hospital and medical research institute in Alexandria, Egypt using an economical nutritional assessment protocol consisted of anthropometric measurements, a biochemical blood measurement (serum albumin) and the seven-point Subjective Global Assessment (SGA**) Settings and Design:** A cross sectional study was done at the dialysis unit of ElMoassat University Hospital and Medical Research Institute in Alexandria, Egypt. **Subjects and Methods:** The study included 160 patients undergoing maintenance hemodialysis. Their nutritional status was assessed using subjective global assessment, anthropometric and biochemical measurement (serum albumin). **Statistical analysis used:** Data were fed to the computer and analysed using IBM SPSS software package version 20.0. **Results:** The present study revealed that among HD patients, 86.3% were mild to moderately malnourished. Mean serum albumin, BMI, TSF and MAMC were significantly lower in malnourished patients compared to well nourished (p= <0.001). MN was more prevalent among HD patients aged ≥45 years (51.4%). Older HD patients (≥45 years) had a significantly lower level of Serum Albumin (3.96± 0.41, p = 0.039), BMI (23.36±4.34, p=0.004), TSF (14.29±4.07, p=0.001) and MAMC (18.18±2.82, p=0.001) when compared with younger HD patients (<45 years). The seven-point SGA correlated positively with objective nutritional markers including Albumin, BMI, TSF, MAMC and Age. **Conclusions:** The study concluded that MN is prevalent among HD patient in Alexandria, Egypt and that the Subjective global assessment is a reliable, precise, and rapid method for estimating the nutritional status in patients on HD.

Keywords: Malnutrition; Nutritional Status; Hemodialysis Patients; Subjective Global Assessment (SGA).

Introduction:

Chronic kidney disease (CKD) is currently a public health problem[(1)]. CKD is a slow, progressive, and irreversible loss of kidney function. Because this loss is slow and progressive, it results in an adaptive process in which the patient remains asymptomatic for some time[(2)]. However, when the kidneys can no longer adequately remove the metabolic degradation products, dialysis treatment should be initiated[(3)]. According to the most recent Egyptian renal registry in 2008, the prevalence of end stage renal disease (ESRD) is 483 per million population and the total recorded number of ESRD patients on dialysis is 40000[(4)].

Despite the benefits of HD in prolonging the survival of patients with CKD, the conditions imposed by the disease and dialysis therapy result in a series of organic changes, with several complications and

nutritional changes[5]. Individuals undergoing dialysis have a significant prevalence of malnutrition (MN) , it is estimated that 50% to 70% of dialysis patients suffer from protein energy malnutrition[6], which is classified as mild, moderate, and severe [7].

The cause of MN is multifactorial and includes inadequate food intake, hormonal and gastrointestinal disorders, dietary restrictions, drugs that alter nutrient absorption, insufficient dialysis, and constant presence of associated diseases, in addition to the hypercatabolic effect of uremia, acidosis, and HD procedure[8]. MN leads to increased susceptibility to infection, impaired wound healing, poor rehabilitation, fatigue, malaise, and increased rates of hospitalization, morbidity and mortality[9,10].

Thus, MN constitute an important risk factor for the outcome of these patients[11], and hence, assessing the nutritional status of HD patients is essential both to prevent MN and to indicate appropriate intervention in malnourished patients[7,12].

Since there is no gold standard for assessing nutritional status in CKD patients, nutrition guidelines recommended the use of a combination of methods to minimize errors with nutritional diagnosis , such as the subjective global assessment (SGA), anthropometric parameters, biochemical analysis, bioelectrical impedance analysis and dual energy X-ray absorptiometry[13,14].While some techniques may work well in research situations, they are often not practical in clinical situations because they require expensive equipment or too much time.

Considering the importance of MN in HD patients, the aim of this study was to determine, the prevalence of MN among HD patients in the dialysis unit of ElMoassat University Hospital and Medical research Institute in Alexandria, Egypt using an economical nutritional assessment protocol consisted of anthropometric measurements, a biochemical blood measurement (serum albumin) and the seven-point SGA[15,16] .

Subjects and Methods:

This cross-sectional study included 160 adult patients with ESRD who had completed a minimum of six months duration on HD in the dialysis unit of El Moassat University Hospital and Medical research Institute in Alexandria, Egypt. Of which 93 were males and 67 were females with a mean age of 42.71 ± 11.68 years. All patients were informed of the purpose of the study and each patient signed a consent form. The study was conducted during the period from March to June 2013, approved by ethics committee of Alexandria Faculty of Medicine and was carried out according to the declaration of Helsinki. The patients' information such as age, sex, and duration of hemodialysis (months) were recorded.

The Nutritional Status was Assessed using:

I. The Seven-Point Subjective Global Assessment tool:

It includes two major categories: history and physical examination.

The history portion of the seven-point SGA is comprised of five sections:

- Weight/weight change;
- Dietary intake;
- Gastrointestinal symptoms;
- Functional capacity;
- Comorbidities as related to nutritional status.

The patient's weight loss from the preceding six months was recorded along with the current weight. All information regarding weight for the SGA was acquired from the patient's medical record. Other information required for the SGA was obtained by interviewing the patient.

The second major category of the seven-point SGA is the physical examination, which includes an evaluation of the patient for:

- Fat and muscle wasting.
- Edema.

The areas below the eye and around the triceps and biceps muscles were evaluated to determine subcutaneous fat loss. Muscle wasting was assessed by examining the temporalis muscle, prominence of the clavicles, the contour of the shoulders (rounded indicated well nourished; squared indicated MN), visibility of the scapula, and interosseous muscle between the thumb and forefinger. The area around the ankles was evaluated to determine edema.

Each section of the seven-point SGA was rated on a scale from one to seven. Based on subjective consideration of all the scores from each category, an overall number was assigned to each patient. A six or seven rating indicated a very mild risk of MN and/or well nourished; a three, four or five rating indicated mild to moderate MN; and a one or two rating indicated severe MN. From those ratings, patients were then classified into one of three groups: 1 = well nourished; 2 = moderate MN; and 3 = severe MN[17]. (Appendix1).

II. Anthropometric measurements

Several anthropometric measurements were obtained after dialysis in order to eliminate edema from affecting the accuracy of the measurements including post-dialysis weight, height, triceps skin-fold (TSF) and mid-arm circumference (MAC). From these, the body mass index (BMI) and mid arm muscle circumference (MAMC) were calculated.

- Weight was measured to the nearest 0.1 kg with a calibrated medical scale.
- Height was measured to the nearest 0.1 cm using a stadiometer.
- The BMI was calculated using the patient' post dialysis weight (kg) divided by the patient's height (cm) squared , patients were classified into underweight (<18.5 kg/m2), normal weight (18.5–24.99 kg/m2), overweight (25–29.99 kg/m2), and obese (>30 kg/m2)[18]
- MAC was measured with a flexible, no stretchable measuring tape. The fistula free arm was located to avoid the possibility of an inaccurate measurement due to fluid retention in the arm with the fistula. The measurement was recorded to the nearest 1 cm. Three measurements of MAC were obtained and then the average was calculated.
- TSF was measured with a body fat calliper and measurement was recorded to the nearest 1 mm. The measurement was repeated thrice and the average was calculated.
- The MAMC was calculated from the MAC and the TSF by the following formula: MAMC(cm) = MAC(cm) – (3.1415×TSF [cm])[13].

III. Biochemical parameters :

Serum Albumin was measured on all patients after the dialysis session. According to the National Kidney Foundation (NKF), serum albumin equal to or greater than 4 g/dL is the outcome goal for HD patients, accordingly albumin values were categorized into either optimal (≥4 g/dL) or sub-optimal (<4 g/dL) [19].

Statistical analysis:

Data were fed to the computer and analysed using IBM SPSS software package version 20.0. Comparisons between groups for categorical variables were assessed using Chi-square test. Student t-test was used to compare two groups for normally distributed quantitative variables. Spearman's rank correlation coefficients were calculated to determine bivariate relationships between results of the seven-point SGA and age, gender Albumin, BMI, TSF, MAMC. The statistical significance for these tests was set at P <0.05. Logistic regression was performed to estimate the magnitude of association between the response variable of MN and the explanatory variables (Albumin, BMI, TSF, MAMC, and age). Odds ratios were tested to estimate the likelihood risk of MN with the explanatory variables. Significance of the obtained results was judged at the 5% level.

Results:

The present study included 160 HD patients with a mean age of 42.71 ± 11.68 years, there were 93 males (58.1%) and 67 females (41.9%). Of the 160 HD patients who participated, 87(54.4%) were younger than 45 years, while the remaining 73 (45.6%) were 45 years and older. Mean BMI was 24.62 ± 5.21; almost one quarter (23.1%) of the HD patients were underweight, 30.6% were normal, 26.9% were overweight and about 19% were obese. One Hundred and thirty eight of HD patients (86.3%) were mild to moderately malnourished according to SGA score, compared to only 22 (13.8 %) of the patients who were well-nourished. Mean serum albumin was 4.03 ± 0.39 and 41.9 % of patients had low albumin (Table I).

This study revealed that the mean serum albumin, BMI, TSF and MAMC were significantly lower in malnourished patients compared to well nourished (p= <0.001). MN was more prevalent among HD patients aged ≥45 years (51.4%, n = 71) as compared to those aged <45 years (48.6%, n = 67), while almost ninety percent of those who were well nourished were under 45 years. Among those who were malnourished, 58.0% were male, more than one quarter (26.8%) were underweight and 47.8% had a suboptimal serum albumin level. No significant difference was found between male and female regarding SGA scoring (P = 0.921) (Table II).

Table (III) shows that older HD patients (≥45 years) had a significantly lower level of Serum Albumin (3.96± 0.41, p = 0.039), BMI (23.36±4.34, p=0.004), TSF (14.29±4.07, p=0.001) and MAMC (18.18±2.82, p=0.001) when compared with younger HD patients (<45 years) (4.08±0.35, 25.68±5.64, 18.52±5.20, and 20.55±2.62 respectively).

The seven-point SGA correlated positively with objective nutritional markers including Albumin, BMI, TSF, MAMC and Age (rs = 0.352, P = <0.001; rs = 0.158, P =0.045; rs = 0.354, P = <0.001; rs = 0.231, P =0.003; and rs = -0.298, P = <0.001, respectively) as shown in table (IV).

In the present study Odds ratios were significant for age, BMI, and TSF. Older HD patients had 20% higher risk for being malnourished than younger HD patients (OR= 1.208, 95% CI =1.012-1.441). Those with lower BMI and TSF had a significantly lower chance to be well nourished (OR=0.786, 95% CI: 0.634 0,975– OR=0.424, 95% CI= 0.239, 0.753 respectively) (Table IV).

Table I: Distribution of studied cases according to different parameters (n = 160)

	No (%)
Age (years)	**42.71 ± 11.68**
<45	87(54.4%)
≥45	73(45.6%)
Sex	
Male	93(58.1%)
Female	67(41.9%)
BMI	**24.62 ± 5.21**
Under weight	37(23.1%)
Normal	49(30.6%)
Over weight	43(26.9%)
Obese grade I	21(13.10%)
Obese grade II	10(6.3%)
SGA	**3.90 ± 1.11**
Mild /Moderate (malnourished)	138(86.3%)
Very Mild (Well nourished)	22(13.8%)
TSF	**16.59 ± 5.16**
MAMC	**19.47 ± 2.95**
Serum albumin	**4.03 ± 0.39**
<4 suboptimal	67(41.9%)
≥4 Optimal	93(58.1%)

Qualitative data were described using number and percent while normally quantitative data was expressed in mean ± SD.

Table II: Comparison between malnourished and well-nourished cases according to different parameters

	SGA		
	Malnourished (n = 138)	**Well-nourished (n = 22)**	**p**
Age (years)	**43.96 ± 11.84**	**34.86 ± 6.63**	0.001*
<45	67(48.6%)	20(90.9%)	0.001*
≥45	71(51.4%)	2(9.1%)	
Sex			
Male	80(58.0%)	13(59.1%)	0.921
Female	58(42.0%)	9(40.9%)	
BMI	**23.88 ± 4.77**	**29.24 ± 5.55**	<0.001*
Under weight	37(26.8%)	0(0.0%)	
Normal	42(30.4%)	7(31.8%)	
Over weight	38(27.5%)	5(22.7%)	<0.001*
Obese grade I	18(13.0%)	3(13.6%)	
Obese grade II	3(2.2%)	7(31.8%)	
TSF	15.43 ± 4.55	23.82 ± 1.71	<0.001*
MAMC	19.03 ± 2.91	22.24 ± 1.17	<0.001*
Serum albumin	**3.98 ± 0.40**	**4.30 ± 0.12**	<0.001*
<4 sub optimal	66(47.8%)	1(4.5%)	<0.001*
≥4 Optimal	72(52.2%)	21(95.5%)	

Qualitative data were described using number and percent and was compared using Chi square test while normally quantitative data was expressed in mean ± SD and was compared using student t-test.

Table III: Comparison between the two age groups according to different parameters

	Age		
	<45 (n = 87)	**≥45 (n = 73)**	**p**
Serum albumin	**4.08 ± 0.35**	**3.96 ± 0.41**	0.039*
<4 suboptimal	32(36.8%)	35(47.9%)	0.198
≥4 Optimal	55(63.2%)	38(52.1%)	
BMI	**25.68 ± 5.64**	**23.36 ± 4.34**	0.004*
Under weight	18(20.7%)	19(26.0%)	
Normal	26(29.9%)	23(31.5%)	
Over weight	18(20.7%)	25(34.2%)	0.015*
Obese grade I	16(18.4%)	5(6.8%)	
Obese grade II	9(10.3%)	1(1.4%)	
TSF	18.52 ± 5.20	14.29 ± 4.07	0.001*
MAMC	20.55 ± 2.62	18.18 ± 2.82	0.001*

Qualitative data were described using number and percent and was compared using Chi square test while normally quantitative data was expressed in mean ± SD and was compared using student t-test.

Table IV: Correlations to determine bivariate relationships between results of the seven-point SGA and albumin, BMI, TSF, MAMC, age.

	SGA	
	r_s	**p**
Serum albumin	0.352	<0.001*
BMI	0.158	0.045*
TSF	0.354	<0.001*
MAMC	0.231	0.003*
Age	-0.298	<0.001*

r_s: Spearman coefficient *: Statistically significant at p ≤ 0.05

Table IV: Multivariate analysis logistic regression for SGA with risk factors

	B	**Sig**	**OR**	**95% CI**	
				Lower	**Upper**
Age	0.189	0.036*	1.208	1.012	1.441
BMI	-0.241	0.029*	0.786	0.634	0.975
TSF	-0.858	0.003*	0.424	0.239	0.753
MAMC	-0.686	0.052	0.503	0.252	1.005
Serum albumin	-3.967	0.132	0.019	0.000	3.300

Discussion:

There is an increasing incidence and prevalence rate of patients on hemodialysis worldwide. MN is common and associated with increased morbidity and mortality in hemodialysis patients [9,10].

The assessment of hemodialysis patient's nutritional status is a challenge[12]. The NKF-K/DOQI Clinical Practice Guidelines for nutrition in patients on maintenance dialysis and for evaluation of protein–energy malnutrition and nutritional status recommended assessment with a combination of valid, and complementary, measures rather than any single measure alone as malnutrition may be identified with greater sensitivity and specificity using a combination of factors[13]. SGA, although subjective, seems to be a valid and reliable method[17,20,21] , it has good correlation with other nutritional markers and significant prognostic value for morbidity and mortality in patients with chronic kidney disease[12,20,21]. Furthermore, the method is recommended by international organizations for nutritional assessment and MN detection in adult dialysis patients [14]. Anthropometric parameters are reliable and valid measurements that indicate nutritional status in HD patients[13,15,22]. Concerning serum albumin, several studies have demonstrated that albumin is a valid indicator of nutritional status in HD patients[19,22].

The present study revealed that among 160 HD patients recruited in this study the majority (86.3 %, n=138) were mildly to moderately malnourished. This result is nearly consistent with findings of other studies. Janardhan et al.[23] found that among 66 patients undergoing hemodialysis in their study in India , the MN rate was 91%, Espahbodi et al.[24] stated that among 105 patients, 98 (93.33%) patients consisted of 56 males and 42 females had mild to moderate MN and three (2.86%) women had severe MN. In another study by Tayyem et al.[25] in Jordan, the MN rate was 61.8 % among 178 patients undergoing hemodialysis. Another study by Afshar et al.[26] detected MN in 40.7% of 54 patients undergoing hemodialysis in capital city of Iran .The differences in prevalence may be because of environmental diversity and different diet regiments in various regions. These findings indicates the urgent need for a strict nutritional and dietary counselling program, to help and advice patients on the most basic ways to improve their nutritional status and to better assess the quality of their hemodialysis. This makes it necessary to assess the nutritional status of renal failure patients periodically and take measures to prevent protein energy MN.

In HD patients, gender and age are related to nutritional status. Poor nutritional status in HD patients varies between genders[27–29]. In the present study, a higher percentage of males (58.0%) were malnourished as compared to 42.0% females. This may be due to the socioeconomic status and life styles of this particular patient sample, as the sample was not randomly selected. Similarly Tayyem and Mrayyan[30] reported that a higher proportion of males (76.2%) were malnourished when compared to 48.9% malnourished females in their study.

This study reported that a higher percentage (51.4 %) of older HD patients (≥45 years) were malnourished this is consistent with results from other studies that have indicated that older HDP tend to be malnourished[31–33]. Old age is often associated with high rates of MN due to difficulties related to this stage of life, such as difficulty buying food and preparing meals, decreased appetite, poor dentition, decreased taste, more chronic or acute diseases, reduced mobility and cognition, and consequently decreased food intake[12]. Particular attention to the nutritional status of older HDP is warranted to decrease the prevalence of MN.

MN has an objective repercussion on anthropometric and laboratory parameters, resulting in low body weight, fat store depletion, body protein loss, and low serum albumin in HD patients[29,34]. The present study reported that serum albumin differ significantly between well and malnourished HD patients and between age groups; mean serum albumin concentrations among malnourished HD patients was <4 g/dL, which the NKF considers sub-optimal and predictive of mortality risk,(8) compared to a serum albumin level ≥4 g/dl among well-nourished HD patients . Our results support previous results of Al Saran et al.[27] , which state that among Saudi HD patients, despite efficient dialysis prescription indicated by mean Kt/v of 1.4 ± 0.15, the mean serum albumin level is low (mean 34 ± 4 g/L). Although this study revealed that according to BMI 26.9 % of HD patients were overweight and 19.13% were obese, which is consistent with the results reported by Dewar et al.[35] , who found in his study population, that 46.4% were overweight or obese. However, this can represent sarcopenic obesity where there is loss of muscle mass and despite this, an excess of adipose tissue. This is seen in disease states where lack of physical activity leads to loss of muscle mass and gain in adipose mass. BMI, TSF, and MAMC were significantly lower in the malnourished HD patients in comparison with the well-nourished HD patients.

Anthropometric measurements (BMI, TSF, and MAMC), age and serum albumin correlated significantly with the seven-point SGA, confirming that they are predictors of the nutritional status of HD patients. Several studies in both eastern and western cultures corroborate these findings [10,26,30,36–38]. As BMI, TSF, MAMC and albumin decreased, the HD patients became malnourished indicating that all these parameters are necessary in determining the nutritional status of HD patients.

