Volume No. 51: (1 & 2)

January 2019

The Journal of the Egyptian Society of Endocrinology, Metabolism & Diabetes

Journal Of The Egyptian Society of Endocrinology, Metabolism & Diabetes.

Editorial & Advisory Board

Instructions to Authors...

The journal publishes reports of clinical and experimental work in all aspects of research in the fields of endocrinology, diabetes and metabolism and related subjects, provided they have scientific ment and represent an important advance in knowledge. The joumal does not publish material that has been printed previously or is under consideration for publication elsewhere. The Editor will consider papers from any country whether or not the author(s) is a member of the society.

Submission

Submit the transcript and 3 copies of the manuscript to the address shown at the end of these instructions. Double-space all material (1cm between lines), leave at least 25-cm margins at top, bottom, and both sides of each page, and begin each of the following on a new page: (1) Title page. (2) Abstract, (3) Text, (4) References, (5) Legends, and (6) Tables. Number all pages and label each page with the name of the first author. The manuscript is to be typed on one side of the paper only. Manuscripts will not be returned. to the authors. A cover letter should include the following statement: This material is original and has not been published previously." Include phone number and mobile phone number of the corresponding author Indicate the date of publication desired. Submission of diskettes or CDs is mandatory.

Submission Fees

Submission fees are 1000 Egyptian Pounds per article provided it does not exceed 8 printed pages. Twenty-five reprints will be provided to the authors. Extra fees will be charged if the article exceeds 8 pages in length, if it contains colored figures or if more than 25 reprints are requested by the authors

Peer Review and Editing

Manuscripts will be sent for peer review without prior editing All manuscripts accepted for publication will be edited in the journal offices. Authors will receive an approval copy of page proofs before publication

Organise all manuscripts as follows

Title Page

Provide a title. Include first name, middle initial, last name, and affiliation of each author. Indicate the name, address, and e-mail address of the author to whom correspondence and reprint requests should be addressed. Provide also a short running title (70 characters and spaces) including first author's name and initials, et al (e.g., SH Asaad-Khalil, et al; Autoantibodies and sibs of children with type 1 diabetes mellitus)

Abstract

Limit the abstract to 250 words. Use a structured format, including Aim, Subjects and Methods, Results, and Conclusions. Provide 3-6 key words for indexing at the end of the abstract Provide a list of abbreviations used throughout the manuscript, arranged alphabetically. at the bottom of the first page.

Text

Articles should be written in clear, concise English according to the Concise Oxford Dictionary Minimize use of abbreviations; any abbreviations used must be defined at first mention (except for units of measurement when used with numbers).Abbreviations may be used in tables and figures for space considerations but must be defined in the accompanying footnotes or legends. The AMA Manual of Style lists standard scientific abbreviations. In general, use generic names for drugs. To maintain anonymity, do not use patient names, initials, or any unnecessary identifying details (Individual cases should be labeled as "case 1," "case 2," and so forth.) The text should be structured as follows:

Introduction: The introduction should contain a clear statement of the aim and novelty of the study. It should include neither results nor conclusions.

Subjects and Methods: Sufficient information should be given to permit repetition of the experimental work. A paper describing experimental work in humans must (a) indicate that informed consent has been obtained from patients when appropriate. (b) include a statement that the responsible ethics committee (institutional review board) has given approval, and/or indicate that the reported investigations have been carried out in accordance with the principles of the Declaration Helsinki as revised in 1996 [JAMA 1997: 277 925-926] Reports of animal experiments must state that the "Principles of laboratory animal care (NIH publication no 85-23, revised 1985) were followed

Results: The results should be stated concisely without comment. The same data should not be presented in both a figure and a table. Express measurements in conventional units (Si units between brackets).

Discussion: The discussion should deal with the interpretation of the results and not recapitulate them. It should deal with the relationship of the new information given in the Results to the corpus of knowledge in that field and should be pertinent to the data presented. The main conclusions should be incorporated in a final paragraph.

References: Number references sequentially as they are cited in the text enclose each reference citation in parentheses at the proper point in the text above the line. Type reference list completely double-spaced. Avoid use of abstracts as reference when possible. Unpublished data should be cited parenthetically in the text and not included in the reference list. For joumal references, Index Medicus journal abbreviations should be used. Include names and initials of all authors (if more than 6, list only the first 3 followed by "et al.") There must be only one reference per number.

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.

Book

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

Chapter in Book

3- Flier JS, Foster DW. Eating disorders: obesity, anorexia nervosa, and bulimia nervosa. In: WilsonJD, Foster DW, Kronerberg HM, Larsen PR, eds Williams Textbook of Endocrinology 9th ed.

Philadelphia: WB Saunders, 1998: 1001-1097.

Tables: Type all tabular material double spaced, each table should be on a separate page and numbered in Roman numbers. Provide a title for each table, and define all abbreviations in a footnote. The exact P value is to be stated in the tables Do not submit tum-page material (tabulations typed across the vertical dimension of the page).

Figures: Submit 3 glossy prints and 3 photocopies for each illustration Provide a legend for each figure; define any abbreviations that appear on the illustrations. Specify stain and magnification for photomicrographs. For any. photographs with a recognizable patient, a release form signed by the patient must be provided. Graphs and diagrams should be submitted as LASER quality computer prints or sharp photographs on white paper. Whenever possible. prints should be cropped to be one column width (86 cm) or be capable of being reduced to such size. Colour illustrations will be accepted; however, the authors will be charged the extra cost Figures should be on a separate page each and should be numbered by Arabic numbers.