The present study showed that the nutrition assessment protocol which consisted of anthropometric measurements, a biochemical blood measurement (serum albumin) and the seven-point SGA can be used as a reliable, rapid, and precise method for nutritional assessment in office, hospital and HD centres. It is preferred in comparison with other time-consuming methods for nutritional assessment

Conclusion:

The present study concluded that malnutrition is prevalent among HD patients in Alexandria, Egypt, using an economical assessment protocol consisted of anthropometric measurements, a biochemical blood measurement (serum albumin) and the seven-point SGA.

The nutritional status of HD patients' needs more attention and it is recommended that periodical monitoring of the nutritional status should be part of the follow-up of dialysis patients, which is fundamental for preventing, diagnosing, and treating MN.

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SUBJECTIVE GLOBAL ASSESSMENT RATING FORM

Patient Name: **ID #:** **Date:**

HISTORY

WEIGHT/WEIGHT CHANGE: ***(Included in K/DOQI SGA)*** — **Rate 1-7**

1. **Baseline Wt:** _____(Dry weight from 6 months ago)
   Current Wt: _____(Dry weight today)
   Actual Wt loss/past 6 mo: _____ **% loss:**_____(actual loss from baseline or last SGA)
2. **Weight change over past two weeks:** _____No change _____Increase _____Decrease

DIETARY INTAKE No Change_____(Adequate) No Change_____(Inadequate)

1. Change: Sub optimal Intake: _____ Protein _____ Kcal _____ Duration_____
   Full Liquid: _____ Hypocaloric Liquid _____ Starvation _____

GASTROINTESTINAL SYMPTOMS ***(Included in K/DOQI SGA-anorexia or causes of anorexia)***

Symptom:	Frequency:*	Duration:+
_____ None	_____	_____
_____ Anorexia	_____	_____
_____ Nausea	_____	_____
_____ Vomiting	_____	_____
_____ Diarrhea	_____	_____

Never, daily, 2-3 times/wk, 1-2 times/wk > 2 weeks, < 2 weeks

FUNCTIONAL CAPACITY — b

Description	Duration:
_____ No Dysfunction	_____
_____ Change in function	_____
_____ Difficulty with ambulation	_____
_____ Difficulty with activity (Patient specific "normal")	_____
_____ Light activity	_____
_____ Bed/chair ridden with little or no activity	_____
_____ Improvement in function	_____

DISEASE STATE/COMORBIDITIES AS RELATED TO NUTRITIONAL NEEDS

Primary Diagnosis_____Comorbidities_____

Normal requirements ____Increased requirements____ Decreased requirements _____

Acute Metabolic Stress: _____None _____Low _____Moderate _____High

PHYSICAL EXAM

_____ Loss of subcutaneous fat (Below eye, triceps, biceps, chest) ***(Included in K/DOQI SGA)*** ____Some areas _____All areas

_____ Muscle wasting (Temple, clavicle, scapula, ribs, quadriceps, calf, knee, interosseous ***(Included in K/DOQI SGA)*** ____Some areas _____All areas

_____ Edema (Related to undernutrition/use to evaluate weight change)

OVERALL SGA RATING

Very mild risk to well-nourished=6 or 7 most categories or significant, continued improvement.

Mild-moderate = 3, 4, or 5 ratings. No clear sign of normal status or severe malnutrition.

Severely Malnourished = 1 or 2 ratings in most categories/significant physical signs of malnutrition.

Original Article

Prospective Validation of the FibroTest Score in Assessing Liver Fibrosis in Hepatitis C Infection with Genotype 4.

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Abstract:

Introduction: Although liver biopsy is still considered the gold standard for assessment of liver fibrosis, it is invasive and may have some risks. FibroTest is used as non-invasive biochemical markers score for assessment of liver fibrosis. Our aim was to evaluate the performance of FibroTest in the diagnosis of hepatic fibrosis in patients chronically infected with hepatitis C virus genotype -4 (HCV-G4). **Methods:** One hundred and twenty two hepatitis with chronic HCV-G4 were properly selected and subjected for both liver biopsy (LB) and FibroTest biochemical markers score. All LBs were examined by two independent pathologists and scored using METAVIR scoring system. Serum samples were taken at the time of the LB to measure the 5 serum biochemical markers required for the FibroTest score (Alpha-2 macroglobulin, haptoglobin, apolipoprotien A1, total bilirubin and gamma-glutamyl transpeptidase). **Results:** According to the METAVIR scoring system, F0 was found in 2.4%, F1 in 45.9%, F2 in 16.4%, F3 in 27.9% and F4 in 7.4% of studied patients. According to the Fibro-Test results F0 was found in 5.7%, F1 in 30.3%, F2 in 19.7%, F3 in 16.4% and F4 in 27.9% of the studied patients. Fibro-Test at the cutoff value of 0.555 could discriminate early from advanced fibrosis with an area under the receiver operator characteristic curve of 0.72, sensitivity of 78%, specificity of 52%, PPV of 66%, NPV of 67% and accuracy of 66%. **Conclusion:** As FibroTest score overestimates the stage of advanced fibrosis, it should not be considered as a reliable surrogate for liver biopsy in hepatitis C infection with genotype 4.

Key Words: Liver fibrosis, Biopsy, FibroTest

Introduction:

Chronic hepatitis C virus (HCV) infection is a major health problem with around two hundred million individuals affected worldwide. Egypt has high prevalence of HCV, where genotype 4 represents over 90% of cases.[1-4] The natural course of chronic hepatitis C is characterized by progressive fibrosis in the inflamed liver and eventually leading to cirrhosis.[5] Assessment of the stage of liver fibrosis is important for diagnosis, treatment, and follow-up both during treatment and after cessation of treatment. So, one of the major challenges is how to best evaluate the degree of fibrosis among the increasing numbers of those patients.

Liver biopsy is considered the gold standard for fibrosis staging and has traditionally been used in the management of hepatitis c patients to provide important information about disease prognosis as well as about the likelihood of response to therapy.[6,7] However, liver biopsy is an invasive procedure and may occasionally be associated with major complications, in addition to the increased rate of false negatives (15-20%) in cases with non- homogenous hepatic fibrosis with sampling error occurring most frequently when small biopsies (<10 mm) are obtained.[8-10]

There has been recent interest in developing serum markers that can reliably predict the presence of fibrosis and subsequently obviate the need for a liver biopsy. FibroTest (FT) is non-invasive score of liver fibrosis that combines the quantitative results of 5 serum biochemical markers (alpha-2 macroglobulin, haptoglobin, apolipoprotein A1, gamm- glutamyl transpeptidase (GGT) and bilirubin) and adjusted with the patient's age and sex in a patented algorithm to generate a measure of fibrosis.[11] FT has been validated in several studies in patients with chronic hepatitis C,[12-15] chronic hepatitis B,[12] alcoholic[16] and non-alcoholic fatty liver disease.[17]

Our aim was to evaluate the performance of FibroTest in an independent prospective cohort of hepatitis C patients with genotype 4.

Patients and Methods:

Patients:

The study included 122 consecutive hepatitis C patients (114 males and 8 females with mean age 41.7±8.1 and age range 20-58 years). All patients were naïve with no history of antiviral treatment. Detection of HCV in the serum was done on the bases of positive HCV RNA by polymerase Chain Reaction (PCR). All the included patients had compensated liver disease. They were selected from outpatients attendees Specialized Medical Hospital, Mansoura University for pretreatment assessment before antiviral therapy in the period from August 2011 to June 2013. The exclusion criteria were co-infection with HBV or HIV or any liver disease other than hepatitis C and patients with decompensated liver disease, cholestasis or hemolysis. Patients with inadequate liver biopsy specimens were also excluded from the study.

Approval:

The study protocol conformed to Medical Sciences Ethics Committee of Mansoura Faculty of Medicine, conducted in accordance with the Declaration of Helsinki and International conference on Harmonization Guidelines, and each participant gave an informed written consent before being enrolled in the study and before performing liver biopsy.

Investigations recommended before interferon therapy included:

- Detection of anti-HCV antibodies, hepatitis B virus surface antigen and core antibodies by immunoassay. Detection of HCV-RNA by polymerase chain reaction (Cobas Amplicor, Roche diagnostics).
- Determination of serum ALT, serum AST, serum albumin, serum bilirubin, serum Alkaline phosphatase, serum creatinine and blood glucose by automated biochemistry analyzer (Cobas Integra 400, Roch diagnostics) and prothrombin time.
- Detection of ANA, AFP and IHA for Schistosomiasis.
- Abdominal ultrasound.
- Ocular fundus examination.
- Electrocardiography for patients above 40 years.

Histo-pathological assessment:

Ultrasound guided true-cut needle liver biopsy was taken under local anesthesia. Liver biopsy specimens were prepared from paraffin blocks and stained with Haematoxyline and eosin stain, Masson's Trichrome, Reticulin, PAS and PAS-diastase stains. All liver biopsies were interpreted by two pathologists who were unaware of the clinical and biochemical data of the patients. Evaluation of the stage of fibrosis was done according to the METAVIR scoring system.[18,19] Fibrosis was staged on a scale of 0-4: F0, no fibrosis; F1, portal fibrosis without septa; F2, few septa; F3, numerous septa without cirrhosis; F4, cirrhosis. Patients with scores F0, F1, F2 were considered to have insignificant or early fibrosis and those with scores F3 and F4 were considered to have advanced fibrosis. A liver biopsy was considered to be adequate if the core sample was >15 mm and had more than 10 portal tracts in the specimen according to the APASL consensus recommendations.[20]

Fibro-Test:

Serum samples were taken at the time of the liver biopsy for measurement of the 5 serum biochemical markers of the FT (Alpha-2 macroglobulin, haptoglobin and apolipoprotien A1, total bilirubin and gamma-glutamyl transpeptidase). The FT was done at Egyptian liver Hospital laboratory (accredited by the Biopredictive Company). The FT score provides a numerical quantitative estimate of liver fibrosis ranging from 0.00 to 1.00, classified on a scale ranging from F0 to F4, corresponding to the METAVIR scoring system. The FT cutoffs for presumed fibrosis stages were 0.00-0.21 (F0), 0.22-0.27 (F0-F1), 0.28-0.31 (F1), 0.32-0.48 (F1-F2), 0.49-0.58 (F2), 0.59-0.72 (F3), 0.73-0.74 (F3-F4), and >0.75 (F4)[12].

Statistical analysis:

The statistical analysis of data was done by using excel program and SPSS program statistical package for social science version 16. The description of data was done in form of mean ±SD for quantitative data, frequency and proportion for qualitative data. The analysis of the data was done to test statistically significant difference between groups. For quantitative data student`s t-test was used to compare between 2 groups. Chi square test was used to compare qualitative data. Area under the receiver operating characteristic curve (AUROC) was drawn to detect the cutoff point with highest sensitivity and specificity to diagnose cirrhosis by the FT score. P is significant if ≤ 0.05.

Results:

This is a cross sectional study conducted on 134 patients selcted from chronic hepatitis C patients attended Mansoura Specialized Medical Hospital, Mansoura University, for pretreatment assessment of antiviral treatment, twelve patients were excluded due to inadequate liver biopsy. The demographic, laboratory, histologic and FT results of the studied patients (122) were described in (Table I). The mean age of the included patients was 41.7±8.1 years with a male predominance (93.4%). The mean ALT was 75.1± 47.8IU/L (range 14-242) and the mean AST was 56.8± 47.8 IU/L (range 15-210). According to the METAVIR scoring system, F0 was found in 2.4 %, F1 in 45.9%, F2 in 16.4%, F3 in 27.9% and F4 in 7.4% of studied patients. According to the FT results, F0 was found in 5.7%, F1 in 30.3%, F2 in 19.7%, F3 in 16.4% and F4 in 27.9% of the studied patients. (Fig 1)

Table II, showed matching of the stages of fibrosis by liver biopsy with that obtained by FT. Among patients with advanced fibrosis the FT was identically matched with the liver biopsy in 18.6%, overestimated the stage of fibrosis in 44.2% and underestimated the stage of fibrosis in 37.7% of cases. Also, in patients with no/mild fibrosis, identical matching was detected in 39.2% of cases with overestimation in 48.1% and underestimation in 12.7%. So, the overall results of the test were identical matching, overestimation and underestimation in 32%, 46.7% and 21.3% respectively.

Significant correlation was found between the FT results and that of liver biopsy (r=0.38, P<0.001). The sensitivity, specificity, Positive predictive value (PPV), Negative predictive value (NPV) and accuracy of the FT score were 78%, 52%, 66%, 67% and 66% respectively (Table III).

The utility of the FT as non-invasive score for assessment of liver fibrosis was statistically evaluated by ROC curve. FT at the cut-off point of 0.555 could discriminate early from advanced stages of fibrosis with an area under.

ROC curve (AUROC) of 0.72, sensitivity of 78%, specificity of 52%, PPV of 66%, NPV of 67% and accuracy of 66% (Figure 2).

Table I: Demographic, laboratory, histologic and FibroTest results of the studied patients:

Variable	Unit/category	Result
Age	Years	41.7 ± 8.1
Sex	Male	114 (93.4%
	Female	8 (6.6%)
Aspartate transaminase	(IU/L)	56.8 ± 47.8
Alanine transaminase	(IU/L)	75.1 ± 47.8
Serum Albumin	gm/dL	4.3 ± 0.3
Platelets	($x10^3$)	171.6 ± 46.1
METAVIR fibrosis stage	0	3 (2.4%)
	1	56 (45.9%)
	2	20 (16.4%)
	3	34 (27.9%)
	4	9 (7.4%)
Fibro-Test fibrosis stage	0	7 (5.7%)
	1	37 (30.3%)
	2	24 (19.7%)
	3	20 (16.4%)
	4	34 (27.9%)

Data expressed as mean ± SD or number (percent) as appropriate.

Table II: Matching of the stages of fibrosis by liver biopsy with that obtained by Fibro-Test:

	Severe fibrosis (F3-F4)	No/mild fibrosis (F0-F2)	Over all
Identical matching	8 (18.6)	31 (39.2)	39 (32)
Over estimation	19 (44.2)	38 (48.1)	57(46.7)
Under estimation	16 (37.7)	10 (12.7)	26(21.3)

Data expressed as number (percent)

Table III: Performance characteristics of the Fibro-Test at a cutoff of 0.555 compared to liver biopsy.

Performance test	Results
Sensitivity	78%
Specificity	52%
Positive predictive value	66%
Negative predictive value	67%
Accuracy	66%

FIBROSIS GRADE

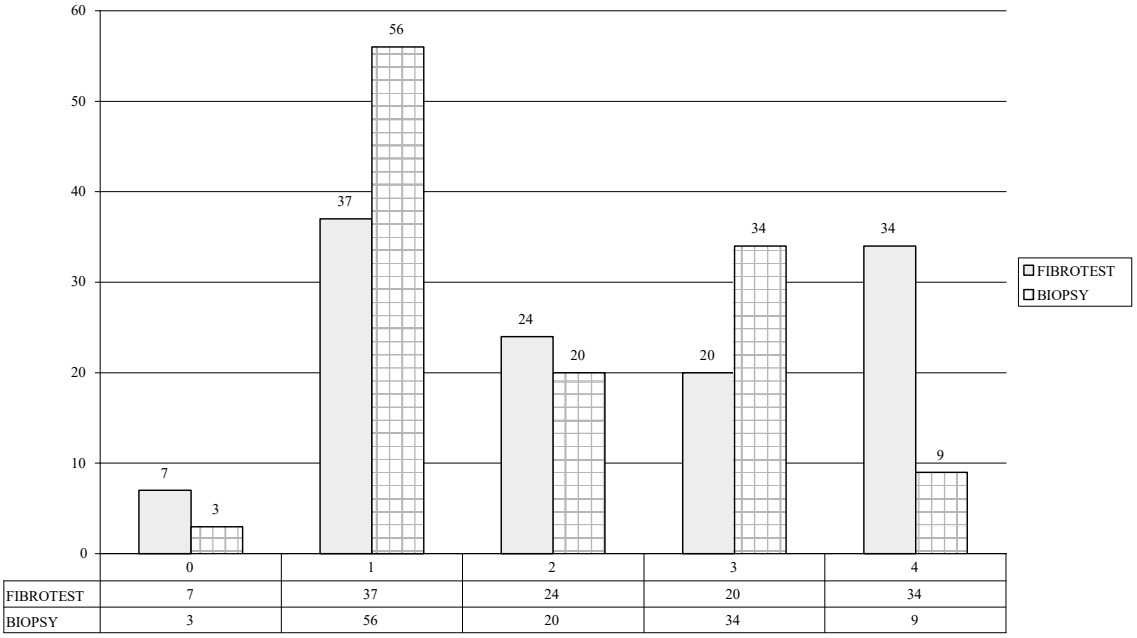

Figure 1: Comparison of fibrosis stages determined by the FibroTest with that of liver biopsy.

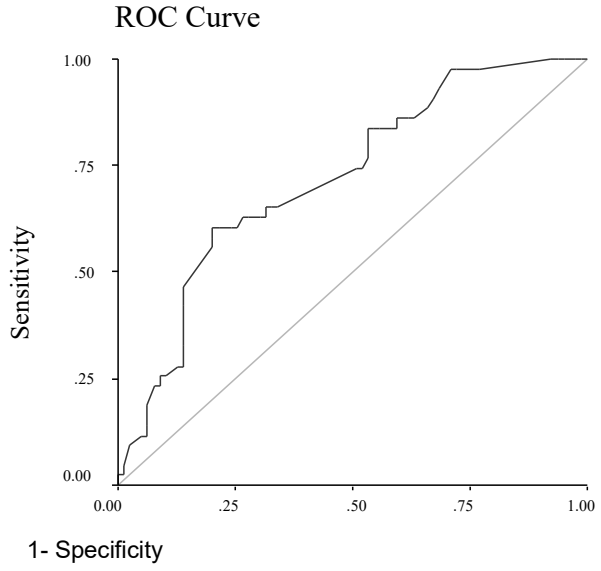

Figure 2: Area under the ROC curve of FibroTest for discrimination of early fibrosis (F0-F2) from advanced fibrosis/cirrhosis (F3, F4). Fibro-Test at cut off point of 0.555 could differentiate mild from severe fibrosis in patients with chronic HCV.

Discussion:

Besides its invasive nature and associated complications, LB has many problems including the following: the specimen obtained represents only 1/50,000 of the total liver volume; the uneven distribution of fibrosis throughout the liver; the inter- and intra-observer variation, as well as the problems related to the specimen obtained in terms of its dimension and the number of portal tracts. Marked reductions in sensitivity for detection of significant fibrosis may occur with biopsies <3 cm in length and with fragmented biopsies. All the above limitations make looking for alternative noninvasive and better markers for hepatic fibrosis a necessity.

This study proved poor matching between the stages of fibrosis obtained by FT and that determined by liver biopsy. While, advanced fibrosis/cirrhosis (F3, F4) was found in liver biopsies in 35% of cases, FT estimated such stages in 44% of patients. Moreover, FT diagnosed evident cirrhosis (F4) in 34 patients (27.9%), while liver biopsy diagnosed cirrhosis in only 9 patients (7.4%). The overestimation of advanced hepatic fibrosis by FT makes it a misleading test for the treating physicians. Furthermore, during management of chronic hepatitis C, diagnosis of F4 carries important therapeutic and prognostic considerations. In that situation, over diagnosis of cirrhosis may have deleterious psychosocial effects with negative impact on either the management decision of individual patients or on the quality of life. In this regard, when FT is applied about 20% of patients will be deprived unnecessarily from treatment as in many areas F4 patients are excluded from therapy.

The results of this study come in accordance with Fontanges et al.[21] who reported that the FT has a NPV of 92% and a PPV of 52% and they attributed discordance of the FT results with the liver biopsy to significant increase of GGT and alpha-2 macroglobulin, with consequent overestimation of fibrosis. Also with Rossi et al.[22] who reported that, the diagnostic yield of the FT is poor and found that FT has a NPV of 85% and a PPV of 78% and the test eliminates the need for liver biopsy in only 26% of patients and does not accurately predict the presence or absence of liver fibrosis.

On the other hand, the results of the current study are discordant with that of the French investigators who invented the test and reported that the FT score in HCV patients gave a 100% NPV for the absence of clinically significant fibrosis or a 91% PPV for its presence and reported that FT could achieve a 46% reduction in the number of liver biopsies required to manage chronic HCV infection.[11] Although the diagnostic accuracy of FT has been previously validated in a meta-analysis[23] yet these results should be considered cautiously as many of the included studies were retrospective and others did not follow the optimal guidelines for liver biopsy, so only few independent studies were included. However, the present study was conducted prospectively with strict adherence to the optimal criteria of the liver biopsy according to APASL consensus recommendations [18]

A possible reason for the poor results of the FT in this study may be related to the difference in HCV genotype in our patients where genotype 4 is the most prevalent whereas the previous studies were done on patients in

Europe and Australia who have predominantly genotypes 1, 2 and 3 infections.[4,24,25]

The ideal blood test for diagnosis of hepatic fibrosis should be accurate, not expensive, simple and reproducible. Applying this to FT in our study, there is overestimation of fibrosis in a significant number of patients, and the test is expensive, not simple and there is a known potential inter-laboratory differences [26].

In conclusion, as FT Score overestimates the stage of advanced fibrosis, it should not be considered as a reliable surrogate for liver biopsy in patients with chronic HCV infection genotype 4. It is recommended that serum biochemical markers may be used either stepwise or in combination with other non-invasive tests such as imaging or elastrogarphy to improve accuracy of liver fibrosis assessment.

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Original Article

Glycated Albumin to Glycated Hemoglobin Ratio in Patients with Liver Cirrhosis and its Relation to Severity of Cirrhosis and Risk of Bleeding of Esophageal Varices.

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Abstract:

Introduction: Esophageal varices (EV) and variceal hemorrhage are serious consequences of portal hypertension in patients with chronic liver diseases. It is considered to be necessary for all cirrhotic patients to evaluate the risk of variceal bleeding. Although several biomarkers have been reported as predictors of the presence of varices, it is still difficult to assess the risk of variceal bleeding without esophagogastroduodenoscopy (EGD). The ratio of glycated albumin (GA) to glycated hemoglobin (HbA1c) was reported to increase with the progression of liver fibrosis. **Aims:** The aim of the study was to investigate whether the GA/HbA1c ratio is related to the severity of cirrhosis and bleeding risk of esophageal varices. **Methodology:** We measured the GA/HbA1c ratio in 80 subjects (20 patient with history of bleeding EV, 20 patient with no history of bleeding EV, 20 patient without EV, and 20 healthy control subjects) and analyzed its relationship with severity of cirrhosis and the presence of varices and its and bleeding risk. **Results:** The GA/HbA1c ratio correlated well with the severity of liver cirrhosis (expressed as Child score). Also, it was significantly higher in the patients who had esophageal varices than that in patients without varices. However, it does not correlate with the risk of variceal bleeding among these patients. **Conclusions:** The GA/HbA1c ratio is correlated with the severity of liver cirrhosis and increased significantly in patients with esophageal varices however, it does not correlate with the risk of variceal bleeding among those patients.

Keywords: Liver cirrhosis; Portal hypertension; Esophageal varices (EV); Glycated albumin (GA); Glycated hemoglobin (HbA1c).

Introduction:

Portal hypertension is a major complication of liver cirrhosis and can be a direct cause of variceal hemorrhage and of bleeding related death. The prevalence of varices in patients with cirrhosis is approximately 60-80% and the risk of bleeding is 25-35%. The incidence of esophageal varices (EVs) increases by nearly 5% per year, and the rate of progression from small to large varices is approximately 5 to 10% per year.(1)

Current guidelines, therefore, recommend that all cirrhotic patients should be screened for varices at diagnosis, with follow up every 2-3 years for patients without varices (depending upon liver disease severity) and 1-2 years for patients with small varices, to assess for enlargement of varices and need for prophylactic treatment.(2) This causes a significant burden and cost to endoscopy units and necessitate patients having repeated unpleasant procedures even when up to 50% may still not have developed esophageal varices 10 years after the initial diagnosis.(3)

If it were possible to predict oesophageal varices by non invasive means, this would restrict testing to the population deemed to be at most risk and reduce the number of endoscopies required. Such a screening test should be simple, quick, reproducible, and cost effective.

In this respect, several clinical, biological, ultrasonographic and elastographic (transient elastography - TE) methods have been proposed (and some of them validated) as noninvasive alternatives to endoscopy.[4]

Glycated proteins are known to reflect the plasma glucose level and glycated hemoglobin (HbA1c), is commonly used as an index of glycemic control in patients with diabetes mellitus[5,6]. Since the lifespan of erythrocytes is about 4 months, theHbA1c level is correlated with the level of glycemia for the past few months.[7]

Another glycated protein, glycated albumin (GA), reflects the plasma glucose level during the past few weeks, because the turnover of albumin is about 3 weeks.[8,9]

Although the ratio of GA/HbA1c is usually close to 3, patients with chronic liver disease (CLD) have a shortened lifespan of erythrocytes due to the hypersplenism, thus resulting in lower HbA1c levels relative to the plasma glucose level. Conversely, the turn-over period of serum albumin in CLD patients is increased to compensate for the reduced albumin production. Therefore, the GA levels in CLD patients are higher relative to the degree of glycemia.[10]

Since the HbA1c level is lower and the GA shows higher values, the GA/HbA1c ratio is assumed to be increased in CLD patients. In fact, the GA/HbA1c ratio in patients with CLD has been reported to show a reciprocal correlation with some indicators of hepatic function, irrespective of the mean plasma glucose levels.[11]

The aim of this work The aim of the study was to investigate whether the GA/HbA1c ratio is related to the severity of cirrhosis and bleeding risk of esophageal varices.

Patients and Methods:

We studied CLD patients (n = 60) and 20 control subjects and was classified into four groups as follows:

Group I: 20 patients with liver cirrhosis and EV which have previously bled.

Group II: 20 patients with liver cirrhosis and EV which have not yet bled.

Group III: 20 patients with liver cirrhosis without EV.

Group IV : 20 normal healthy control subjects.

Exclusion Criteria:

Any patient with malignancy, diabetes mellitus, endocrinal diseases, cardiovascular disease, pulmonary diseases, renal disease, collagenic diseases, on immunosuppressive therapy, also patients with history of EV band ligation, or sclerotherapy were excluded from this study. None of them had had primary prophylactic treatment for variceal bleeding or any surgical treatment for portal hypertension.

All patients were subjected to the following:

- History taking and thorough clinical examination.
- Electrocardiogram and chest X- ray: to exclude chest or cardiac disorders.
- Laboratory investigations: including prothrombin time, activity, and calculation of the international normalized ratio (INR), serum glucose, urea, creatinine, total proteins, albumin, total and direct fractions of bilirubin concentrations, as well as activities of alanine and aspartate aminotransferases, alkaline phosphatase and gamma glutemyl transpeptidase. Serum hepatitis-C virus antibodies detection using enzyme immunoassay. serum hepatitis-B virus surface antigen (HBsAg) using enzyme immunoassay. Complete urine analysis to exclude proteinuria.[12,13]
- Serum HbA_1c.[14]
- Serum GA and calculation of the GA to HBA_1c ratio.[15]

- Screening for patients will be scored according to Child-Pugh classification.[16]
- Abdominal ultrasound.
- Upper GIT endoscopy: was performed at our institution according to the standard procedures. If esophageal varices were detected, their size was graded based on the Beppu (Japanese) grading system.[17]
- Data were collected, revised and transferred into statistical package for social science (SPSS/ version 10). Results were expressed as means and standard deviation. Statistical tests used in this study were (t. test, Fisher Exact test, Mann Whitney test, Monte Carlo test & chi.square). A level of 5% was considered as the cutoff level of significance.

Results:

As regarding demographic data, the study consisted of 57 (73.5%) males and 23 (26.5%) females, their age ranged from 25 to 71 and 70 % of them were from rural areas with no statistically significant difference between different groups.

Presence of jaundice, splenomegaly, ascites, hepatic encephalopathy, spider angioma and/or palmer erythema was found to be statistically higher in cirrhotic patients with esophageal varices than in those without varices (p value = 0.004, < 0.001, < 0.001, 0.023, 0.025 and 0.028) respectively.

Laboratory investigations were done including liver functional tests (ALT, AST, GGT, alkaline phosphatase, albumin, total bilirubin, and prothrombin time). In addition, the HbA1c and GA levels were also measured in all subjects.

Serum bilirubin was found to be statistically higher in cirrhotic patients with esophageal varices than those without varices with p value (<0.001). Serum albumin was found to be statistically lower in cirrhotic patients with esophageal varices than those without varices with p value (<0.001).

Platelet count and PT activity were found to be statistically lower in cirrhotic patients with esophageal varices than those without varices with P value (<0.001) and in group I than group II with P value (0.041 and 0.032 respectively).

Child score was found to be statistically higher in cirrhotic patients with esophageal varices than those without varices with p value (<0.001).

Portal vein (PV) diameter was found to be statistically higher in cirrhotic patients with esophageal varices than those without varices with p value (0.002).

Spleen diameter was found to be statistically higher in cirrhotic patients with esophageal varices than those without varices with p value (<0.001) and in bleeders than non bleeders (p = 0.014). (Table I)

Esophageal Varices were graded according to Beppu (Japanese) grading system. In group I, no patient had F1 esophageal varices (EV), while 55% of patients in groups II were found to have F1 EV. F2 EV were found in 80% versus 40% of patients in groups I & II respectively. F3 EV were found in 20% versus 5% of patients in groups I & II respectively. There was statistically significant difference between both groups p value (<0.001). (table II)

In group I, 100% versus 20% of patients in group II were found to have **red colour signs**. There was a statistically significant difference between both groups with p value (<0.001).

North Italian Endoscopic Club (NIEC) index was calculated to estimate the esophageal variceal risk of bleeding. It ranged between 38.60 and 49.70 with a mean of 43.09 ± 3.74 in group I patients. In group II patients, it ranged between 24.90 and 43.30

with a mean of 32.80 ± 5.73 There was a statistically significant difference between both groups, being higher in group I p value (<0.001).

GA/HbA1c Ratio:

The GA/HbA1c ratio in patients with CLD has been reported to show an inverse correlation with several parameters of hepatic function.

We examined whether the GA/HbA1c ratio differed in patients with or without varices. Comparing the 60 cirrhotic patients with 20 control subjects there was a statistical significance between the two groups (p =0.041). Also in comparison patients with varices divided into 20 patient with history of bleeding (group I), 20 patient without history of bleeding (group II) and 20 patients without varices (groupIII), GA/HbA1c ratio was significantly higher in patients with varices than that in patients without varices (p = 0.008) suggesting that the increased GA/HbA1c ratio also correlates with the presence of varices. However, there was no statistically significant difference between those bleeders and non bleeders .

There was a significant positive correlation between GA/HbA1c ratio and Child score (r_s 0.263 and P = 0.042) as shown in (figure 1).

There was no correlation between NIEC index and GA/HbA1c ratio, ($r_{s=}$ 0.090, p=0.582).

The diagnostic performance of GA/HbA1c ratio was compared to that of endoscopy, considered the gold standard, in patients presenting with esophageal varices. The receiver operator characteristic (ROC) curve analysis generated a ratio cut off value (COV) of 6.35 that could discriminate cirrhotic patients with and without esophageal varices, with an area under the curve of 0.711 (p=0.008). The ratio COV of 6.35 gave a diagnostic specificity of 85% , a sensitivity of 60%, and predictive values (positive and negative) of 89% and 52% respectively, making this method a rather specific one capable of detecting esophageal varices in cirrhotic patients (85%), than estimating the degree of its severity among such patients (60%) as shown in figure (2).

Table (I): Comparison between the studied groups according to (portal vein and splenic diameter).

	Group I (n=20)	**Group II (n=20)**	**Cirrhotic with EV (n=40)**	**Cirrhotic without EV (n=20)**
PV diameter (mm)				
Min. – Max.	14.0 – 18.0	13.80 – 16.0	13.80 – 18.0	12.0 – 16.0
Mean ± SD.	15.59 ± 1.18	15.14 ± 0.77	15.36 ± 1.01	14.11 ± 1.36
^{MW}p	p_1=0.276		p_2= 0.002*	
Spleen (cm)				
Min. – Max.	17.0 – 22.0	14.0 – 21.60	14.0 – 22.0	12.0 – 19.90
Mean ± SD.	19.04 ± 1.60	17.37 ± 2.42	18.21 ± 2.19	13.88 ± 2.18
^{t}p	p_1=0.014*		p_2<0.001*	

Table (II): Comparison between the studied groups according to EV and risk signs of bleeding

	Group I (n=20)		Group II (n=20)		P
	No.	%	No.	%	
EV					
No	0	0.0	0	0.0	
F1	0	0.0	11	55.0	$^{FE}p<0.001^{*}$
F2	16	80.0	8	40.0	
F3	4	20.0	1	5.0	
Red colour sign					
No	0	0.0	12	60.0	$<0.001^{*}$
Yes	20	100.0	8	20.0	
NIEC					
Min. – Max.	38.60 – 49.70		24.90 – 43.30		$<0.001^{*}$
Mean ± SD.	43.09 ± 3.74		32.80 ± 5.73		

EV: Esophageal Varices

NIEC: North Italian Endoscopic Club

Red colour signs: red wale markings, cherry-red spots, Haematocystic spot and diffuse redness.

SD: Standard Deviation

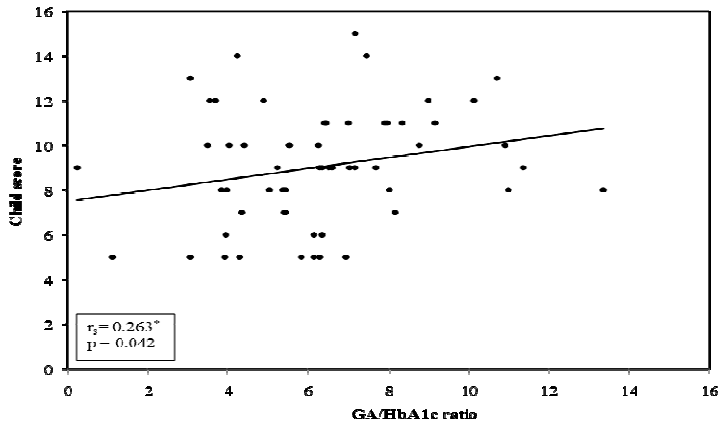

Figure (1): Correlation between GA/HbA1c ratio and Child score in cirrhotic patients group

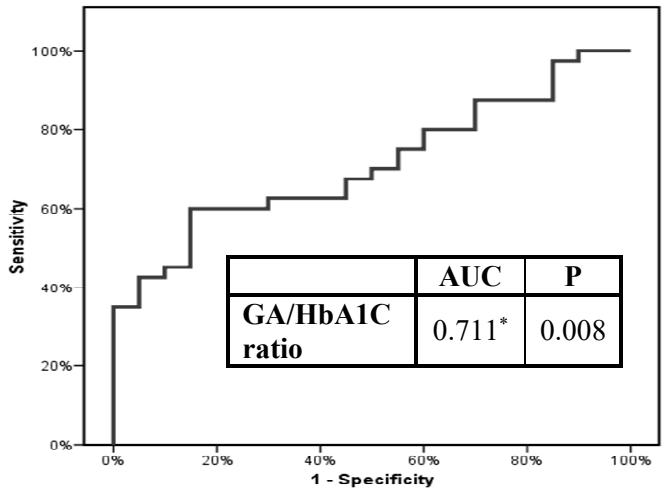

Figure (2): ROC curve for GA/HbA1C ratio to differentiate between cirrhotics with no varices [Group 3] and cirrhotics with EV [Group 1 and 2].

Discussion:

Gastro-esophageal variceal hemorrhage is a major complication of portal hypertension resulting from cirrhosis.[18] Variceal hemorrhage occurs in 25 to 35 percent of patients with cirrhosis and accounts for 80 to 90 percent of bleeding episodes in these patients.[3] Up to 30 percent of initial bleeding episodes are fatal and a higher percent of survivors have recurrent bleeding after the first variceal hemorrhage.[19] Therefore, early detection of esophageal varices and timely introduction of beta-blockers and band ligation for primary prevention of bleeding, decrease the morbidity and may improve quality of life in liver cirrhosis patients. For optimal management, it is important to understand which patients are most likely to have bleeding.

EGD is the gold standard method for evaluating varices to determine whether a patient should be treated to prevent a first variceal hemorrhage.[20,21] However, the need for repeating EGD is a burden for the patient with a high cost and a small, but significant, risk of complications.

Therefore, many non-invasive or minimally invasive methods have been proposed to evaluate the presence/size of such varices. On the other hand, the Baveno IV International Consensus Workshop on methodology of diagnosis and treatment concluded that no study reached a high enough level of significance to warrant the widespread use of such noninvasive markers of esophageal varices. [20]

Regarding some radiological markers for example doppler ultrasonography[22], computed tomography[23], capsule endoscopy[24], fibroscan[25] have been proposed to predict the presence or size of the varices. However, none of the available methods completely meets the criteria of an ideal (accurate, simple, inexpensive and easily reproducible) method.[4,26]

In the present study, we have shown that the GA/HbA1c ratio in cirrhotic patients increases with the severity of cirrhosis and correlated to the presence of esophageal varices but not its risk of bleeding.

In our results the receiver operator characteristic (ROC) curve analysis generated a ratio cut off value (COV) of 6.35 that could discriminate cirrhotic patients with and without esophageal varices, (specificity of 85%, a sensitivity of 60%, and predictive values positive and negative of 89% and 52% respectively, making this method a rather specific one capable of detecting esophageal varices in cirrhotic patients.

Similar to our results, Y Sakai et al[27] found that GA/HbA1c ratio was significantly higher in patients with varices than that in patients without varices ($p<0.001$).

In contrast to our findings, however, they found that GA/HbA1c ratio was significantly higher in patients in the “high-risk varices group”, suggesting that the GA/HbA1c ratio is associated with an increased risk of variceal hemorrhage.

The difference between these results and our work may be due to several causes. The first cause was the small sample size (only 47 patients) in *Sakai*'s study. The second cause was the patients selection CTP- class A liver cirrhosis only, although according to Zaman[28] et al the risk of development of esophageal varices is three times more in child classes B and C than child A. This narrowed the scope of patient selection. Also, they only excluded patients with poorly controlled DM. In our work we study patients with all Child classes so we had more chance to select different patient varieties. Also we totally exclude patients with DM to avoid any bias in the result of GA/HbA1c ratio.