Proofs

A proof is sent with the manuscript to the corresponding author. Typographical errors only should be corrected. One corrected proof should be returned immediately with the manuscript to the Editorial office by express mail. If the proof is not returned within four weeks, papers will be published without the author's corrections Any changes to the text or figures are liable to be charged to the authors

Revised Manuscripts

Revised manuscripts accepted for publication should be submitted in both hard copy and on two 3.5-inch diskette form. Use Windows operating system and Word as word processing software. Electronic submission of articles is possible but ONLY in the form of Word attachment file and must be followed by 3 printed copies of the manuscript. If a paper is not received back in the Editorial office within four weeks, it will be treated as a new article.

Conflict of Interest and Acknowledgments

Authors are required to disclose any potential conflict of interest Acknowledgments should list brief statements of assistance, financial support, and prior publication of the study in abstract form, if applicable.

Send cover letter and materials to:
The Journal of the Egyptian Society of Endocrinology, Metabolism & Diabetes
Prof. Samir Helmy Assaad-Khalil,
Editor-in-Chief
Professor of Internal Medicine
Unit of Diabetes and Metabolism
Faculty of Medicine, Alexandria University
Alexandria, Egypt.
Tel.: (203) 4874204
Fax: (203) 4833321
e-mail: assaadkhalil@hotmail.com
website: jesemd.alexu.edu.eg
ISSN: 1110-1245 (print)
ISSN: 2535-1745 (Online)

Journal Of The Egyptian Society of Endocrinology, Metabolism & Diabetes.

Contents

Letter from The Editor

Dear Colleague,

In this volume, we have included a very important editorial article which deals with an important issue of health care for persons with diabetes i.e. Complications of Diabetes Mellitus. Two colleagues from the University Hospital of Alexandroupolis, Athens; Greece Prof. Nikolaos Papanas and Prof. Stella Papachristou are sharing their experience with an article entitled: Skin AGEs and Complications ofDiabetes Mellitus. 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

ISSN: 1110-1245 (Print)
ISSN: 2535 – 1745 (On Line)
Website: www.jesemd.com
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

Editorial Article

Skin AGEs and Complications of Diabetes Mellitus

Stella Papachristou, Nikolaos Papanas

Diabetes Centre-Diabetic Foot Clinic, Second Department of Internal Medicine, University Hospital of Alexandroupolis, Alexandroupolis, Greece

Address correspondence to: Prof. Nikolaos Papanas, Diabetes Centre-Diabetic Foot Clinic, Second Department of Internal Medicine, University Hospital of Alexandroupolis, G. Kondyli 22c, Alexandroupolis, Greece, email: papanasnikos@yahoo.gr

According to the International Diabetes Federation (IDF) statistics, it is estimated that every 7 seconds someone is dying due to diabetes mellitus (DM) or its complications.[1] Major complications of DM include cardiovascular disease, diabetic nephropathy, diabetic neuropathy, and retinopathy.[2] These complications are also linked with substantial morbidity and poor quality of life.[1,2] Therefore, timely diagnosis of DM and its complications is crucial. In this context, skin advanced glycation end products (skin AGEs) have received increased attention during the last years.[3]

During hyperglycaemic state, glucose forms a non-enzymatic process with plasma proteins, lipids, or nucleic acids, which is termed the "Maillard reaction".[4] This reaction results in the formation of endogenous AGEs. In addition to them, exogenous AGEs are of extreme importance. Indeed, dietary AGEs are by-products of heat-processed food consumption and are partially absorbed as such.[5] Smoking is also a major source of exogenous AGEs.[6] AGEs are metabolised via extracellular proteolysis as well as intracellular uptake and degradation in tissue macrophages, and Kupffer cells in the liver.[7] Once degraded intracellularly, AGEs are further metabolised to second generation AGEs and excreted by kidneys.[7]

AGEs accumulate with increasing age. Nevertheless, their accumulation is exacerbated by many pathologic conditions, such as DM, cardiovascular disease, Alzheimer's disease etc. In such conditions, AGEs are not merely a disease manifestation, but instead a factor contributing to the pathophysiology.[8]

Measurement of AGEs can be accomplished by several different methods. These include ELISA using monoclonal or polyclonal antibodies, high performance liquid chromatography (HPLC), and mass spectrography.[9] For tissue-bound AGEs, the gold standard remains as tissue biopsy, but it is invasive and very demanding.

In recent years, AGE-related autofluorescence has been used as a non-invasive option to measure AGEs. This method uses the autofluorescence, which is inherent in some major AGEs (e.g. pentosidine).[10] In order to achieve quick, inexpensive and non-invasive measurements, the AGE reader (Diagnoptics, Groningen, The Netherlands) was produced.[10,11] This is a portable device that illuminates a skin surface of approximately 4 cm^2 at the volar side of the dominant arm, guarding against surrounding light with an excitation light source. Emission and reflected extraction light are measured with a spectrometer using a glass fibre. [10,11]

Increased skin autofluorescence (SAF) has been linked with microvascular complications of DM such as nephropathy, neuropathy and to a lesser extent retinopathy.[10-12] In subjects with type 2 DM, this method was also able to predict likelihood of developing these microvascular complications except for retinopathy.[12]

SAF can contribute to the timely and accurate identification of the future risk for diabetic

peripheral neuropathy (DPN).[13] In high-risk subjects, close monitoring, multifactorial treatment and education should be intensively offered to avert further risk of complications, notably foot ulcerations.[13,14]

In conclusion, skin AGEs are increasingly being appreciated for the timely diagnosis of chronic DM complications. For this purpose, they merit further widespread utilisation, to improve patient outcomes.[13-15]

Funding: None

Conflicts of interest: None

References

1) **Global report on Diabetes** https://www.who.int/diabetes/global-report/en/ Last accessed 04 July 2019

2) **Pafili K, Papanas N, Maltezos E.** Treatment of diabetic complications: how can we learn by seeking and blundering? Angiology 2015; 66: 301-3.