Bando et al[29] have previously reported that the GA/HbA1c ratio in patients with CLD has an inverse correlation with some indicators of hepatic function, regardless of the mean plasma glucose level, suggesting that the increase in the GA/HbA1c ratio reflects the reduction of liver function caused by the progression of liver cirrhosis. In accordance with this study we found that the GA/HbA1c ratio was significantly increased in the patients with advanced cirrhosis expressed in the term of Child score. The ratio showed a significant positive correlation with Child score among all patients ($p =0.042$).

Few researchers studied the glycated albumin to glycated hemoglobin ratio in

relation to hepatitis C virus related liver fibrosis, hepatitis B virus related liver fibrosis and NASH they found that the GA/HbA1c ratio was positively correlated with the fibrosis stage of the liver.[11,30,31]

Finally, to summarize, our results indicate that the GA to HbA1c ratio might be a promising marker for the severity of liver cirrhosis. This – in turn- has its expected impact on the severity of portal hypertension and hense the formation of EV. However, the relation of GA to HbA1c ratio to the risk of bleeding was not proved in our work. Further studies with higher patient number will be needed in the future to clarify point of controversy.

Conclusions:

From this study we can conclude that GA/HbA1c ratio is a new promising marker for predicting EV in cirrhotic patients. However, it does not correlate with the risk of variceal bleeding among these patients. Also it correlats well with the severity of liver cirrhosis (expressed as Child score).

Recommendations:

From this study, we recommend early detection of cirrhotic patients who have high GA/HbA1c ratio because they are more liable for development of esophageal varices and hence, more frequent endoscopic follow up for those patients would be indicated however, further studies in the future with higher number of patients will be needed to determine the presence or absence of a relationship between GA/HbA1c ratio and the risk of bleeding among cirrhotic patients with esophageal varices.

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Original Article

Effect of Dehydroepiandrosterone and Muscular Exercise on Insulin Sensitivity and Tumour Necrosis Factor Alpha in High Fat Diet Fed Rats

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Abstract:

Background: Visceral obesity is considered as an inflammatory condition with production of pro-inflammatory mediators such as IL-6 and TNF-α that play a role in the induction of insulin resistance and type 2 diabetes in obese individuals. Both high fat diet and lack of physical activity are risk factors for obesity. DHEA is a precursor of steroid hormones that may play a role in improving insulin resistance in obese individuals. **Aim** of the present study was to investigate the possible effects of muscular exercise, DHEA and their combination on insulin sensitivity, TNF-α in high fat diet fed rats. **Materials and Methods:** The study was conducted on 40 adult male Albino rats with body weight of approximately 100-150g. All rats were fed on high fat diet (60% fat) for 14 weeks. Body weight was measured at the end of the 8th week and those who had 10% increase in their body weight or more had continued the last 6 weeks of the experiment. During the last 6 weeks of experiment, rats were distributed randomly as follows: **Group I (Control, n=10):** This group received oral sesame oil every day for 6 weeks. **Group II (DHEA, n=10):** This group received DHEA orally dissolved in sesame oil every day for 6 weeks. **Group III (Exercise, n=10):** This group had an exercise protocol of swimming for 5 days/week for 6 weeks. **Group IV (Combined, n=10):** They received DHEA as group II and had the exercise program as group III for 6 weeks. At the end of the study, all rats were sacrificed and visceral fat was weighed. Fasting serum glucose, insulin and TNF-α were measured. Insulin sensitivity was calculated by QUICKI (quantitative insulin sensitivity check index). **Results:** rats of the combined group showed significant decrease in bodyweight and visceral fat than all the other groups. They also showed significant improvement in fasting serum glucose level, insulin and TNF-α levels than the other 3 groups. The combined group was significantly more insulin sensitive than the other 3 groups. There was no significant difference between the DHEA group and the Exercise group regarding all the parameters measured though both were significantly improved than the Control group. **Conclusion:** our results support that the combination of DHEA and exercise showed better results for body weight, visceral fat and insulin sensitivity than DHEA or exercise alone. Both modalities showed better results for serum TNF-α than either alone.

Keywords: obesity, DHEA, exercise, insulin sensitivity, TNF-α, visceral fat.

Introduction:

Obesity is a growing epidemic worldwide; its prevalence has been rising tremendously over the last 30 years (WHO, 2013).[1] It results from the imbalance between energy intake and energy expenditure. [2] It is a pro-inflammatory condition in which hypertrophied adipocytes and adipose tissue-resident immune cells (primarily lymphocytes and macrophages), both contribute to increased circulating levels of pro-inflammatory cytokines. [1,3]

Abbreviations:

T2DM, type 2 diabetes mellitus; **HFD**, high fat diet; **TNF-α**, tumour necrosis factor alpha; **IL-6**, interleukin 6; **IRS**, insulin receptor substrate; **DHEA**, dehydroepiandrosterone; **QUICKI**, quantitative insulin sensitivity check index; **RMR**, resting metabolic rate; **PPAR**, peroxisome proliferator-activated receptor; **GLUT4**, glucose transporter 4; **PI3K-PKB**, phosphatidylinositol 3-kinase-protein kinase B; **AMPK**, AMP-activated protein kinase; **PGC-1α**, peroxisome proliferator-activated receptor-γ coactivator-1α .

Insulin resistance is a common feature of obesity and is strongly associated with the etiology of type 2 diabetes mellitus (T2DM), hypertension and coronary heart disease.[4,5] It is defined as the decreased peripheral tissue response to insulin-mediated cellular actions and the term "insulin resistance" refers to reduced whole-body glucose uptake in response to physiological levels of insulin.[6] It correlates strongly with central-visceral obesity but not with lower body (i.e., hip, lower extremity) obesity.[7]

Among all environmental influences, both high-fat diet (HFD) and lack of or decline in daily physical activity are the most important factors for obesity development.[8] Excessive intake of dietary fat promotes adipocyte hypertrophy, altering their normal endocrine function to an inflammatory pathologic condition that increases the secretion of tumour necrosis factor alpha (TNF-α) and interleukin-6 (IL-6), and concomitantly reducing adiponectin secretion.[9] TNF-α is a potent pro-inflammatory cytokine, secreted mainly by adipose tissue-resident macrophages in obese individuals. TNF-α induces insulin resistance mainly by defective phosphorylation of insulin receptor substrate 1 (IRS-1).[1] This reduces the expression of glucose transporters and adiponectin in adipocytes, which contributes to the development of insulin resistance.[10] TNF-α also indirectly induces insulin resistance by altering adipocyte differentiation and adipocyte lipid metabolism.[11] Moreover, it inhibits the conversion of preadipocytes to mature adipocytes allowing further recruitment of uncommitted cells and thus possible expansion of adipose tissue mass.[1] TNF-α also has an enhancing effect on the production of other inflammatory cytokines, such as IL-6. [12]

Dehydroepiandrosterone (DHEA) is a natural steroid that serves as a precursor of male and female sex hormones. It is produced from the adrenal gland, and together with its sulfated form (DHEA-S), are the most abundant steroid hormones in humans.[13] Patients with metabolic syndrome have lower DHEA and DHEA-S levels.[14,15] The obesity and type 2 diabetes patients show lower concentrations of DHEA and other sex steroid hormones.[16] While some studies indicated that DHEA administration leads to improving insulin sensitivity, reducing fat mass and normalizing glucose metabolism,[17,18] others demonstrated no improvement in insulin sensitivity or body composition. [19]

Exercise training is considered as an important environmental factor associated with body weight regulation and has shown to control obesity and weight gain and to improve insulin sensitivity and help in reducing the risk of diabetes.[20,21] Research groups have already demonstrated that intermittent swimming exercise is more efficient than continuous swimming exercise in decreasing adiposity in rats fed a high fat diet.[22]

The aim of the present study was to investigate the possible effects of muscular exercise, DHEA and their combination on insulin sensitivity, TNF-α in high fat diet fed rats.

Materials and Methods:

The study was conducted on 40 adult male Albino rats (10-12 weeks old), with bodyweight of approximately 100-150g. Rats were housed at room temperature with a 12-h light–dark cycle; 5 rats per cage; with free access to food and water. The duration of the study was 14 weeks. All rats were fed on HFD [23] (60% fat, 20% carbohydrate and 20% protein) with vitamin and mineral mix for the 14 weeks. The HFD was prepared every 3 days to ensure its freshness.[24] All rats were subjected to body weight measurement at the end of the eighth week of the experiment and those who had 10% increase in their body weight or more had continued the last 6 weeks of the experiment.[25]

During the last 6 weeks of experiment, rats were distributed randomly into 4 groups as follows: **Group I (Control, n=10):** This group received sesame oil (1mg/Kg body

weight) administered orally by gastric tube in the morning at 9 am every day for 6 weeks and served as a control. **Group II (DHEA, n=10):** This group received DHEA (1mg/kg body weight) (bought from LKT Laboratories, Inc) dissolved in sesame oil administered orally by gastric tube in the morning at 9 am every day for 6 weeks.[26] **Group III (Exercise, n=10):** The animals of this group had an exercise program consisted of swimming in individual tanks filled with water and maintained at 28-32°C.[27] They swam for 10, 20, and 30 minutes twice daily on the first, second, and third days respectively to adapt. The swimming period was maintained at 30 minutes twice, [28] separated by 45-min rest period.[29] The exercise protocols were performed for 5 days/week for 6 weeks.[27] Rats were continuously monitored during swimming to prevent them from drowning.[30] **Group IV (Combined, n=10):** This group received DHEA in the same dose as group II and had the exercise program as group III for 6 weeks.

Sampling: At the end of the study, rats of all groups were fasted overnight, weighed, anaesthetized using ether then euthanized by decapitation.[30] Visceral fat mass was assessed by weighing the epididymal, mesenteric, and retroperitoneal fat pads.[31] Blood was collected from trunk after decapitation into a clean dry non-heparinized Wasserman tubes for separation of serum. [30] Samples were allowed to clot for 10-20 mins at room temperature before centrifugation for 20-mins at the speed of 2000-3000 r.p.m. A fraction of the sera was taken and used for estimation of fasting glucose levels immediately.[32] The rest of the sera were aliquoted and stored at-20°C until assayed. Samples were centrifuged again after thawing before the assay.

The following parameters were assessed: 1.Serum insulin levels were assayed with enzyme-linked immunosorbent assay (ELISA) kits (supplied by WKEA MED supplies, USA).[32] 2.Insulin sensitivity was assessed using the QUICKI method (quantitative insulin sensitivity check index). This method was calculated using fasting glucose and insulin values.[33,34] QUICKI = 1 / [log (I_0) + log (G_0)], Where I_0 is fasting insulin (μU/ml), and G_0 is fasting glucose (mg/dl). 3. TNF-α was assessed with ELISA kits. (supplied by WKEA MED supplies, USA) [35]

Statistical analysis:

The values of the measured parameters were expressed as mean ± SD. The difference between the studied groups was determined using ANOVA test (F-test) and least significant difference (LSD). Correlations between two quantitative variables were assessed using Pearson correlation coefficient (r). $P < 0.05$ values were considered significant. All statistical analyses were processed using SPSS for windows Version 20.0.

Results:

Comparison between body weights (gm) in the studied groups.

At the beginning of the study, the body weight was 132 ± 2.34 gm.

At the end of the eighth week, all rats exhibited increase in body weight which ranged between 190 ± 19.7 gm with a mean % of increase 43.7% ± 14.9%. Then rats were distributed randomly into 4 groups and there was no significant difference between these groups.

At the end of the 14th week, the body weight was measured. There was a significant difference between the four groups where F=7.908 and P= <0.001. The DHEA group had a significant decreased body weight than the Control group where P= 0.007, while it had a significant increased body weight than the Combined group where P=0.050. The Exercise group had a significant decreased body weight than the Control group where P= 0.022, while it had a significant increased body weight than the Combined group where P=0.020. The Combined group had a significant decreased body weight than the Control group where P=<0.001. There was no significant difference between the DHEA group and the Exercise group.

Comparison between visceral fat (gm) in the studied groups at the end of the study:

At the end of the study, visceral fat (epididymal, mesenteric, and retroperitoneal) was weighed. The mean values of the Control group, DHEA group, Exercise group and Combined group were 5.15 ± 0.92 gm, 4.11±0.63 gm, 3.91 ± 0.63 gm and 3.14 ± 0.52 gm respectively. There was a significant difference between the four groups where F= 14.366 and P= <0.001.

The DHEA group had a significant decreased visceral fat than the Control group where P= 0.002, while it had a significant increased visceral fat than the Combined group where P=0.003. The Exercise group had a significant decreased visceral fat than the Control group where P= <0.001, while it had a significant increased visceral fat than the Combined group where P=0.018. The Combined group had a significant decreased visceral fat than the control group where P=<0.001. There was no significant difference between the DHEA group and the exercise group.

Comparison between insulin sensitivity in the studied groups at the end of the study:

At the end of the study, the fasting serum glucose and insulin levels were measured. The mean values of serum glucose for the Control group, DHEA group, Exercise group and Combined group were 133 ± 8.61 mg/dl, 113 ± 6.62 mg/dl, 113 ± 8.37 mg/dl and 98.7 ± 8.04 mg/dl respectively. There was a significant difference between the four groups where F= 31.626 and P= <0.001.

The mean values of serum insulin for the Control group, DHEA group, Exercise group and Combined group were 22.7 ± 2.37 μU/ml, 16.9 ± 0.90 μU/ml, 16.6 ± 1.13 μU/ml and 14.4 ± 2.22 μU/ml respectively. There was a significant difference between the four groups where F= 39.770 and P= <0.001.

The fasting serum glucose and insulin levels were significantly higher in the Control group than other studied groups, while they are significantly lower in the Combined group than the other groups. There was no significant difference between the DHEA group and the Exercise group.

Insulin sensitivity was calculated using the QUICKI method. There was a significant difference between the four groups where F= 32.714 and P= <0.001.

The DHEA group had a significant increased insulin sensitivity than the Control group where P= <0.001, while it had a significant decreased insulin sensitivity than the Combined group where P=<0.001. The Exercise group had a significant increased insulin sensitivity than the Control group where P= <0.001, while it had a significant decreased insulin sensitivity than the Combined group where P=<0.001. The Combined group had a significant increased insulin sensitivity than the control group where P=<0.001. There was no significant difference between the DHEA group and the Exercise group.

Comparison between serum tumour necrosis factor alpha (TNF-α) (ng/L) in the studied groups at the end of the study:

At the end of the study, the serum TNF-α level was measured. There was a significant difference between the four groups where F= 37.599 and P= <0.001.

The DHEA group had a significant lower serum TNF-α level than the Control group where P=< 0.001, while it had a significant higher serum TNF-α level than the Combined group where P=<0.001. The Exercise group had a significant lower serum TNF-α level than the Control group where P= <0.001, while it had a significant higher serum TNF-α level than the Combined group where P=<0.001. The Combined group had a significant lower serum TNF-α level than the Control group where P=<0.001. There was no significant difference between the DHEA group and the Exercise group.

Correlation study:

I- Correlation study between visceral fat and serum TNF-α:

A significant positive correlation was detected between visceral fat and fasting serum TNF-α in total cases where r=0.934 and p= <0.001.

II- Correlation between visceral fat and insulin sensitivity measured by QUICKI:

A significant negative correlation was detected between visceral fat and insulin sensitivity measured by QUICKI in total cases where r= -0.919 and p= <0.001.

Table I : Comparison between body weights (gm) in the studied groups.

	Control (n=10)	DHEA (n=10)	Exercise (n=10)	Combined (n=10)	F	p
Baseline						
Mean ± SD.	132 ±2.5	132 ± 2.5	132 ± 2.2	131 ± 2.4	0.434	0.730
At end of 8 weeks						
Mean ± SD.	196 ± 20.6	181 ± 17.1	194 ± 19.2	187 ± 21.3	1.159	0.339
Mean % of change	48.5	37.3	46.7	42.4		
At end of 14 weeks						
Mean ± SD.	244 ± 27.5	217 ± 16.6	221 ± 16.4	198 ± 22.4		
p_1		0.007*	0.022*	<0.001*	7.908*	<0.001*
p_2			0.654	0.050*		
p_3			0.020*			

F: F test (ANOVA), SD= standard deviation.
p1: p value for Post Hoc test (LSD) for comparing between Control and each other group.
p2: p value for Post Hoc test (LSD) for comparing between DHEA group with each other group.
p3: p value for Post Hoc test (LSD) for comparing between Exercise group with Combined group.
*: Statistically significant at p ≤ 0.05

Table II: Comparison between insulin sensitivity in the studied groups at the end of the study:

	Control (n=10)	DHEA (n=10)	Exercise (n=10)	Combined (n=10)	F	p
Insulin sensitivity measured by QUICKI						
Mean ± SD.	0.288 ± 0.006	0.305 ± 0.004	0.306 ± 0.006	0.318 ± 0.010		
p_1		<0.001*	<0.001*	<0.001*	32.714*	<0.001*
p_2			0.761	<0.001*		
p_3			<0.001*			

F: F test (ANOVA), SD= standard deviation.
p1: p value for Post Hoc test (LSD) for comparing between Control and each other group.
p2: p value for Post Hoc test (LSD) for comparing between DHEA group with each other group.
p3: p value for Post Hoc test (LSD) for comparing between Exercise group with Combined group.
*: Statistically significant at p ≤ 0.05

Table III: Comparison between serum tumour necrosis factor alpha (TNF-α)(ng/L) in the studied groups at the end of the study:

	GroupI (Control) (n=10)	GroupII (DHEA) (n=10)	GroupIII (Exercise) (n=10)	Group IV (DHEA+Exercise) (n=10)	F	p
TNF -α						
Mean ± SD.	518± 43.8	407 ± 49.3	389 ± 53.4	293 ± 42.0		
p_1		<0.001*	<0.001*	<0.001*	37.599*	<0.001*
p_2			0.396	<0.001*		
p_3			<0.001*			

F: F test (ANOVA), SD= standard deviation.
p1: p value for Post Hoc test (LSD) for comparing between Control and each other group.
p2: p value for Post Hoc test (LSD) for comparing between DHEA group with each other group.
p3: p value for Post Hoc test (LSD) for comparing between Exercise group with Combined group.
*: Statistically significant at p ≤ 0.05

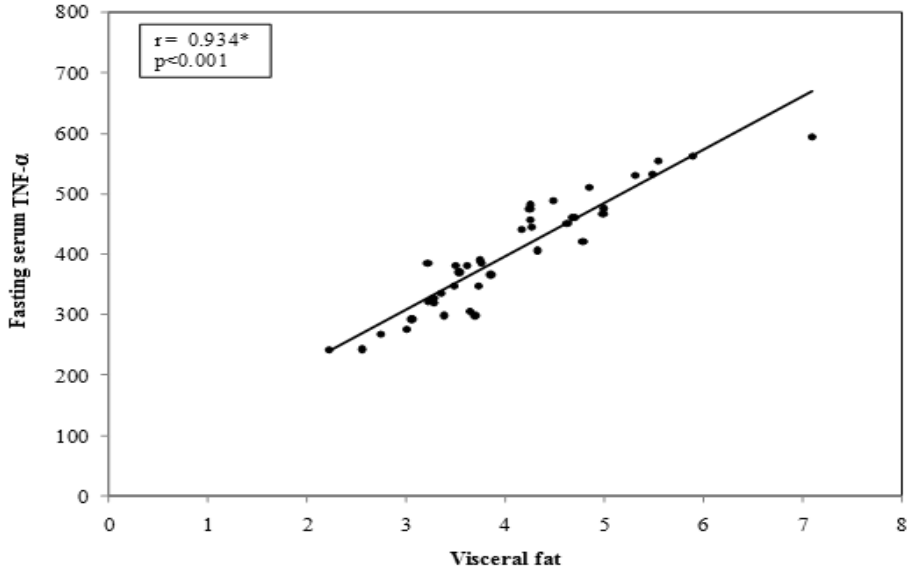

Fig.1: Correlation between visceral fat and fasting serum TNF-α in total cases.

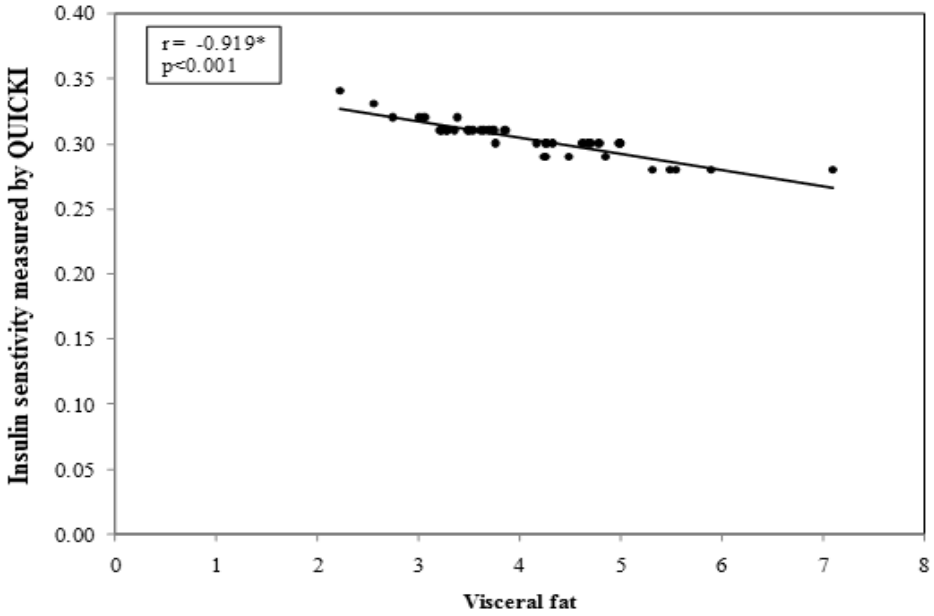

Fig.2: Correlation between visceral fat and insulin sensitivity measured by QUICKI in total cases.

Discussion:

Obesity is a complex metabolic disorder that is one of the most prevalent public health problems.[36] Excess adiposity is an established risk factor for metabolic diseases including insulin resistance, T2DM, hypertension, nonalcoholic fatty liver disease, polycystic ovarian diseases, and several types of cancer.[37] HFDs are known to lead to a positive fat balance and consequently to adipose mass accumulation.[38] Adult mice fed a HFD (60% fat) developed glucose intolerance, insulin resistance, increased expression of TNF-α, decreased expression of adiponectin and hyperlipidemia.[39] Our results showed that after 8 weeks of HFD (60% fat), all rats exhibited increase in body weight which ranged between 190 ± 19.7 gm with a mean % of increase 43.7% ± 14.9%. Chen et al[40] demonstrated that mice fed on HFD (45% fat) for 12 weeks had achieved a higher body

weight, visceral adiposity, fasting plasma glucose and insulin levels than rats fed on a low fat diet (10% fat) for the same period.

Six weeks of combined DHEA treatment and swimming exercise induced significant lower body weight and visceral fat than results observed from DHEA administration alone or intermittent swimming alone for the same period of time. This may be due to DHEA administration alone had shown to reduce adiposity by increasing resting metabolic rate (RMR) which is the largest component of the daily energy budget in most human societies. DHEA also down-regulates adiposity through the reduction of peroxisome proliferator-activated receptor gamma (PPARγ) in adipocytes.[41,42,43] Exercise also enhances glucose/lipid metabolism in skeletal muscle which is involved in white adipose tissue reduction. Also, both aerobic and resistance training in human subjects had also shown to increase the RMR so increasing energy expenditure during rest as well as during exercise.[10,44] Therefore, 6-weeks of combination treatment may have promoted additive reductions in body weight and visceral fat. According to Sato et al[26], body weight and abdominal fat were reduced by 6-weeks DHEA administration combined with treadmill training in rats fed on high sucrose diet for 20 weeks. They hypothesized that DHEA administration and exercise training, both can up regulate fatty acid metabolism which can help in adipose tissue reduction.[45,46]

Our results showed that 6-weeks of combined DHEA treatment with exercise training induced significant decrease in serum TNF-α level than results observed from DHEA administration alone or exercise training alone for the same period of time. This may be due to the synergistic effect of DHEA and exercise together in decreasing the body weight and visceral fat as our results showed that there is a positive correlation between visceral fat and TNF-α levels.[47] Similarly, Hostamilgil et al[48] reported that the adipose-tissue expression of TNF-α was positively related to BMI and decreased in proportion to the loss in body weight.

Several studies showed that DHEA reduced TNF-α serum concentrations and its induced inflammatory response.[32,49] DHEA may have lowered the circulating levels of inflammatory cytokines such as TNF-α and IL-6 as demonstrated by Weiss et al through activation of PPARα in aging humans causing a decrease in release of these inflammatory cytokines by various cell type.[50] Brignardello et al[51] showed that DHEA treatment reduced mRNA expression of TNF-α, TNF-receptor I (TNF-RI) and TNF-receptor II (TNF-RII) in peripheral blood mononuclear cells. A reduction in mRNA expression of TNF-α indicated a down regulation of this system.

Exercise also showed reductions in the expression of TNF-α in white adipose tissue.[52] Studies suggested that long-term exercise reduces adipose inflammation via suppression of macrophage infiltration and a switch from the inflammatory M1 macrophages to the anti-inflammatory M2 macrophages.[53,54] Weight reduction through exercise decreased the volume and number of adipocytes and also reduced the number of endothelial and macrophage cells that are lodged inside adipose tissue that produce pro-inflammatory mediators. Increased production of anti-inflammatory mediators by adipocytes and decreased hepatic production of fibrinogen and other pro-inflammatory mediators were other consequences of exercise-induced weight reduction. Weight loss also influenced the immune system by reducing the number of mononuclear cells in the circulation; these are important sources of pro-inflammatory cytokines.[55,56]

We also demonstrated that 6-weeks of DHEA treatment combined with swimming exercise induced significant decrease in fasting serum glucose and insulin levels than results observed from DHEA administration alone or exercise training alone for the same period of time. The combined interventions were more beneficial for insulin sensitivity than either DHEA administration or exercise training alone. This may be due to the significant decrease in body weight and

visceral fat than the DHEA group or the exercise group. As evidenced by the present study, visceral fat was negatively correlated with insulin sensitivity.[57,58] The improvement in insulin sensitivity may be also correlated to the improvement in the fasting serum TNF-α level as demonstrated by Plomgaard et al.[59] Similarly, Sato et al[26] had demonstrated that the combination of DHEA administration and treadmill running had additively or synergistically improved blood glucose levels and activated the glucose uptake in skeletal muscle because both modalities significantly increased basal muscular DHEA and dihydrotestosterone levels which were positively correlated with insulin sensitivity.[60]

In patients with obesity and T2DM, skeletal muscles have reduced glucose metabolic capacity due to insulin resistance and the ability of insulin to stimulate glucose transporter 4 (GLUT4) translocation decreases, resulting in a reduced GLUT4 content at the plasma membrane.[61,62] DHEA treatment enhanced glucose transport rates through GLUT4 transporter translocation to the cell surface. This effect seems to involve stimulation of insulin receptor substrate tyrosine phosphorylation and the associated phosphatidylinositol 3-kinase-protein kinase B (PI3K-PKB) activity.[63,64] Veras et al[65] had demonstrated that DHEA supplementation in ovariectomized female rats fed a HFD had maintained glucose-induced insulin secretion and pancreatic islet function. This effect was mediated by an increase in the phosphorylated PKB.

Exercise also had an “insulin-like effect” to facilitate glucose transport from the circulation into the working muscles.[66] Many studies had shown that exercise training can increase mitochondrial proliferation and boost the expression of GLUT4, and can in turn enhance lipid and glucose metabolic capacities.[67,68] Some demonstrated that exercise-induced, contraction-mediated GLUT4 translocation from an intracellular pool to the muscle membrane where glucose uptake takesplace, is independent of insulin and occurs through calcium/calmodulin-dependent protein kinase IV and, secondarily, through AMP-activated protein kinase (AMPK), which induces expression of peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α), a transcriptional coactivator that is essential for mitochondrial biogenesis.[68,69] Exercise improved HFD-induced metabolic dysfunction, including insulin resistance, fat accumulation, and mitochondrial dysfunction may also be due to the decreased expression of protein kinase Cβ in both skeletal muscle and liver as demonstrated by Rao et al.[70]

Despite positive findings in most rodent models and some of human studies, indicating that DHEA treatment improved glucose tolerance and insulin sensitivity, other DHEA studies in humans had yielded inconclusive results.[71,72] One possibility to explain these discrepancies was that one or many metabolites of DHEA, rather than DHEA itself, may be necessary for its full action in human physiology. DHEA undergoes extensive conversion to multiple products by phase 1 reactions involving the cytochrome P450 system. Studies showed that these phase 1 products, which frequently decline in elderly who had been the major participants in human DHEA treatment studies, can be more potent than parental DHEA.[73,74] It is also possible that qualitative changes in DHEA metabolism between rodents and humans may account for these differences.[75,76]

In conclusion, on the basis of this study, we support other results showing that combined treatment of DHEA and exercise showed better results for body weight, visceral fat and insulin sensitivity than DHEA or exercise alone. We report that combination of both modalities may have a better effect in reducing serum TNF-α than either alone.

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Original Article

Comparative Study of Changes of Irisin, Interleukin-6 (IL-6) and Adiponectin Levels in Acute and Chronic Exercise in Young and Old Rats (Muscle– Adipose Tissue Crosstalk)

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Abstract:

Introduction: Although the existence of health promoting effects of regular physical exercise is beyond doubt, our knowledge about the underlying molecular, cellular and systemic mechanisms involved in such effects is still fragmentary. Skeletal muscle secretes several bioactive proteins that mediate many exercise-induced benefits. Irisin is a myokine that presumably is an important link within the causal chains leading from physical activity to better health. **Aim:** To investigate the effects of acute and chronic exercise on circulating levels of irisin, intrleukin -6 (IL-6) and adiponectin in young and old rats. **Material and methods:** The present work was carried on 48 adult male Wistar (albino) rats that were divided into 2 groups according to the age: group I (young rats group) that consisted of 24 rats subdivided into 3 subgroups (each of 8 rats): group IA: included non- exercising rats and acting as control one, group IB: included rats which performed acute exercise in the form of 2 sessions of 90 min. swimming, group IC: included rats which performed chronic exercise in the form of swimming 1 hour/day , 5 days /week for 8 weeks. Group II(old rats group) that consisted of 24 rats subdivided into 3 subgroups (each of 8 rats): group IIA that involved rats that did not perform any exercise (control group), group IIB which involved rats which performed acute exercise as previously mentioned and group IIC that involved rats which performed chronic exercise as mentioned before. Serum levels of irisin (myokine), interleukin-6 (IL-6) and adiponectin were estimated in all rats of the studied groups. **Results:** The present study revealed a statistical significant increase in the mean values of serum levels of irisin in acute exercise and chronic exercise of both young and old rats but, this rise is significantly higher in acute exercise than chronic one, and is significantly higher in young rats than old rats. Regarding mean values of serum levels of IL-6 and adiponectin, the present work showed a statistical significant elevation of their levels in rats performing acute exercise, but their levels in young rats were significantly higher than old ones. On the other hand, there was a significant reduction of their levels in rats performed chronic exercise and this reduction was significantly more in old rats. **Conclusion:** Irisin release was more prominent in acute exercise in young rats that could indicate that age and type of exercise may be the primary predictors of its release.IL-6 levels were elevated in acute exercise and reduced in chronic one reflecting that acute form of exercise is a short term inflammatory process whereas chronic one produces anti- inflammatory effect. Adiponectin values were raised in acute exercise in young rats and down regulated in chronic one in old rats and these results reflect the association between adiponectin and age and muscle mass. Muscle - adipose tissue crosstalk is mediated by several bioactive proteins. In the future, the discovery of presence and beneficial functions of more unknown bioactive factors could strengthen the development of sports science and exercise physiology.

Keywords: Irisin- interleukin-6- adiponectin- acute and chronic exercise- young and old rats.

Introduction:

Skeletal muscles support physical activity and generate large energy with muscle contraction. Growing evidence has shown that muscle cells secrete many bioactive proteins which have regulatory role in the muscle and other organs via endocrine, autocrine and

Abbreviations:

BAT: Brown adipose tissue. **FNDC5:** Fibronectin domain containing protein C 5. **IL-6:** Interleukin-6. **PCG-1α:** Peroxisome proliferator – activated receptor gamma co-activator - 1α. **WAT:** White adipose tissue.

paracrine actions; this is the so called myokine theory. These secreted proteins are changed in response to exercise and suggested to mediate acute and chronic effects obtained by exercise.(1)

Although the existence of health promoting effects of regular physical exercise is beyond doubt, our knowledge about the underlying molecular, cellular and systemic mechanisms involved in such effects is still fragmentary. In 2012 a new muscle- derived messenger substance (myokine) was described. This newly identified myokine was named irisin after the Greek messenger goddess iris.(2)

In their original work, Bostrom et al(3) unfolded an impressive line of evidence for the health related benefits of irisin, starting with the observation of improved health and longevity in transgenic mice with mild overexpression of peroxisome proliferator – activated receptor gamma coactivator - 1α (PCG- 1α) in muscle, which is a model for well-known induction of PCG- 1α by physical exercise. The ensuing experiments led to the characterization of a membrane protein (fibronectin domain containing protein C 5) (FNDC5) that is expressed under control of PCG- 1α in skeletal muscle. The extracellular part of FNDC5 i.e irisin cleaved and secreted into the extracellular space causes similar systemic effects to those seen in the mouse model namely increased energy expenditure and weight loss and improved glucose homeostasis. (4)

A remarkable aspect about irisin is that the amino acids sequence is 100% identical among most mammalian species which suggests a highly conserved function. (5) It was reported that irisin has potent effect on converting white adipose tissue (WAT) into brown adipose tissue (BAT) both in vivo and in vitro.(6) This evidence opened up some questions about the physiological roles of irisin.

It has been shown that exercise produces a short term inflammatory response and skeletal muscle could be a source of pro and anti- inflammatory cytokines and it has been considering as an important source of interleukin-6(IL-6).(7)

Adiponectin is an adipose tissue discovered hormone that has anti- atherogenic, anti- inflammatory and anti- diabetic effects and it is associated with health status and glucose and free fatty acids metabolism. Moreover, acute and chronic exercises affect body composition, carbohydrates and lipid metabolism. (8)

Based on these data, the present study was designed to investigate the effects of acute and chronic exercise on circulating levels of irisin, intrleukin -6 (IL-6) and adiponectin in young and old rats.

Material and Methods

This study was carried on 48 adult male Wistar (albino) rats that housed under the same environmental conditions. They were given standard diet and they had free access to tap water. The study protocol was approved by *Ethics Committee of Faculty of Medicine, Alexandria University.*

The animals were divided into 2 main groups (each of 24 rats) as follows:

Group I (young rats group): That included 12 months old rats. Their weight ranged from 220- 250 g and they were divided into 3 subdivisions (each of 8 rats) as follows:

IA: Included non-exercising normal rats that act as control for young rats group.

IB: Included rats which performed acute exercise by 2 sessions of 90 min. swimming. (9)

IC: Included rats which performed chronic exercise by I hour swimming/day, 5 days /week for 8 weeks. (10)

Group II (old rats group): That involved 24 months old rats. Their weight ranged from 350- 400 g and they were divided into 3 subdivisions (each of 8 rats) as follows:

IIA: Involved non-exercising normal rats that act as control for old rats group.

IIB: Involved rats which performed acute exercise as mentioned before. (9)

IIC: Involved rats which performed chronic exercise as mentioned previously. (10)

Acute exercise protocol: **(9)**

Rats were adapted to swimming for 10-20 min. for 3 days to reduce water induced stress. The animals swam in groups of two or three rats in plastic cylindrical pool of 45 cm in diameter that were filled to a depth of 60 cm, for two 90 min. long bouts, separated by a 45 min. rest period. The water temperature was maintained between 34-35°C.

Chronic exercise protocol: [10]

After initial training as mentioned in acute exercise, the rats underwent chronic exercise for I hour/day, 5days/week for 8 weeks.

Blood sample collection:

Blood was collected 8-16 hours after acute exercise and 24 -36 hours after the final session of chronic exercise.[9,10] Blood samples were drawn from retro-orbital venous plexus under ether anesthesia and serum was separated for biochemical estimation.

The following parameters were assessed in all rats of the studied groups:

1- Determination of serum levels of irisin [11]

The serum levels of the myokine (irisin) were determined using a commercial enzyme-linked immunosorbent assay kit following the manufacturer's instructions (Irisin EIA kit EK-067-16; Phoenix Pharmaceuticals, Inc. Burlingame, CA, USA) of a spectrophotometric reader at a wave length of 450 nm. The test provided a range of detection of 0.1- 1000 ng/ml and exhibited a coefficient of variation of 6-10 % inter- and intra- assay. The serum was kept at -80 ºC.

2- Determination of serum levels of interleukin-6 (IL-6). [12]

The serum levels of IL-6 were estimated by ELISA using reagents kit of Genzyme Corporation (Cambridge, MA).

3- Determination of serum levels of adiponectin. [13]

Quantitative determination of serum adiponectin was performed using the mouse/rat adiponectin ELISA kit (B-Bridge International, Inc.) according to the manufacturer's instructions.

Statistical Analysis:

Data were expressed as mean± SD. Data analysis was performed using the statistical package for social sciences (SPSS) (version 20) software. Student's paired t test was performed to compare variables between the two studied groups. Pearson's correlation coefficient (r) was used to detect the association between different parameters. Significance was set at $P < 0.05$ and values of $P< 0.01$was considered highly significant.

Results:

Comparison of mean values of serum levels of irisin (ng/ml) in the studied groups. (Table I, figure 1)

The data of the present study revealed that there was a statistical significant elevation of mean serum values of irisin in rats performing both acute and chronic exercise (group IB,IC and group IIB ,IIC) than normal non exercised rats in young and old rats of group I and group II, respectively (group IA, IIA) (P =0.0006 and 0.0010, respectively). In addition, serum levels of irisin were statistically higher in young rats than old ones as well as in acute exercise than chronic exercise (P = 0.001).