3) **Meerwaldt R, Links T, Graaff R, et al.** Simple non-invasive measurement of skin autofluorescence. Ann N Y Acad Sci 2005; 1043: 290-8.

4) **Singh VP, Bali A, Singh N, Jaggi AS.** Advanced glycation end products and diabetic complications. Korean J Physiol Pharmacol 2014; 18(1): 1-14.

5) **Uribarri J, Woodruff S, Goodman S, et al.** Advanced glycation end products in foods and a practical guide to their reduction in the diet. J Am Diet Assoc 2010; 110(6): 911-6.

6) **Cerami C, Founds H, Nicholl I, et al.** Tobacco smoke is a source of toxic reactive glycation products. Proc Natl Acad Sci USA 1997; 94(25): 13915-20.

7) **Smedsrod B, Melkko J, Araki N, Sano H, Horiuchi S.** Advanced glycation end products are eliminated by scavenger-receptor-mediated endo-cytosis in hepatic sinusoidal Kupffer and endothelial cells. Biochem J 1997; 322 (Pt 2): 567-73.

8) **Nin JW, Jorsal A, Ferreira I, et al.** Higher plasma levels of advanced glycation end products are associated with incident cardiovascular disease and all-cause mortality in type 1 diabetes: a 12-year follow-up study. Diabetes Care 2011; 34(2): 442-7.

9) **Goh S-Y, Cooper ME.** The role of advanced glycation end products in progression and complications of diabetes. J Clin Endocrinol Metab 2008; 93(4): 1143-52.

10) **Lutgers HL, Graaff R, Links TP, et al.** Skin autofluorescence as a noninvasive marker of vascular damage in patients with type 2 diabetes. Diabetes Care 2006; 29(12): 2654-9.

11) **Smit AJ, Gerrits EG.** Skin autofluorescence as a measure of advanced glycation endproduct deposition: a novel risk marker in chronic kidney disease. Curr Opin Nephrol Hypertens 2010; 19(6): 527-33.

12) **Stirban A, Gawlowski T, Roden M.** Vascular effects of advanced glycation endproducts: Clinical effects and molecular mechanisms. Mol Metab 2013; 3(2): 94-108.

13) **Huijberts MS, Schaper NC, Schalkwijk CG.** Advanced glycation end products and diabetic foot disease. Diabetes Metab Res Rev 2008; 24(Suppl 1): S19-S24.

14) **Stirban A, Tschöpe D.** Vascular effects of dietary advanced glycation end products. Int J Endocrinol 2015; 2015: 836498.

15) **Stirban A, Heinemann L.** Skin autofluorescence - A non-invasive measurement for assessing cardiovascular risk and risk of diabetes. Eur Endocrinol 2014; 10(2): 106-10.

16) **Stirban A, Pop A, Fischer A, Heckermann S, Tschoepe D.** Variability of skin autofluorescence measurement over 6 and 12 weeks and the influence of benfotiamine treatment. Diabetes Technol Ther 2013; 15(9): 733-7.

Original Article

Relationship Between E – Selectin Gene Polymorphism and Carotid Artery Atherosclerosis in End Stage Renal Disease

Rasha Gawish[1], Manar Elgelany[1], Mohamed Abd-Elkader[1], Mona Sobhy[2], Omnia Gamal El-din[3]

(1) Department of Internal Medicine, Nephrology & Transplantation Unit, Faculty of Medicine Alexandria university.
(2) Department of Medical Biochemistry Department, Faculty of Medicine, Alexandria University.
(3) Department of Radio Diagnosis, Faculty of Medicine, Alexandria University, Alexandria, Egypt

Corresponding author: Rasha Gawish Email: gawishrasha@gmail.com

Abstract:

Background: Adhesion molecules like the members of selectin family participate in the interaction between leukocytes and the endothelium. They are also involved in the pathogenesis of atherosclerotic processes. E-selectin is a cell surface glycoprotein that mediates the adhesion of leucocytes to vessels endothelium, an important early step in the atherosclerotic process. End-stage renal disease (ESRD) is a highly atherogenic disease, but it is unknown whether genetic polymorphism(s) in the E-selectin gene plays a role in the severity of arterial damage in this condition. **Objective:** The aim of the study was to test the relationship between the Leu 554 Phe polymorphism of the E-selectin gene and the severity of carotid atherosclerosis in End stage renal disease patients. **Methods:** In this study, we tested whether the Leu554Phe variant in the E-selectin gene is linked to carotid atherosclerosis as estimated by intima-media thickness (IMT), cross-sectional area and carotid stenosis using carotid ultrasonography in 70 well-characterized ESRD patients. The frequency of this polymorphism was also measured in a population sample of 30 healthy controls of the same geographical area using genetic analysis of Leu554Phe polymorphism of E-selectin gene by restriction fragment length polymorphism **Results:** A total of 14.3% patients had the CC genotype, 78% had the CT genotype, 7.14% had the TT genotype and this distribution did not differ from that in the control population. Intima-media thickness (IMT) (P =0,926) and cross-sectional area (P = 0.567) were not significantly higher in patients with the T-allele than in those without this allele. Furthermore, the degree of carotid stenosis was not significantly higher (P = 0.593) in patients with T-allele than in CC patients. On multivariate analyses including the traditional and non-traditional risk factors, the Leu554Phe polymorphism was confirmed as not an independent correlate of IMT (P = 00926), cross-sectional area (P = 0.567) and carotid stenosis (P = 0.593). **Conclusion:** In ESRD, the Leu554Phe polymorphism of E-selectin gene is not associated with the severity of carotid atherosclerosis, suggesting that genetically-determined alterations in the E-selectin molecule doesn't render ESRD patients with this gene variant particularly susceptible to the detrimental effects of inflammation on the arterial wall.

Key Words: E- selectin, Leu554Phe variant, carotid stenosis, ESRD,

INTRODUCTION

Atherosclerosis is a multifactorial disease that involves the interaction of environmental and genetic factors; it is the single most common cause of death and disability worldwide [(1)]. Accelerated atherosclerosis has been noticed in patients with CKD. It was also noticed that atherosclerotic lesions developed in the early stages of CKD [(2).]

Cardiovascular diseases are 3.5–100 times higher in patients who are on maintenance dialysis than the general population [(3)]. End-stage renal disease patients have also much greater mortality from myocardial infarctions (MIs) [(4)]. The mortality of patients receiving dialysis reaches 60 % in the first year after a first myocardial infarction.

Numerous pathophysiologic observations led to the postulation of the response-to-injury hypothesis of atherosclerosis, which initially stated that endothelial desquamation was the key event in atherogenesis [(5)]. Now, it is considered that the pathophysiology of atherosclerosis is due to endothelial dysfunction rather than endothelial denudation. Whichever process is at work, each characteristic lesion of atherosclerosis represents a different stage in a chronic process of inflammation that takes place in the arterial wall [(6).]

The first morphologic phenomenon observed in plaque formation is the adherence of monocytes to the endothelial surface [(7)], monocytes adherence are triggered by a number of adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), P-Selectin and E-Selectin. Moreover, there are T-lymphocytes that tend to accumulate in atherosclerotic lesions [(8).]

This adhesion is followed by monocytes migration into the intima, this migration is induced by the presence of certain mediators called chemo attractants in the intima as monocyte chemoattractant protein-1 (MCP-1) which is released from the endothelium [(9)].

In the intima, monocytes are activated, converted to macrophages and may divide. Macrophage cell division is triggered by macrophage colony stimulating factor (M-CSF), which can augment expression of macrophage scavenger receptors leading to uptake of modified lipoprotein particles and formation of lipid laden macrophages or foam cells [(10)].

The macrophage foam cells produce a range of inflammatory cytokines including tumor necrosis factor alpha, metalloproteinases and the pro coagulant tissue factor that potentially stimulates thrombus formation when in contact with blood [(11)].

Atherosclerosis in chronic kidney disease:

The increased risk of atherosclerosis in CKD patients is the result of interaction between traditional risk factors and specific uremic and dialysis related risk factors [(12)]. Moreover, the prevalence of the traditional risk factors is greater in dialysis dependent patients than in the general population [(13)].

Cardiovascular risk factors in chronic kidney disease:

Risk factors can be divided into the following:

I- Traditional risk factors [(14)] :

Traditional risk factors include the following: age , sex, family history , obesity, cigarette smoking, hypertension, diabetes mellitus dyslipidemia and sedentary life style

II- Non traditional risk factors [(15)]:

They include homocysteine ,lipoprotein (a), asymmetric dimethyl arginine (ADMA), advanced glycation end products (AGEs) ,oxidative stress , infections and inflammation.

III- Other potential risk factors specific to ESRD

Risk factors related to ESRD include disturbance of glucose metabolism, anaemia [(16)], calcium-phosphate disorders [(17)], coagulation equilibrium imbalance [(18)], vitamin E-deficiency [(19)], hypoalbuminaemia [(20)],haemodynamic overload (plasma volume expansion, arteriovenous fistula) [(21)].

IV- Risk factors related to dialysis [(21)]:

Endotoxin, bioincompatibility of membranes, fluctuation in blood pressure and fluctuation in serum electrolytes level.

Inflammation and atherosclerosis

Atherosclerosis is considered as a chronic inflammatory process as suggested by Ross. Inflammation mediates all stages of atherosclerosis from initiation through progression and thrombotic complications.

Inflammation determines the fragility of the fibrous cap of the plaque, and its thrombogenic potentiality. T cells stimulate matrix metalloproteinases production such as proteinase, collagenase and also the production of the pro-coagulant tissue factors .Macrophage-derived matrix metalloproteinase digests the extracellular matrix of the plaque resulting in fibrous cap instability and plaque rupture. This in turn leads to the exposure of the tissue factor-rich lipid core to the circulating blood components, and thrombosis occurs [(22)].