Comparison of mean values of serum levels of IL-6 (pg/ml) and adiponectin (ng/ml) in the studied groups. (Tables II, III, figures 2, 3)

The results of the present work showed a statistically significant rise of mean serum levels of IL-6 and adiponectin in young and old rats performing acute exercise (group IB and IIB) than those non- exercised young and old rats (group IA, IIA). Moreover, these levels of IL-6 and adiponectin were statistically higher in young rats (group IB) than those of old rats (group IIB) (P = 0.001 and 0.000, respectively).

Contrarily, mean values of serum levels of both IL-6 and adiponectin were statistically significantly reduced in young and old rats undergoing chronic exercise (group IC,IIC) than those young and old rats performing acute exercise (group IB ,IIB) as well as non- exercised rats of group IA and IIA (P= 0.001). In addition, the reduction of these levels was significantly more in old rats (group IIC) than young ones (group IC) (P= 0.0004 and 0.0001, respectively).

Correlation between serum levels of irisin (ng/ml), IL-6 (pg/ml) and adiponectin ng/ml) in young and old rats performed acute and chronic exercise (Tables IV, V)

The present study showed that there was a statistical significant positive correlation between serum levels of irisin, IL-6 and adiponectin in rats performed acute exercise in both young and old rats (r= 0.602, 0.608, P=0.14, 0.13). However, it was found that values of serum irisin had a statistical significant negative correlation with those

of IL-6 and adiponectin in rats undergone chronic exercise in young and old rats (r= -0.499, -0.501, P=0.049, 0.048). On the other hand, serum levels of IL-6 and adiponectin were significantly positively correlated with each other in rats performed chronic exercise in young and old rats(r=0.604, P= 0.013).

Table (I): Comparison of mean values of serum levels of irisin (ng/ml) in the studied rats.

Subgroup (Each n=8)	Group I (Young rats)(n=24)	Group II (Old rats)(n=24)	P1
A (Non-exercising rats)			
Range	310.2- 510.2	298.7- 489.9	0.2911
Mean± SD	422.26 ±74.29	400.75±78.40	
B (Rats which performed acute exercise)			
Range	799.5- 991.9	747.9- 874.5	0.0006**
Mean± SD	931.98±66.47	814.08 ±48.56	
C (Rats which performed chronic exercise)			
Range	688.9- 797.8	591.6- 710.4	0.0010**
Mean± SD	751.23 ±50.89	655.30 ±50.27	
P2	0.001*	0.001*	
P3	0.001*	0.001*	
P4	0.001*	0.001*	

n= Number of rats.
P1 Comparison between the two groups in each subgroup. **P2** Comparison between subgroup A and subgroup B.
P3 Comparison between subgroup A and subgroup C. **P4** Comparison between subgroup B and subgroup C.

Table (II): Comparison of mean values of serum levels of IL-6 (pg/ml) in the studied rats.

Subgroup (Each n=8)	Group I (Young rats)(n=24)	Group II (Old rats)(n=24)	P1
A (Non-exercising rats)			
Range	3.47- 4.98	3.22 - 4.87	0.2316
Mean± SD	4.34 ±0.62	4.10 ±0.66	
B (Rats which performed acute exercise)			
Range	12.56-16.1	6.88 - 10.2	0.0000**
Mean± SD	14.58 ±1.37	8.56±0.98	
C (Rats which performed chronic exercise)			
Range	2.32 - 3.27	1.32- 2.47	0.0004**
Mean± SD	2.73 ±0.41	1.88 ±0.38	
P2	0.001*	0.001*	
P3	0.001*	0.001*	
P4	0.001*	0.001*	

n= *Number of rats*
P1= *Comparison between the two groups in each subgroup.* ***P2=*** *Comparison between subgroup A and subgroup B.*
P3= *Comparison between subgroup A and subgroup C.* ***P4 =*** *Comparison between subgroup B and subgroup C.*

Table (III): Comparison of mean values of serum levels of adiponectin (ng/ml) in the studied rats.

Subgroup (Each n=8)	*Group I (Young rats)(n=24)*	*Group II (Old rats)(n=24)*	P1
A (Non-exercising rats)			
Range	19.34- 32.72	19.22 - 32.48	0.4552
Mean± SD	25.98 ±4.60	25.71±4.79	
B (Rats which performed acute exercise)			0.0000**
Range	59.97- 78.45	35.88-55.48	
Mean± SD	70.25 ±6.31	48.98±6.44	
C (Rats which performed chronic exercise)			0.0001**
Range	10.6-16.32	8.32- 10.22	
Mean± SD	13.46 ±2.12	9.32±0.74	
P2	0.001*	0.001*	
P3	0.001*	0.001*	
P4	0.001*	0.001*	

n= Number of rats

P1= *Comparison between the two groups in each subgroup.* ***P2 =*** *Comparison between subgroup A and subgroup B.*

P3= *Comparison between subgroup A and subgroup C.* ***P4=*** *Comparison between subgroup B and subgroup C.*

Table (IV): Correlation between serum levels of irisin (ng/ml), IL-6 (pg/ml) and adiponectin (ng/ml) in young and old rats performing acute exercise (group IB and IIB).

	Irisin	**IL-6**
IL-6		
Pearson correlation (r)	0.602*	
Sig.(2 tailed)	0.014	
Adiponectin		
Pearson correlation (r)	0.608*	0.878**
Sig.(2 tailed)	0.013	0.000

*Correlation is significant at the 0.05 level (2-tailed). ** Correlation is significant at the 0.01 level (2-tailed).

Table (V): Correlation between serum levels of irisin (ng/ml), IL-6 (pg/ml) and adiponectin (ng/ml) in young and old rats performing chronic exercise (group IC and IIC).

	Irisin	**IL-6**
IL-6		
Pearson correlation (r)	-0.499*	
Sig.(2 tailed)	0.049	
Adiponectin		
Pearson correlation (r)	-0.501*	0.604*
Sig.(2 tailed)	0.048	0.013

*Correlation is significant at the 0.05 level (2-tailed). ** Correlation is significant at the 0.01 level (2-tailed).

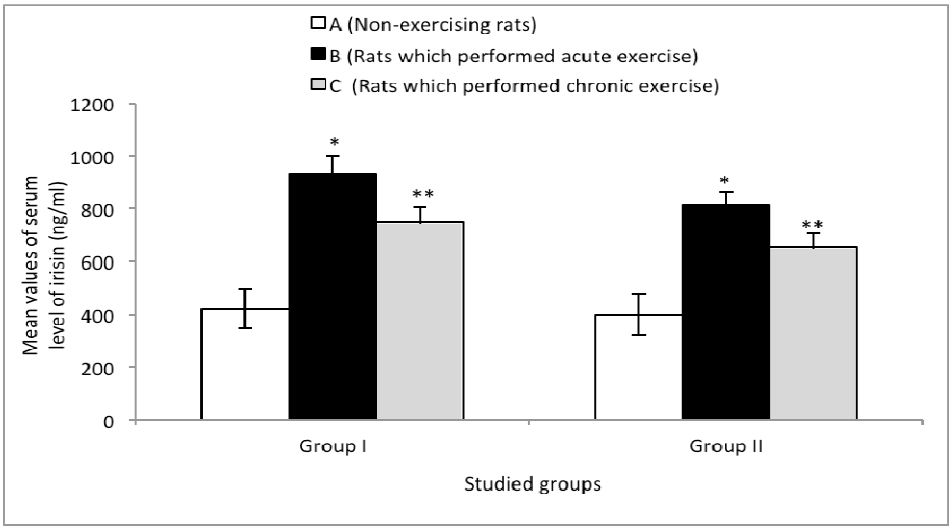

Fig.1: Comparison of mean values of serum levels of irisin (ng/ml) in the studied rats.
(Group I: Young rats group Group II: Old rats group)

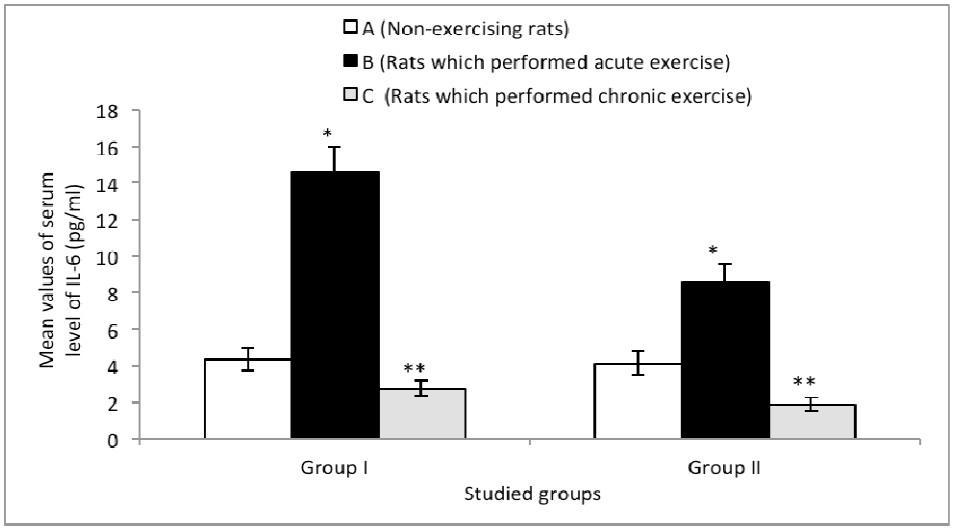

Fig.2: Comparison of mean values of serum levels of IL-6 (pg/ml) in the studied rats.
(GroupI: Young rats group Group II: Old rats group)

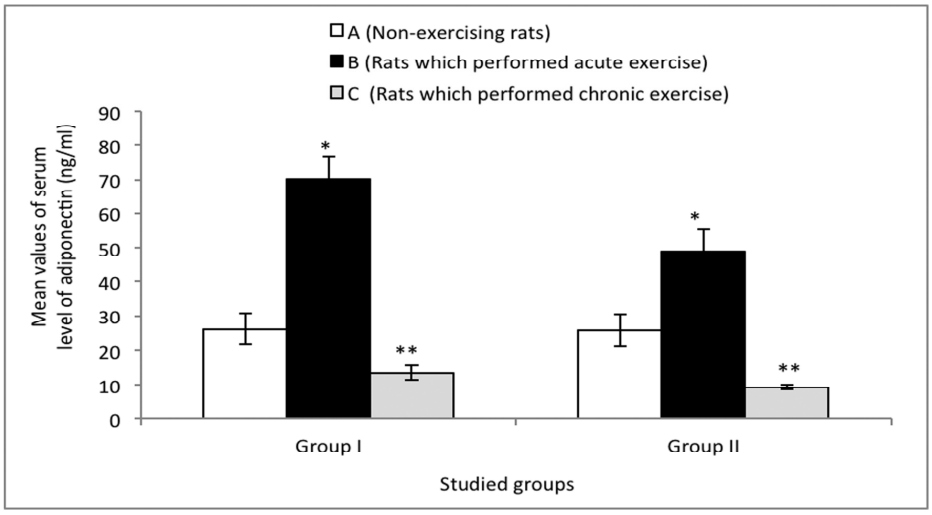

Fig.3: Comparison of mean values of serum levels of adiponectin (ng/ml) in the studied rats.
(GroupI: Young rats group Group II: Old rats group)

Discussion:

Accumulating evidence has demonstrated the mechanisms underlying the benefits of acute and regular exercise. A single bout of exercise drastically changes various physiological parameters such as hormone secretion, blood flow and the activity of the nervous and immune system, in addition to altering the expression /activity of certain genes and proteins in skeletal muscle and other tissues. Further, regular exercise adaptively improves normal body functions including energy metabolism, muscle strength, brain-nervous system, endocrine system and immune functions even in resting state. (14)

Several key bioactive proteins in skeletal muscle and adipose tissues are involved in the development of this adaptation and contribute in the promoting health benefits along with maintaining physiological homeostasis and sports performance during exercise.(15) So, the goal of the present study was to explore and compare changes in serum levels of some skeletal muscle bioactive proteins as irisin and IL-6 as well as adipose tissues proteins as adiponectin in two models of exercise (acute and chronic) in young and old rats.

The recent discovery of FNDC5/irisin protein that is liberated by muscle tissue in response to exercise might be an important finding with regard to the curative and protective role of exercise. The most striking aspect of this myokine is its capacity to drive brown- fat development of white fat and thermogenesis. However, the nature and secretion form of this new protein is controversial.(16)

The present study revealed that serum levels of irisin were statistically significantly elevated in rats undergone acute and chronic exercise in comparison to sedentary non exercised ones; moreover, they were significantly higher in acute exercise than those in cases of chronic exercise and in young rats than its levels in old ones (reflecting the muscle mass). These results reflecting its crucial role in acute form of exercise and this role is more prominent in younger ages. These findings are supported by results of a study done by Montzoros et al(17) who showed significant acute elevation of circulating irisin in response to exercise with a greater increase after maximal workload and they stated that irisin release could be a function of muscle energy demand. Also, our results were in agreement with Julia et al(18) who found that irisin was elevated in skeletal muscle and serum of mice immediately after acute exercise of running wheel and treadmill performance in comparison to sedentary non exercised group.

The FNDC5 gene is expressed in human muscle. Age and muscle mass are the primary predictors of circulating irisin, with young male athletes having several folds higher irisin levels than middle-aged obese women. Circulating irisin levels increase in response to acute exercise whereas FNDC5 mRNA and circulating irisin levels decrease after surgically induced weight loss in parallel to decrease in body mass.(19)

Also, in a study performed by Aydin et al(20), irisin levels were raised with exercise being higher in younger rats undergone water exercise than in old rats.

In contrast to our findings, Norheim et al(21) reported that there was no enhancing effect of long term training on circulating irisin levels and little or no effect of training on browning of subcutaneous white adipose tissue. On the other hand, in a study done to evaluate circulating levels of irisin in response to acute and chronic whole body vibration exercise in humans, it was found that acute bouts of whole vibration exercise are effective in increasing circulating irisin levels but chronic training does not change levels of baseline irisin levels.(22)

The present work revealed significant raised serum levels of IL-6 in cases of acute exercise which was more significant in young rats and a significant reduction in chronic exercise. Skeletal muscle is a source of pro- and anti-inflammatory cytokines, and recently, it has been recognized as an important source of IL-6. Acute physical exercise is known to induce a pro-inflammatory cytokine profile in plasma. (23)

Acute exercise produces a short-term inflammatory response, whereas long term exercise demonstrates a long term- anti-inflammatory effect, thus, down regulates

skeletal muscle production of cytokines involved in the onset, maintenance and regulation of inflammation.[24] Although many types of cells are capable of producing cytokines, the main source of exercise-induced IL-6 production appears to be the exercising muscle. The primary function of additional IL-6 may be to regulate the supply of carbohydrates as muscle reserves of glycogen become depleted and since the production of cytokines is greater with endurance exercise, it seems unlikely that they play an important role in the hypertrophy of muscles and bones. [25]

Similarly, Pedersen BK [26] reported that strenuous exercise induces increased pro-inflammatory and anti-inflammatory as IL-6 and IL-1 and he stated that concentration of IL-6 is related to the intensity of the exercise and IL-6 is known to have growth factor abilities, also increased IL-6 levels triggers anti-oxidant defense in the body.

Furthermore, it has been demonstrated that IL-6 has many biological roles during exercise such as induction of lipolysis, suppression of TNF-α and stimulation of cortisol production and the IL-6 gene is rapidly activated during exercise and the activation of this gene is further enhanced when muscle glycogen content is low. [27]

Regarding serum levels of adiponectin, the current work showed significant higher levels in acutely exercised rats which were more significantly higher in young rats than old ones. Inversely, these levels were significantly reduced in chronic exercise and more significantly reduced in old rats. These results could reflect the association between adiponectin and muscle mass and age. Adiponectin plays an important role in the control of metabolic dysfunction and it improves glucose homeostasis, insulin sensitivity and fatty acids oxidation as well as anti-atherogenic and anti-inflammatory effects.[28]

In a study done by Bouassida[8] to evaluate the effects of physical exercise and physical training on adiponectin levels, it was found that adiponectin concentrations present a delayed increase (30 min.) after short term intense exercise (60 min.) but these levels did not change in response to long term training (12weeks) and stated that most training studies which improve fitness level and affect body composition could increase adiponectin concentrations. Also, it was reported that aerobic interval training resulted in significant increase in adiponectin levels [29]

In addition, Yokoyama et al[30] assessed the effect of aerobic exercise (including bicycle ergometer for 40 min. /day for 5 days and walking for 10,000 steps/day) on plasma adiponectin levels and insulin resistance in type 2 diabetes and they found that plasma adiponectin are significantly correlated with anthropometrical changes induced by aerobic exercise and marked weight reduction results in a significant increase in adiponectin levels in obese subjects. Therefore, adiponectin may play a central role in operating insulin action when an improvement of insulin sensitivity is achieved mainly by fat reduction. Exercise may indirectly increase plasma adiponectin when there is reduction in body weight or fat mass.

Saunders et al [31] showed that both acute and short term aerobic exercise resulted in a significant increase in plasma adiponectin levels in inactive, abdominally obese men independent of intensity. Moreover, in a study done to evaluate the effect of different intensities of running on serum adiponectin in male rats, it was found that serum adiponectin concentrations rose when running intensities in one exercise session increased and during recovery period , these levels decreased with the same pattern in different exercise intensities.[32]

On the other hand, Lim et al [33] showed that circulating adiponectin levels do not change with acute exercise and exercise training regardless the intensity. Therefore, they concluded that there are no meaningful acute or chronic effects of exercise on adiponectin levels.

Conclusion: Irisin release was more prominent in acute exercise in young rats that could indicate that age and type of exercise may be the primary predictors of its release.IL-6 levels were elevated in acute exercise and reduced in chronic one reflecting that acute form of exercise is a short term inflammatory process whereas chronic one produces anti-inflammatory effect. Adiponectin values were raised in acute exercise in young rats and

down regulated in chronic one in old rats and these results reflect the association between adiponectin and age and muscle mass. Muscle - adipose tissue crosstalk is mediated by several bioactive proteins.In the future, the discovery of presence and beneficial functions of more unknown bioactive factors could strengthen the development of sports science and exercise physiology.

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Original Article

Effect of High Fat Diet and Exercise on Memory Performance in a Rat Model of Alzheimer Disease: Role of Nuclear Factor Kappa Beta and Neprilysin

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Abstract:

Background: The pathophysiology of the Alzheimer disease (AD) is complex and involves multiple pathways of neuronal damage. Recently attention has been focused on the role of diet and exercise in the development and progression of neurodegenerative diseases. High caloric consumption and sedentary lifestyles are presumed to attribute to these disorders. The exact mechanisms underlying such association are still not fully elucidated. **Aim:** The aim of the present work was to study the possible effects of high fat diet (HFD) and exercise on memory performance as well as to assess the hippocampal levels of beta amyloid (β amyloid), neprilysin (NEP) and nuclear factor-kappa beta (NF-κB) proteins in a rat model of AD. **Material and methods:** The study was conducted on 32 adult male albino rats. AD was induced in all rats by a single dose of intracerebroventricular (ICV) injection of colchicine. The rats were divided into 2 groups. Group I rats (standard diet group) included 16 Alzheimer rats fed on standard diet for 10 weeks and were further subdivided into group IA and group IB. Group IA (sedentary standard diet) included 8 rats with normal daily activity while group IB (exercise standard diet) included 8 rats exposed to voluntary exercise for the last six weeks of experimental period. Group II rats (HFD group) included 16 Alzheimer rats fed on high fat diet for 10 weeks and were further subdivided into group IIA and group IIB. Group IIA (sedentary HFD) included 8 rats with normal daily activity while group IIB (exercise HFD) included 8 rats exposed to voluntary exercise for the last six weeks of experimental period. Biochemical measurement (β amyloid, NEP, NF-κB, lipid profile, glucose and insulin) and modified Morris water maze were used to assess the effect of voluntary exercise and HFD on memory retention. **Results:** Alzheimer rats fed on HFD showed higher level of β amyloid and NF-κB and poor memory performance when compared to Alzheimer rats fed on standard diet. In addition, Alzheimer rats subjected to voluntary exercise showed lower levels of β amyloid and NF-κB and better memory performance than sedentary rats which are probably attributed to increased neprilysin (β amyloid degrading enzyme). **Conclusions:** The present study highlights the importance of diet control and voluntary exercise in AD to slow down the progression of memory dysfunction by decreasing β amyloid levels and inflammatory markers as NF-κB. The increased NEP levels seen with exercise may contribute to this improvement.

Keywords: Alzheimer disease, high fat diet, amyloid hypothesis, neprilysin, NF-κB.

Introduction:

Dementia is a syndrome of acquired cognitive defects in multiple domains sufficient to interfere with social or occupational functioning.(1) There are a number of diseases that cause dementia, but the most common is Alzheimer disease (AD).(2) AD is the most common neurodegenerative disorder of the elderly. It is an irreversible, progressive brain disorder related to changes in nerve cells that result in the death of brain cells. It is characterized by cognitive dysfunctions and behavioral and social deterioration.(3)

Abbreviations:

AD, Alzheimer disease; **Beta-amyloid**, β amyloid; **HFD,** high fat diet; **NEP,** neprilysin; **NF- κB,** nuclear factor-kappa beta.

The pathophysiology of AD is complex and involves multiple distinct and overlapping redundant pathways of neuronal damage. Hippocampus, limbic system and cortex are the primary neuronal injury regions involved in disease pathophysiology. Its heterogeneous etiology makes it difficult to define clinically the most important factors in determining the onset and progression of the disease.[4] The histological hallmarks are the senile plaques and neurofibrillary tangles. Plaques are dense, mostly insoluble deposits of beta amyloid (β amyloid) protein and cellular material outside and around neurons. Neurofibrillary tangles are intracellular aggregates of the microtubule-associated protein tau which has become hyperphosphorylated.[5,6]

Many hypotheses have been raised regarding the pathophysiology of AD. The most common is the amyloid hypothesis. The amyloid hypothesis emphasizes that increased β amyloid production or failure of its clearance induce gradual β amyloid accumulation throughout life, resulting in the formation of amyloid plaques, which induce oxidative stress (OS) and inflammatory responses resulting in synaptic damage, tau tangles, and then neuronal loss.[7,8] The resulting oxidative stress and inflammation are characterized by the release of cytokines and reactive oxygen species (ROS) known to activate the nuclear factor-kappa β (NF- κB) which further activates inflammatory processes. In addition, β amyloid levels in brain are influenced not only by its production, but also by different clearance mechanisms including its clearance to blood and CSF, phagocytosis by microglia, and enzymatic degradation.[9] Neprilysin (NEP) is one of the most important β amyloid degrading enzymes whose levels were found to decrease in AD.[10]

There is currently no cure for AD; however, there are multiple drugs that have been proven to slow disease progression and treat symptoms. Investigations for novel therapeutic approaches targeting the presumed underlying pathogenic mechanisms are a major research focus. Antiamyloid agents and tau-related therapies are under clinical trials.[11,12] In addition to pharmaceutical agents, attention has recently been directed towards the role of other therapeutic adjuncts including exercise and diet control. There are studies showing that exercise decrease the risk of neurodegenerative disease such as AD.[13,14] Moreover, high fat diet (HFD) was reported to affect expression of genes that might influence inflammatory processes as well as genes that are important for maintaining synaptic function and plasticity in rodents.[15]

The aim of the present work was to study the possible effects of high fat diet and exercise on memory performance as well as to assess the hippocampal levels of β amyloid, NEP and NF-κB proteins in a rat model of AD.

Materials and Methods:

The study was conducted on 32 adult male wistar albino rats, with a body weight ranging from 125-150 grams (3-4 months). The rats were kept under standard laboratory conditions, maintained on a 12-h light–dark cycle. Rats were divided into two groups. The first group included 16 rats fed on standard diet (10% fat, 70% carbohydrate, and 20% protein) for ten weeks[16] and the second group included 16 rats fed on HFD (60% fat, 20% carbohydrate, and 20% protein) for ten weeks.[16] AD was induced in all rats by a single dose of ICV injection of colchicine divided between the right and left ventricles.[17] The rats were anesthetized with sodium pentobarbital (45 mg/kg, ip).[18] Central administration of colchicine results in cell death associated with cognitive impairment, which resembles the microtubule dysfunction in AD. Each rat received 15 micro gram colchicine dissolved in artificial cerebrospinal fluid (ACSF) (ACSF; in mM: 147 NaCl, 2.9 KCl, 1.6 MgCl2, 1.7 CaCl2 and 2.2 dextrose) (7.5 micro gram each side in 5 µL of ACSF).[18] The injection was 0.8 mm posterior to the bregma, 1.8 mm lateral to sagittal suture. A 10 µL Hamilton microsyringe was used for injection. To promote diffusion, the microsyringe was left in place for a period of 2 min following injection. After injection, all rats received gentamicin (5 mg/kg, ip) to prevent sepsis.[19] Special care was taken during the postinjection period to provide food and water inside the cage of the rat.

Two weeks after injection of colchicine, Alzheimer was confirmed by passive avoidance test.[20] Memory retention deficit was evaluated through passive avoidance apparatus. The apparatus consists of equal

size light and dark compartments (30x20x30 cm). A 40-W lamp was fixed 30 cm above its floor in the center of the light compartment. The floor consisted of metal grid connected to shock scrambler. The two compartments were separated by a trap door that could be raised to 10 cm. On day 13 after colchicine injection, rats were placed in the light compartment and the time lapse, before each rat entered the dark compartment and had all four paws inside it, was measured in seconds and termed as initial latency (IL). Immediately after the rat entered the dark chamber with all the four paws inside the dark chamber, the trap door was closed and an electric foot shock (0.8 mA) was delivered for 3 sec. Five sec later, the rat was removed from the dark chamber and returned to its home cage. Rats that had an IL of more than 60 sec were excluded from the study. [20] Twenty four hours later, the latency time was again measured in the same way as in acquisition trial, but the foot shock was not delivered and the latency time was recorded to a maximum of 300 sec. Rats had latency time more than 300 sec were excluded from the study. The remaining rats were termed Alzheimer rats. [21] Therefore, in the present study, the successful induction of AD was detected from the latency time in passive avoidance test.

Then **Group I rats (standard diet group)** which included 16 Alzheimer rats, fed on standard diet, were further subdivided into group IA and group IB. Group IA (sedentary standard diet) included 8 rats with normal daily activity while group IB (exercise standard diet) included 8 rats were exposed to voluntary exercise for the last six weeks of experimental period. [22] In group IB the cage was changed to a 2.4 times larger than standard cage and was equipped with a running wheel that freely rotates against a 100 g resistance. [22] **Group II rats (HFD group)** which included 16 Alzheimer rats, fed on HFD, were further subdivided into group IIA and group IIB. Group IIA (sedentary HFD) included 8 rats with normal daily activity while group IIB (exercise HFD) included 8 rats exposed to voluntary exercise for the last six weeks of experimental period. Behavioral studies were used to assess the effect of voluntary exercise and HFD on memory retention. Rats were subjected to daily water maze training followed by memory retention test using modified Morris water maze.

I- Memory assessment using modified Moris water maze:

Water maze task was performed for all rats. The water maze consisted of a dark circular pool, 125 cm in diameter, 55 cm in height, filled with opaque water (about 22° ± 3°C) to a depth of 20 cm, a submerged circular black platform (10 cm in diameter) was placed 20 cm away from the edge in a fixed location and 1 cm below the water surface. The pool was divided into 4 quadrants by 4 starting points marked on its wall: North, South, East, and West (N-S-E-W). The platform provided the only escape from the water. Several cues were placed outside the maze in a fixed position relative to the pool as a window, colored curtain and a red flag; they helped the rat to locate the position of the escape platform hidden below the water surface.[23] The path taken by the rat was recorded by a digital video camera that was mounted above the center of the pool to record the distance traveled and time taken by each rat to reach the platform. The distance was measured by aid of a grid with 10 cm squares placed over the pool (Figure 1).[24]

All experimental rats were subjected to daily water maze training before testing of memory retention on the last day. Each rat received four trials per day during three daily sessions. During the training session each rat was placed in the water facing the wall of the pool at one of the four designated starting points (N-S-E-W) and allowed to swim and find the hidden platform located in the NW quadrant (target quadrant) of the maze. Each of four starting positions was used once in four training sessions. ([25]) During each trial, each rat was given 120 sec to find the hidden platform. After mounting the platform, rats were allowed to remain there for 20 sec, and were then placed in a holding cage for 30 sec until the start of the next trial. After completion of training, the returned to their home cages. If rats couldn't find the platform in 120 sec they were placed on it by the examiner and left there for 20 sec.

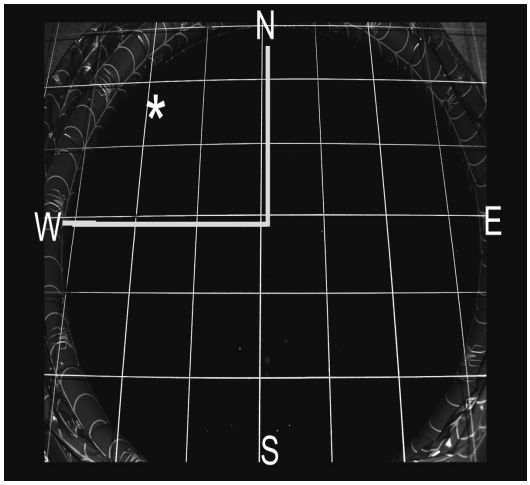

Figure 1: Water maze pool divided into 4 quadrants with 4 starting points (N-S-E-W), the mark points to the location of the hidden platform in the target quadrant of the pool (N-W). A grid with 10 cm squares was placed over the pool to measure the distance swum by the rat. [24]

At the end of the training period, memory retention testing was performed 24 hours after last day of training for each group, to test for memory consolidation. Retention testing consisted of 60 sec of a free swimming period for each rat with the hidden platform removed from the pool. The better the memory is consolidated the more time will the rat swim around the location of the platform and less escape trials will be attempted from the wall of the pool. The time spent in the target quadrant [expressed as percent of the time spent in the pool (total of 60 sec)], the distance swum in the target quadrant (expressed as percent of the distance swum in the pool) and the number of escape trials from the edges of the pool were calculated.[25]

II- Biochemical assessment:

Measurement of serum metabolic parameters:

On the last experimental day for each group; 14 hour fasting blood samples were collected, rats were sacrificed by decapitation immediately after behavioral assessments. Blood samples were collected from the retro-orbital venous plexus of the rat via inserting a capillary haemotocrit tube under light ether anaesthesia.[26] Blood was collected into a clean dry non-heparinized Wassermann tubes for separation of serum. The serum was separated by centrifugation at 3000 rpm for 15 min. Aliquots of serum were stored in eppendorf tubes at -20°C for measuring glucose and insulin levels and at 4°C for measuring the lipid profile parameters.

Serum glucose (mg/dl)[27], total cholesterol (mg/dl)[28], Triglyceride (mg/dl)[29] and HDL-cholesterol (mg/dl)[30] levels were assayed by enzymatic colorimetric method according to the manufacturer instructions (Bio–diagnostic; Dokki, Giza, Egypt, www.bio-diagnostic.com). Moreover, serum LDL-cholesterol (mg/dl) level was determined using the Friedewald formula.[31] Besides, serum insulin level was determined by enzyme-linked immunosorbent assay (ELISA)[32] according to the manufacturer instructions (WKEA Med Supplies Corp, US, www.wkeamedsupplies.com).

Measurement of hippocampal β amyloid peptide1-42, NEP and NFκB-p65 by ELISA

The whole brain was removed and washed with ice cold saline. Hippocampus was dissected through the following way: a small curved forceps was placed between the cerebral halves in a closed position, and then the forceps was repeatedly opened and closed. Once an opening was obtained along the midline, the forceps was opened and directed to both sides to separate the cortex

from the hippocampus. This movement was repeated on either side until the upper part of the hippocampus was visible (identified by its whitish color). The cortex was gently picked up with the forceps and the hippocampus was freed from the cortex without damaging the cortex. The hippocampus was cut and separated from the fornix. The two halves of the hippocampus were separated and weighted. [33] Hippocampi from each rat were stored at a temperature of -80 °C for biochemical analysis.

For determination of cytoplasmic proteins (β amyloid 1-42 and NEP), the hippocampus from each rat in this study was homogenized in homogenization buffer (10 mM HEPES, pH 7.9, 1.5 mM MgCl2, 10 mMKCl, 500 µM DTT) supplemented with complete protease and phosphatase inhibitor cocktails. The lysate was incubated on ice for 1 hour followed by addition of 5 µl 10% NP-40 and then vortexed for 10 sec. The lysate was then centrifuged for 20 sec at 14,000 rpm and the supernatant containing cytoplasmic proteins was collected and stored at -20°C for future use. Furthermore, for determination of nuclear fraction of NFκb-p65, The resulting pellet after centrifugation was incubated in nuclear protein extraction buffer (20 mM HEPES, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 500 µM DTT) supplemented with complete protease and phosphatase inhibitor cocktails and vortexed for 10 sec every 10-15 min for a total of 1 hour. The resulting lysate was then spun at 4°C for 5 min at 14,000 rpm and the supernatant containing nuclear proteins was collected and stored at -20°C for future use.[34] The protein concentration of each sample was measured in supernatants of hippocampus using Lowry method[35] and was expressed in mg/ml. Total tissue proteins level was used for normalization of ELISA results. Supernatants from hippocampus homogenate were used for determination of: β amyloid peptide Aβ1-42 (pg/mg protein)[36], NEP (pg/mg protein)[37] and NFκb-p65 (ng/mg protein)[38] proteins concentrations by ELISA, using rat-specific antibody, according to the manufacturer instructions. (WKEA MED SUPPLIES CORP, US, www.wkeamedsupplies.com).

Statistical Analysis:

The values of the measured parameters were expressed as median and interquartile range (IQR). The distributions of quantitative variables were tested for normality using Kolmogorov-Smirnov test. It revealed the data were abnormally distributed. The non-parametric tests were used. The difference between the studied groups was determined using Mann Whitney test. Spearman correlation coefficient was performed for evaluating the behavioral, neurochemical and biochemical variables. $P \leq 0.05$ values were considered significant. All statistical analyses were processed using SPSS for windows Version 20.

Results:

I- Memory retention testing in the different studied groups

Water maze memory retention testing after 6 weeks of voluntary exercise in exercise groups (group IB and IIB) resulted in a significant increase in the percent of the time spent in the target quadrant of the pool as compared to sedentary groups (group IA and IIA) where $P < 0.05$ respectively. In addition, the percent of time spent in the target quadrant of the pool was significantly less in sedentary HFD (group IIA) than sedentary standard diet (group IA) where $P<0.05$. (Table I)

In addition, voluntary exercise in exercise groups (group IB and IIB) resulted in a significant increase in the percent of the distance swum in the target quadrant of the pool as compared to sedentary groups (group IA and IIA) where $P < 0.05$ respectively. In addition, the percent of distance swum in the target quadrant of the pool was significantly less in sedentary HFD (group IIA) than sedentary standard diet (group IA) where $P<0.05$. (Table I)

Moreover, voluntary exercise in exercise groups (group IB and IIB) resulted in a significant decrease in the number of escape trials as compared to sedentary groups (group IA and IIA) where P<0.05 respectively. In addition, the number of escape trials was significantly more in sedentary HFD (group IIA) than sedentary standard diet (group IA) where P < 0.05. (Table I)

II- Serum lipid profile, glucose and insulin levels in the different studied groups

Serum cholesterol levels were significantly higher in sedentary HFD group (group IIA) than sedentary standard diet group (group IA) where P < 0.05. However, there was no significant difference between exercise HFD group (group IIB) and exercise standard group (group IB) where P =0.141. In addition, serum cholesterol levels were compared between group IIB and IIA to assess the effect of exercise. Exercise in group IIB resulted in decreased cholesterol level compared to group IIA. However this difference didn't reach statistical significant where p= 0.092 (Table II).

Serum triglyceride levels were significantly higher in sedentary HFD group (group IIA) than sedentary standard diet group (group IA) where p < 0.001. However, there was no significant difference between exercise HFD group (group IIB) and exercise standard group (group IB) where P =0.226. Exercise resulted in a significant decrease in triglyceride level in group II B compared to group IIA where p<0.001. (Table II)

Serum HDL-cholesterol levels were significantly less in sedentary HFD group (group IIA) than sedentary standard diet group (group IA) where p<0.001. However, there was no significant difference between exercise HFD group (group IIB) and exercise standard group (group IB) where P =0.563. In addition, serum LDL-cholesterol levels were significantly higher in sedentary HFD group (group IIA) than sedentary standard diet group (group IA) as well as between exercise HFD group (group IIB) and exercise standard group (group IB) where p<0.05 and p<0.001 respectively. (Table II)

To assess insulin resistance by Homeostasis model assessment for insulin resistance (HOMA-IR), HOMA-IR was calculated as follows:

HOMA-IR = [fasting glucose (mM) x fasting insulin (μU/mL) / 22.5].[39]

HFD resulted in a significant increase in HOMA-IR in HFD group (group II) compared to standard diet group (group I) (group IIA with group IA and group IIB with IB) where p<0.001 respectively. Exercise in group IIB resulted in a significant decrease in HOMA-IR when compared to group IIA where p<0.05 indicating improvement of insulin resistance by exercise. Result of fasting serum glucose and insulin levels are shown in table II.

III- Hippocampal β amyloid 1-42 level (pg/mg protein), neprilysin level (pg/mg protein) and nuclear factor-kappa B level (ng/mg protein) in the different studied groups

The hippocampal β amyloid level was found to be higher in sedentary HFD (group IIA) than in sedentary standard diet group (group IA) where p<0.05. The hippocampal β amyloid level was also increased in group IIB compared to group IB. However, this difference didn't reach statistical significance where p= 0.082. In addition, hippocampal β amyloid level was compared between subgroup B and subgroup A in both groups to assess the effect of exercise. Exercise in group IB resulted in a significant decrease of β amyloid level compared to group IA where p < 0.05. Moreover, exercise in group IIB resulted in a significant decrease of hippocampal β amyloid level compared to group IIA where p < 0.05. (Table III)

Hippocampal NEP level was found to be higher in exercise standard diet group (group I B) compared to sedentary standard diet group (group IA) with p < 0.05. In addition, hippocampal NEP level was found to be higher

in exercise HFD (group II B) compared to sedentary HFD group (group IIA) with p< 0.05. On the other hand, there was no significant difference between groups IA and group IIA where P = 0.239 or between groups IB and group II B where P = 0.751regarding NEP levels indicating that HFD perse did not significantly influence NEP levels. (Table III)

In addition, Hippocampal NF-κB level was found to be less in exercise standard diet group (group I B) compared to sedentary standard diet group (group IA) with p<0.05. In addition, hippocampal NF-κB level was found to be less in exercise HFD (group II B) compared to sedentary HFD group (group IIA) with where p < 0.05. Moreover, HFD increase hippocampal NF-κB in sedentary HFD (group IIA) than sedentary standard diet group (group IA) where p<0.05 as well as between exercise HFD (group IIB) than exercise standard diet (group IB) where p<0.05. (Table III)

Correlation analysis:

A significant positive correlation was found between level of hippocampal β amyloid and serum cholesterol among all studied groups where r = 0.397 and p<0.05 (Figure 2) and a significant negative correlation was found between level of hippocampal β amyloid and NEP among all studied groups where r = -0.359 and p<0.05 (Figure 3). In addition, a significant positive correlation was found between level of hippocampal β amyloid and nuclear NF-κB among all studied groups where r=0.514 and p<0.05 (Figure 4). Furthermore, a significant positive correlation was found between level of hippocampal NF-κB and HOMA-IR among all studied groups where r =0.417 and p<0.05 (Figure 5).