Adhesion Molecules:

The adherence of circulating leucocytes to endothelial cells and the subsequent trans-endothelial migration to the vascular intima is one of the key events in the initiation of atherosclerosis [(23)]. These processes are mediated chiefly by different cellular adhesion molecules (CAMs), which are released on the surface of vascular endothelial cells in response to pro-inflammatory cytokines, oxidants or oxidized LDL-cholesterol [(24)]. Increased expression of CAMs have been described on endothelial cells, vascular smooth muscle cells and macrophages in human atherosclerotic plaques. Soluble types of these adhesive molecules are thought to be shed from activated cells and can be measured in peripheral blood. Plasma concentrations of these soluble forms of CAMs are elevated in septicemia, autoimmune diseases and allograft rejection [(24)].

Cell adhesion molecules fall mainly under four broad families selectins, immunoglobulin superfamily, integrins and cadherins [(25)]. The members of immuoglobulin superfamily are related to immunoglobulins and are often expressed on cell surface. They are the biggest group of adhesion molecules including more than 70 members, e.g., intercellular adhesion molecules (ICAM), and vascular cell adhesion moleucle-1 (VCAM-1) [(25)].

Members of the selectin family play an important role in the adhesion of leukocytes to activated endothelium. This produces characteristic rolling motion of the leucocyte on the endothelial surface. The selectins can be classified according to their site of expression into: P-selectin (platelet), L-selectin (leucocyte) and E selectin (endothelium) [(26)].

E-selectin:

E-selectin was described as an antigen that was induced on cultured human umbilical vein endothelium after stimulation by interleukin 1 (IL-1) and that was contributed to the adhesion of neutrophils, previously described as endothelial leucocyte adhesion molecule-1 [(27)].

E-selectin has a molecular weight of approximately 94.000 dalton. It is composed of an array of discrete protein domains; an amino terminal, C-type lectin domain, a single epidermal growth factor (EGF) like domain and from 4-9 short consensus repeats (SCR), a single membrane spinning region and a cytoplasmic tail with a 589 amino acids as shown in Figure (1) The terminal lectin domain and an epitope within the EGF like region are important for ligand binding [(28)]. The human E-selectin gene *(SELE)* is located on chromosome 1q. It consists of 14 exons and 13 introns, spanning approximately 13kb [(29)] .

E-selectin is expressed only by cytokines activated endothelial cells, cytokines such as interluekin-1 and tumor necrosis factor alpha induce ICAM, VCAM, and E-selectin. Interleukin-1 mRNA and tumor necrosis factor protein have been demonstrated in smooth muscle cells and macrophages of atherosclerotic plaques. At the endothelial cells, E-selectin as other selectins mediate leukocyte rolling which is the initial step in leukocytes adhesion to the endothelial cells [(30)].

E-selectin is maximally released 2-4 hours after cell has been activated. Within the next 24-48 hours, E-selectin is removed from the cytoplasmic membrane by shedding into the circulation [(31)] Plasma E-selectin levels were increased in patients with hypertension, hyperchlosterolemia and diabetes mellitus, all the three disorders are important risk factors for atherosclerosis, it was expressed by endothelial cells overlying atherosclerotic plaques as well as by adventitial vessels [(32)].

Several lines of evidence indicate that besides environmental factors, the genetic background has an important influence on inflammation-induced arterial damage. The E

selectin genomic location was previously known to be linked to blood pressure. Moreover the polymorphism in the E-selectin gene (Leu554Phe) hinders the E selectin shedding from the endothelium was believed to be associated with coronary atherosclerosis [33].

In patients with ESRD, it is still unknown whether the genetic background plays a role in atherosclerosis and inflammation. It is still a relevant question because in uremia, the factors predisposed to atherosclerosis do not correspond to those in the general population. Adhesion molecules have a crucial rule in the development of atherosclerosis, Therefore attention was given on Leu554Phe polymorphism as it enhances the anchoring of E-selectin to the endothelium [34]. This alteration in amino acids sequence in E-selectin gene that might amplify the inflammation process on the vessel wall.

AIM OF THE STUDY

The aim of the study was to test the relationship between the Leu554Phe polymorphism of the E-Selectin gene and the severity of carotid atherosclerosis as estimated by intima- media thickness (IMT), cross-sectional area and carotid stenosis using carotid ultrasonography in End stage renal disease (ESRD) patients.

SUBJECTS AND METHODS

The present study was conducted on 100 subjects selected from the kidney dialysis unit, El-Mowasah hospital, Alexandria Main University Hospital.

The design of the study consist of two groups:

Group (I): included 70 dialysis patients of age ranged between 20 to 70 years old ,who have been on regular dialysis treatment (RDT) for at least 6 months and who were free of overt infections (fever, infected vascular access , peritonitis or exit-site infection) but suffered from other chronic disease as hypertension , diabetes and cardiac diseases.

Group (II): included 30 healthy age and sex matched as patients who were considered as control group for genetic data analysis, and who were free from acute or serious chronic diseases such as cancer, hypertension, diabetes and Coronary heart disease.

The following was done to all the enrolled subjects:

I- Thorough history taking and Complete clinical examination including:

1. Body mass index.

The patients were grouped according to their body mass index (BMI) into underweight (<18.5 kg/m2), average weight (18.5–24.9 kg/m2) and overweight (>24.9 kg/m2).

2. Vital signs.

- **Heart rate**

It is measured by palpation of the radial artery pulsation, number of heart beats per minute.

- **Blood pressure**

In hemodialysis patients pre dialysis and post dialysis BPs were calculated as the average value of all recordings [12 measurements (i.e. 3 per week)] taken during the month preceding the study. The mean value of pre dialysis and post dialysis BPs was then obtained for each patient and it was considered for global statistical assessment.

II- Laboratory investigations including:

1- Fasting blood glucose is performed during the mid-week non-dialysis day [35].

2- Blood urea and serum creatinine.

3- Haemoglobin level [36].

4- Fasting Lipid profile (Total cholesterol, serum triglycerides, low density lipoproteins cholesterol and high density lipoproteins cholesterol) [37].

5- Serum calcium, phosphrous and PTH [38].

6- CRP, measured by nephelometry (high sensitivity)[39].

7- Genetic analysis of Leu55Phe polymorphism of E-selectin gene by restriction fragment lengh polymorphism [40].

I- Carotid artery ultrasonography for vascular assessment:

Doppler studies were performed in longitudinal and transverse planes using anterior, lateral and posterior approaches by both B-mode imaging and power Doppler imaging. Assessment of atherosclerosis is done by measurements of:

1- Carotid intima media thickness [41]:

Measurement of carotid IMT bilaterally was done by B-mode 7 MHZ probe [ultrasound examination was done using LOGIQ 7 PRO, GE (General Electric Medical System).

2- Degree of stenosis [42].

3- Cross section area, was calculated by the standard formula [43].

Statistical Analysis of the data [44]:

Data were statistically described in terms of mean ± SD, median (interquartile [IQR] range) or frequencies (number of cases) and percentages when appropriate. Comparison of numerical variables between the study groups was done using Student's t test for independent samples in comparing two groups when normally distributed and Mann-Whitney U test for independent samples when not normally distributed .

Comparison of numerical variables between more than two groups was done using the one-way analysis of variance (ANOVA) test with post hoc multiple two-group comparisons in normal data, and the Kruskal Wallis test with post hoc multiple two-group comparisons in non-normal data. Association between qualitative data was done using the Chi-square test .

Correlation between various variables was done using the Pearson moment correlation equation for linear relation in normally distributed variables and Spearman's rank correlation equation for non-normal variables. P-Values less than 0.05 were considered statistically significant. All statistical calculations were done using computer programs SPSS (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA) version 15 for Microsoft Windows.

RESULTS

I- Demographic data:

This study included 100 subjects, which were divided into two groups.

Group (I): included 70 dialysis patients, who have been on regular dialysis treatment (RDT) for at least 6 months and who were free of overt infections but suffer from other chronic disease as hypertension , diabetes and cardiac diseases, which were considered as risk factors of atherosclerosis. This group was then subdivided according to the genotype distribution of Leu554Phe E selectin gene polymorphism on basis of absence or presence of risk allele (T) into **(TT/CT)** versus **(CC).**

Group (II): included 30 healthy age and sex matched as patients who were considered as control group for genetic data analysis, and who were free from acute or serious chronic diseases **Table (1)**.

II- Genotype distribution:

The genotype distribution of the Leu554Phe polymorphism of the E-selectin gene did not deviate from Hardy–Weinberg equilibrium either in patients (CC genotype, 14.3%; CT genotype, 78.6%; TT genotype, 7.1%) or in control healthy subjects (CC genotype, 13.3%; CT genotype, 83.3%; TT genotype, 3.3%) **Tables (2)**.

IV- Carotid artery ultrasonography for measuring the indicators of atherosclerosis:

1- Carotid intima media thickness (IMT):

In Patients, IMT ranged 0.60 – 1.6 mm with a mean of 1.08 ± 0.67 mm while in control, it ranged between 0.61 – 0.89 mm with a mean of 0.76 ± 0.10mm, there were statistically significant difference between two groups as regard IMT, where P<0.001 (P significant as P<0.05).**Table (3)**

2- Cross section area:

In Patients, cross section area ranged 1.227-40.7 mm^2 with a mean of 21±19.7 mm^2 while in control, it ranged between 13.85-46.56 mm^2 with a mean of 30.205 ± 16.355 mm^2, There were statistically significant difference between two groups as regard cross section area, where P<0.001 (P significant as P<0.05). **Table (3)**

3- Degree of stenosis:

In Patients, degree of stenosis ranged 0.0 – 20.0%with a mean of 2.29 ± 4.87 %while in control ,it ranged 0.0 – 10.0% between with a mean of 0.50 ± 2.01% ,There were

statistically significant difference between two groups as regard degree of stenosis, where P=0.040 (P significant as P<0.05). **Table (3)**

V- E-selectin Leu554Phe polymorphism and carotid atherosclerosis

When patients were categorized on the basis of the presence/absence of the risk allele (i.e. TT and CT vs CC patients), no significant difference emerged between the two groups as for demographic and somatometric data or for clinical and biochemical parameters. **Table (4)**

Relation of presence of the risk allele (TT/CT) and indicators of carotid atherosclerosis:

1) Intima media thickness:

Patient's intima Thickness in the Patients with CT or TT genotypes ranged between 0.60 – 6.1 mm with a mean of 0.888 ± 0.712 mm while in Patients with CC genotypes it ranged between 0.60 – 1.8 mm with a mean of 0.850 ± 0.375 mm. There were no statistically significant difference between patients with presence of risk allele (TT/CT) and patients with the absence of risk allele (CC) as regard IMT where P=0.926 (P significant as P<0.05).**Table (5).**