Table I: Comparison between different studied groups regarding memory retention testing

	Group IA	Group IB	Group IIA	Group IIB	MW test (P value)
Percent of time spent in target quadrant	11.67 (2.92) #	15.0 (6.67)	4.84 (5.84) #•	8.50 (3.0)	P1 < 0.05*
					P2 < 0.05*
					P3 < 0.05*
					P4=1.000
Percent of distance swum in target quadrant	14.15 (4.12) #	26.88(24.39)	12.52 (7.10) #•	20.07 (6.85)	P1 < 0.05*
					P2 < 0.05*
					P3 < 0.05*
					P4=0.074
Escape trials	9.0 (2.5) #	6.5 (2.75)	12.50 (4.50) #•	8.50 (3.0)	p1< 0.05*
					P2 < 0.05*
					p 3< 0.05*
					P4=0.133

Data are presented as a median (IQR) *significant # A significant vs B • Significant vs group I

$^{MW}p_1$: p value for Mann Whitney test for comparing between IA with IB

$^{MW}p_2$: p value for Mann Whitney test for comparing between IIA with IIB

$^{MW}p_3$: p value for Mann Whitney test for comparing between IA with IIA

$^{MW}p_4$: p value for Mann Whitney test for comparing between IB with IIB

Table II: Comparison between different studied groups regarding serum metabolic parameter

	Group IA	Group IB	Group IIA	Group IIB	MW test (P value)
Serum cholesterol (mg/dl)	54.0 (19.75)	64.5 (23.0)	89.5 (17.75)•	73.0 (34.5)	P1 =0.461 P2=0.092 P3 < 0.05* P4=0.141
Serum triglyceride level (mg/dl)	19.0 (9.50)	18.50(12.0)	52.5 (41.5)#•	21.5 (11.0)	P1 =0.636 P2<0.001* P3<0.001* P4=0.226
Serum HDL-cholesterol (mg/dl)	45.5(10.75)	42.0(15.25)	29.0(9.25)•	39.5(25.75)	P1 =0.635 P2=0.090 P3<0.001* P4=0.563
Serum LDL cholesterol (mg/dl)	26.0(10.25)	22.0(5.00)	52.5(28.25)•	46.5(14.0)•	P1 =0.370 P2=0.367 P3=0.003* P4<0.001*
Fasting serum glucose level (mg/dl)	95.0(16.50)	91.50(11.25)	140.0 (20.75)•	140.0 (13.25)	P1 =0.526 P2 =0.631 P3 <0.001* P4 =0.951
Fasting serum insulin level (MIU/ml)	3.65(1.33)	3.15(1.33)	8.40(1.33) #•	6.75(2.18)•	P1 =0.552 P2 < 0.05* P3< 0.001* P4< 0.001*
HOMA-IR	0.82 (0.31)	0.69 (0.32)	2.89 (0.89) #•	2.31 (0.85)•	P1 =0.399 P2 < 0.05* P3 < 0.001* P4 < 0.001*

Data are presented as a median (IQR) *significant

A significant vs B • Significant vs group I

$^{MW}p_1$: p value for Mann Whitney test for comparing between IA with IB

$^{MW}p_2$: p value for Mann Whitney test for comparing between IIA with IIB

$^{MW}p_3$: p value for Mann Whitney test for comparing between IA with IIA

$^{MW}p_4$: p value for Mann Whitney test for comparing between IB with IIB

Table III: Comparison between different studied groups regarding hippocampal levels of β amyloid, Neprilysin and Nuclear NF-κB

	Group IA	Group IB	Group IIA	Group IIB	MW test (P value)
Hippocampal β amyloid level (pg/mg protein	97.50(45.00)#	43.5 (51.75)	190.0 (129.75)#•	89.50 (84.75)	P1 < 0.05* P2 < 0.05* P3 < 0.05* P4=0.082
Hippocampal NEP level(pg/mg protein	33.5 (9.50)#	57.5 (135.0)	39.0 (28.75)#	135.0 (235.0)	P1 < 0.05* P2 < 0.05* P3=0.239 P4=0.751
Hippocampal NF-κB level (ng/mg protein	609.5 (252.5)#	380.0 (161.25)	700.0 (148.0)#•	512.0 (241.25)•	P1 < 0.05* P2 < 0.05* P3 < 0.05* P4 < 0.05*

Data are presented as a median (IQR) *significant # A significant vs B • Significant vs group I

$^{MW}p_1$: p value for Mann Whitney test for comparing between IA with IB

$^{MW}p_2$: p value for Mann Whitney test for comparing between IIA with IIB

$^{MW}p_3$: p value for Mann Whitney test for comparing between IA with IIA

$^{MW}p_4$: p value for Mann Whitney test for comparing between IB with IIB

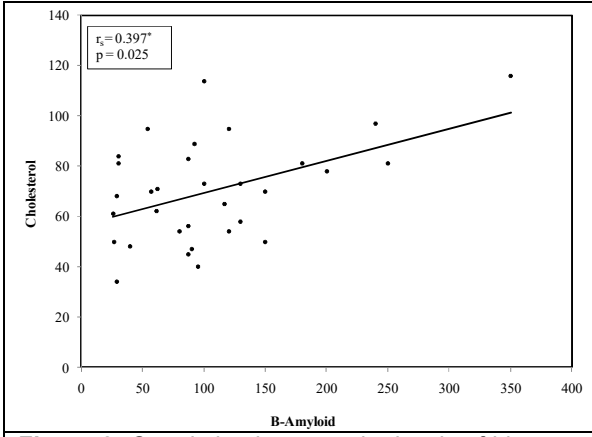

Figure 2: Correlation between the levels of hippocampal β amyloid and serum cholesterol in all studied groups

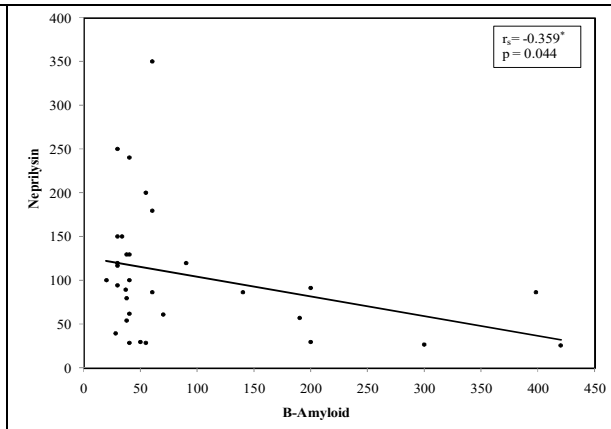

Figure 3: Correlation between the levels of hippocampal β amyloid and NEP in all studied groups

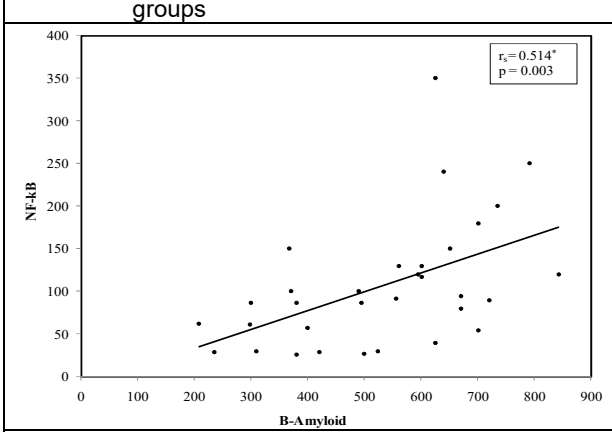

Figure 4: Correlation between the hippocampal levels of β amyloid and NF-κB in all studied groups

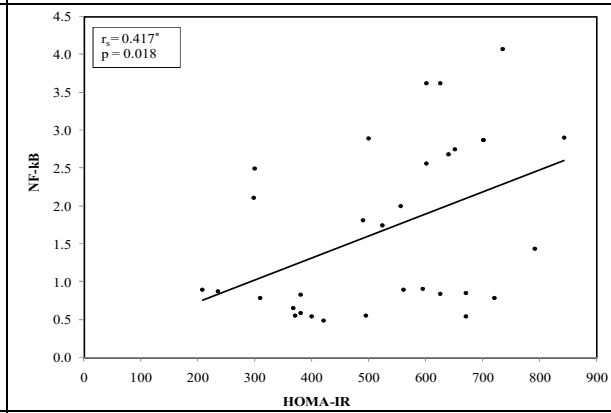

Figure 5: Correlation between the levels of HOMA-IR and the hippocampal NF-κB in all studied groups

Discussion:

Alzheimer disease is a common neurodegenerative disease. It is characterized clinically by progressive deterioration of memory, and pathologically by β amyloid plaques and neurofibrillary tangles.[40] Recent attention has been focused on the role of diet and exercise in the development and progression of neurodegenerative diseases. High caloric consumption and sedentary lifestyles are presumed to attribute to these disorders.[41] Therefore, it has become quite essential to investigate the effect of HFD and the possible preventive role of voluntary exercise on the progression of AD.

In the present work, results of water maze memory retention testing revealed that exercise (in rats exposed to voluntary exercise) caused a significant increase in the percent of the time spent and percent of the distance swum in the target quadrant of the pool as compared to the sedentary groups. It also caused a significant decrease in the number of escape trials from the edges of the pool as compared to the sedentary groups. Therefore, memory retention testing revealed an improvement in memory consolidation in Alzheimer rats subjected to exercise compared to sedentary Alzheimer rats. Moreover, HFD caused a significant decrease in the percent of the time spent and percent of the distance swum in the target quadrant of the pool in sedentary Alzheimer rats as compared to the sedentary standard diet group. It also caused a significant increase in the number of escape trials from the edges of the pool in HFD sedentary Alzheimer rats as compared to the sedentary standard diet Alzheimer rats. Therefore, memory retention testing revealed a deteriorating effect of HFD on memory consolidation. Such findings prompted us to assess the levels of β amyloid, NEP and nuclear NF-κB in the different groups to investigate the underlying mechanism of HFD and exercise on memory in AD.

In the current study, β amyloid was significantly higher in the HFD sedentary subgroup than sedentary standard diet subgroup. These findings are consistent with Pedrini et al[36] who compared the effect of four different diets on β amyloid. They found β amyloid to be higher in HFD than other diets. These finding are in agreement also with Pasinetti et al[42] who found that rats fed on high saturated fats diets had a learning and memory impairments and were associated with greater cerebral β amyloid deposition. In addition, Julien et al[43] examined the effect of HFD on transgenic mice model of AD. They demonstrated that consumption of a HFD not only increased β amyloid 40 and 42 concentrations in parieto-temporal cortex homogenates from transgenic mice, but also increased total tau protein compared to controls fed on standard diet.

In the present study, the sedentary HFD group showed increased levels of total cholesterol and triglycerides and LDL-cholesterol and decreased level of HDL-cholesterol in comparison to sedentary standard diet. Furthermore, the exercise HFD showed a decrease in cholesterol and triglyceride towards normal. Meissner et al[44] showed that voluntary running wheel increased cholesterol turnover in healthy mice owing to an increased fecal bile acid excretion and a decreased intestinal cholesterol absorption. In addition, positive correlation was found in this study between level of hippocampal β amyloid and serum cholesterol. Similar result obtained from Reiss[45] who found that high levels of cholesterol were associated with increased risk of AD and patients taking cholesterol-lowering drugs (statins) had a lower prevalence of AD. Therefore, the present study indicates that HFD may affect the progression of AD through increasing brain β amyloid levels. Accumulating data suggest that cholesterol may affect more directly the amyloid cascade by promoting amyloidogenic processing of amyloid precursor protein. Amyloid precursor protein is a transmembrane protein whose proteolysis generates β amyloid. Alterations in cholesterol metabolism might also promote neuronal degeneration by perturbing membrane fluidity and signal transduction.[46]

The present work additionally conveyed the effect of HFD on insulin resistance. HOMA-IR was significantly higher in HFD than standard diet groups and HOMA-IR level was improved by exercise. Similar observation was found by Touati et al[47] who studied the effect HFD for 12 weeks and compared it by a group that was fed same diet and exposed to

treadmill exercise. They concluded that exercise reduced adiposity, improved glucose, insulin levels and plasma lipid profile, and exerted an antihypertensive effect and that the exercise training induces these beneficial effects without the requirement for dietary modification.

Although the precise role of β amyloid in disease progression remains somewhat controversial, many efforts to halt or reverse disease progression have focused on reducing its synthesis or enhancing its removal. NEP, one of amyloid degrading enzyme, has been the main target of Alzheimer research last year's.[48]

In the present study, voluntary exercise resulted in a significant increase in NEP levels in exercise groups when compared to sedentary groups. There was no significant difference between Alzheimer rats fed on standard diet and Alzheimer rats fed on HFD regarding NEP level. In addition, a significant negative correlation was found between hippocampal NEP level and β amyloid levels indicating that NEP induced by voluntary exercise can decrease brain β amyloid.

These finding are similar to Iwata et al[49] who reported that administration of the adeno-associated virus vector mediated NEP gene into an AD mouse model caused reduced β amyloid oligomers, with concurrent alleviation of abnormal learning and memory function. These findings were also consistent with park et al[50] who found that the administration of recombinant soluble NEP by intracerebral injection into AD mice significantly reduced accumulation of β amyloid and improved the behavioral performance on the water maze test. On the other hand, Meilandt et al[51] suggested that NEP may not cleave some naturally occurring oligomeric β amyloid.

Brain β amyloid clearance is also accomplished through efflux into the periphery.[52] It has been hypothesized that a dynamic equilibrium exists between the brain and peripheral β amyloid (plasma β amyloid) which is the basis for several anti- β amyloid vaccination approaches.[53,54] It should be noted that the route of administration of NEP is very important in degradation of brain β amyloid. Peripheral administration of recombinant NEP does not affect brain β amyloid as much as plasma β amyloid. This is explained by the fact brain-to-plasma clearance mechanisms for β amyloid are saturated under normal conditions and that an additional driving force in the form of reduced plasma β amyloid concentrations cannot enhance clearance from brain. Recombinant NEP as anti- β amyloid therapeutic would therefore not be expected to achieve significant improvement.[55]

While the causes of Alzheimer disease remain poorly defined, neuroinflammation appears as a central event in AD pathophysiology.[56] NF-κB is known as the master controller of inflammation.[57] A significant positive correlation was found between the level of hippocampal β amyloid and NF-κB. The high level of nuclear NF-κB in AD was explained by Granic et al[58] who found that the pathological hallmarks of AD (β amyloid and hyperphosphorylated tau) are capable of inducing nuclear NF-κB activation via various mechanisms. One common mechanism is the activation of the advanced glycation end products (AGE/RAGE) signaling pathway. β amyloid and tau can undergo a non-enzymatic glycation and form AGEs. These AGEs bind to RAGEs and can trigger nuclear NF-κB dependent gene transcription. In addition, AGEs have been reported to generate reactive oxygen intermediates, leading to the activation of cytokines including IL-1 and TNF, which in turn induce the translocation of NF-κBto the nucleus resulting in further inflammation.

In order to show the effect of exercise on expression of NF-κB in the brain, Ma Y et al[59] investigated the effects of treadmill training on the recovery of neurological function and the expression NF-κB in the ischemic rat brain after middle cerebral artery occlusion-reperfusion. Their results indicated that treadmill training promoted functional recovery and reduced the overexpression of NF-κB in rat brain tissue after ischemia. It has also been shown in this study that exercise can reduce nuclear NF-κBlevels.

In the last decade, it has been clear that exercise beneficially affects the brain functions. However, the effect of exercise appears to be very complex. That could include neurogenesis via neurotrophic factors, increased capillarization, decreased oxidative damage, and increased proteolytic degradation by proteasome and NEP that results in decreased accumulation of

carbonyl and β amyloid protein. Exercise also improved redox state and decrease free ROS. [60] Data from our study indicate that exercise modulates the expression of NF-κB which plays a pathogenic role in AD.

As regards the effect of diet on expression of hippocampal NF-κB in AD, the HFD resulted in a significant increase of NF-κB compared to standard diet. Several studies indicate that caloric restriction suppresses NF-κB activation and immune response.[61-63] Caloric restriction inhibits NF-κB signaling possibly through a ROS dependent mechanism.[61] Additionally, caloric restriction could downregulate the expression of 56 inflammatory genes, many of which are transcriptionally regulated by NF-κB.[62] Even short term caloric restriction (10 days) resulted in decreased NF-κB activity in kidneys of aged mice.[63] Therefore, caloric restriction, a known mediator of improved life and health span, functions, at least in part, via NF-κB and inflammatory suppression.

To date, a link between Alzheimer disease and metabolic disorders has been established. The patients with type 2 diabetes are found at increased risk of developing AD and vice versa.[64] Inflammation plays critical roles in the pathogenesis of AD and type 2 diabetes. Overproduction of pro-inflammatory cytokines (TNF-alpha and NF-κB) is a key feature of the pathophysiology of type 2 diabetes and neuronal dysfunction in AD. In this study a positive correlation was found between HOMA-IR and hippocampal nuclear NF-κB. In addition, insulin resistance in Alzheimer rats fed on HFD subjected to voluntary exercise was decreased than sedentary rats pointing to the importance of exercise in reducing insulin resistance. This goes in agreement with Granic et al[58] who supposed that the link between diabetes mellitus and AD is due to NF-κB activation. Since insulin resistance in the peripheral tissue can lead to glucose intolerance, neuronal inflammatory processes triggered by the NF-κB pathway may be propagated even further via stimulation of the AGE/RAGE signaling pathway. Therefore, they expected that alleviating symptoms of type 2 DM may be an effective way to treat AD.

To summarize, Alzheimer rats fed on HFD showed higher level of β amyloid and nuclear NF-κB and poor memory performance when compared to Alzheimer rats fed on standard diet. In addition, Alzheimer rats subjected to voluntary exercise showed lower levels of β amyloid and NF-κB and better memory performance than sedentary rats which are probably attributed to increase NEP (β amyloid degrading enzyme).

Conclusions:

The present study highlights the importance of diet control and voluntary exercise in AD to slow down the progression of memory dysfunction by decreasing β amyloid levels and inflammatory markers as NF-κB. The increased NEP levels seen with exercise may contribute to this improvement**.**

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