2-Cross section area:

Patient's Cross section in the Patients with CT or TT genotypes ranged between 1.227-40.7 mm^2 with a mean of 21±19.7 mm^2 while in Patients with CC genotypes it ranged between 5.123-40.1 mm^2 with a mean of 20.996±19.836 mm^2. There were no statistically significant difference between patients with presence of risk allele (TT/CT) and patients with the absence of risk allele (CC) as regard cross section area, where P=0.567 (P significant as P<0.05).**(Table (5)**

3- Degree of stenosis:

Patient's degree of stenosis in the Patients with CT or TT genotypes ranged between 0 – 20 % with a mean of 2.25 ± 4.642 % while in Patients with CC genotypes it ranged between 0 – 20 % with a mean of 2.5 ± 6.346%. There were no statistically significant difference between patients with presence of risk allele (TT/CT) and patients with the absence of risk allele (CC) as regard degree of stenosis, where P=0.593 (P significant as P<0.05).**(Table (5)**

E- Selectin gene and carotid atherosclerosis multiple regression analyses:

Since the association between E-selectin genotypes and carotid atherosclerosis could be confounded by other risk factors, multiple regression analyses were performed adjusting for all variables which were associated with the three indicators of carotid atherosclerosis. In these analyses, mentioned risk factors (cofounders) were confirmed as a positive correlate of IMT, cross sectional area and the severity of carotid stenosis **(Table 6).**

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

	Cases (n = 70)		Control (n = 30)		Test of Sig.	p
	No.	%	No.	%		
Sex						
Male	40	57.1	13	73.3	χ^2=	0.205
Female	30	42.9	17	56.7	1.608	
Age (years)						
Min. – Max.	22.0 – 74.0		27.0 – 55.0		t=	0.055
Mean ± SD.	51.06 ± 13.52		47.30 ± 5.84		1.941	
Median	52.50		48.50			

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

p: p value for comparing between the two groups

Table (II): Comparison between the two studied groups according to E-selectin gene polymorphism

	Cases (n = 70)		Control (n = 30)		χ^2	^{MC}p
	No.	%	No.	%		
E-selectin						
TT	5	7.1	1	3.3		
CC	10	14.3	4	13.3	0.437	0.918
CT	55	78.6	25	83.3		
HWE	0.053		0.139			

χ^2: **Chi square test** MC: Monte Carlo test

p: p value for comparing between the two groups

HWE: p value for comparing observed and expected data according to Hardy-Weinberg between the two groups

Table (III): Comparison between the two studied groups according to the indicators of atherosclerosis

	Cases (n = 70)	Control (n = 30)	U	p
INT thickness (mm)				
Min. – Max.	0.60 – 1.6	0.61 – 0.89		
Mean ± SD	1.08 ± 0.67	0.76 ±0.10	397.50*	<0.001*
Median	1.0	0.77		
Cross section (mm^2)				
Min.– Max.	1.227– 40.7	13.85– 46.56	3.871*	
Mean ± SD.	21 ± 19.7	30.205± 16.355		<0.001*
Median	16.20	17.0		
Degree of stenosis %.				
Min. – Max	0.0 – 20.0	0.0 – 10.0	863.0*	0.040*
Mean ± SD.	2.29 ± 4.87	0.50 ± 2.01		
Median	0.0	0.0		

U: **Mann Whitney test**t: **Student t-test**

p: p value for comparing between the two groups *: Statistically significant at p ≤ 0.05

Table (IV): Main demographic, somatometric, clinical and biochemical characteristics of the study population divided on the basis of E-selectin Leu554Phe polymorphism

	Patients with CT or TT genotypes (n=60)	Patients with CC genotypes (n=10)	P-Value
Age	51.38±13.581	49.10±13.658	0.817
Sex: *n (%)*			
Male	35(58.3%)	5(50%)	0.735
Female	25(41.7%)	5(50%)	
BMI	25.258±2.316	25.300±2.611	0.518
Smoker *n (%)*	10(16.7%)	4(40%)	0.104
DM *n (%)*	21(35%)	5(50%)	0.483
Systolic	128±18.208	116±21.705	0.988
Diastolic	81.33±9.994	72±12.293	0.878
Duration of RDT	6.277±4.032	6.200±3.190	0.654
HR	65.10±6.262	64.50±4.972	0.510
Hemoglobin	10.682±1.392	10.730±0.874	0.118
WBCs	6.543±3.286	6.350±3.177	0.785
Cholesterol	168.27±45.217	147.30±28.706	0.104
Triglyceride	177.40±109.46	140.00±50.323	0.465
LDL	106.27±24.378	112.20±17.492	0.276
HDL	60.42±9.230	66.30±4.191	0.095
Ca	8.658±0.983	9.190±0.690	0.436
PO_4	5.243±2.072	5.74±2.560	0.361
Ca X PO_4	45.660±19.731	52.625±23.405	0.322
PTH	553.692±405.294	685.840±316.997	0.228
Albumin	3.833±0.275	3.620±0.561	0.617
Creatinine	10.026±2.644	9.630±2.008	0.475
Urea	129.95±26.602	128.5±28.602	0.934
FBG	90.242±16.273	88.00±13.333	0.265
CRP	8.086±8.814	6.455±6.852	0.569
IMT	0.888±0.712	0.850±0.375	0.926
Cross-sectional area	21±19.7	20±19.836	0.567
Degree of carotid stenosis	2.25±4.642	2.50±6.346	0.593

Data are mean ±SD or percent frequency, as appropriate

Table (V): Distribution of studied sample according to different parameters

	Patients with CT or TT genotypes (n=60)	**Patients with CC genotypes (n=10)**
Int. Thickness(mm)		
Min.	0.60	0.60
Max.	6.1	1.8
Mean	0.888	0.850
S.D	0.712	0.375
P Value	**0.926**	
Cross section (mm^2)		
Min.	1.227	5.123
Max.	40.7	40.1
Mean	21	20.996
S.D	19.7	19.836
P Value	**0.567**	
Degree of stenosis%		
Min.	0	0
Max.	20	20
Mean	2.25	2.50
S.D	4.642	6.346
P Value	**0.593**	

Table (VI): Multiple regression analyses

	Beta regression coefficients	**Partial *R***	**P-Value**
IMT (Multiple R= 0.744, P <0.001*)			
BP	0.007	0.339	0.005*
$Ca \times PO_4$	0.015	0.672	<0.001*
Cross-sectional area (Multiple R= 0.934, P <0.001*)			
Age	0.010	0.545	<0.001*
BP	0.022	0.759	<0.001*
BMI	0.020	0.251	0.043*
FBS	0.254	0.454	<0.001*
Carotid stenosis (Multiple R= 0.909, P <0.001*)			
Smoking	0.633	0.259	0.033*
BP	0.029	0.906	<0.001
LDL	0.022	0.759	<0.001
Serum urea	0.20	0.251	0.043

DISCUSSION

There are 17 mutations that have been detected in adhesive molecules, five of which resulted in an amino acid substitution. In E-selectin, exchange at Ser ^{128} Arg in epidermal growth factor (EGF) domain and Leu ^{554} Phe in membrane domain, and a DNA mutation from guanine to thymine (position 98) are examples of mutations due to amino acids substitutions [45].

In this study, Leu^{554}Phe E-selectin polymorphism has been studied, in which the mutation in exon 10 leads to the substitution of leucine (Leu) by phenylalanine (Phe) at amino acid 554, (Leu = allele-C; Phe=allele-T). This mutation is believed to delay the shedding of E-selectin from vascular endothelium [45].

In the present study, the distribution of *SELE* rs5355 C and T genotypes did not show significant difference in both ESRD patients on dialysis and control groups. The genotype distribution of our studied Egyptian population group was identical to those reported by MS Issac et al. [46] however, they were different from those reported previously in Iranian [47] and Italian populations [48].

Three well established indicators of carotid atherosclerosis were studied , namely intima media thickness (IMT), cross-sectional area [49], and arterial stenosis [50], These indicators reflect different aspects of the atherosclerotic process. Our statistics showed lack of association between *SELE* rs5355 C>T genotypes and presence of carotid atherosclerosis in ESRD patients. This finding is in concordance with results reported previously by Isaac MS et al. [46] This result is contrary to results found by Testa et al, [47] who reported that IMT, CSA, and the degree of carotid stenosis were significantly higher in patients with CT and TT genotypes (i,e., in patients with T allele) when compared to CC patients.

Robert Bernat et al 2012, studied the relationship of Leu554Phe polymorphism for atherosclerosis and Long-Term Outcome after Percutaneous Coronary intervention (PCI) with Stenting, The study included 190 patients with standardized clinical follow-up over 5 years, which were initially treated with PCI, The long term clinical outcome was defined by the major adverse cardiac events MACE: death, target vessel revascularization (PCI or coronary bypass grafting, CABG) and myocardial infarction. T allele carriers of Leu554Phe polymorphism were shown to have a significantly lower repeat revascularization rate in comparison with the CC genotype. There was no significant difference between the incidence of in-stent restenosis and Leu554Phe polymorphism [51].

Srivastava et al, 2018 analysed the Leu554Phe polymorphism and expression of E-selectin gene in 250 patients with essential hypertension and 250 normal healthy controls. A significant association of E-selectin genotypes (CT + TT) with essential hypertension ($P < .0001$, Odds ratio = 2.2 [1.58-3.24] at 95% CI) was observed [52].

Marteau et al 2004, studied the association between E selectin polymorphism (Leu554Phe), blood pressure and obesity.478 men and 546 women were selected from volunteers for a free health check-up. These individuals underwent two examinations (t_0 and t_{+5}) and they were not taking medications that can affect blood pressure. At t_0, no relationship was observed between Leu554Phe polymorphism and blood pressure. However at t_{+5}, systolic blood pressure (SBP) was greater in individuals carrying the T allele, and the Leu554Phe polymorphism was associated with SBP in interaction with BMI ($P < 0.001$ in men and $P < 0.05$ in women). There was a steeper increase in SBP with BMI greater than 25 Kg/m^2 in carriers of the T allele than in CC homozygotes. Similar results were observed for diastolic blood pressure in men ($P = 0.0103$).These results suggest a BMI- specific effect of Leu554Phe polymorphism of the E-selectin gene on blood pressure [53].

CONCLUSION

The main conclusion that emerged from the present results was the following:

1. E-selectin Leu^{554}Phe polymorphism can be of value as a genetic marker to assess risk of atherosclerosis in ESRD.

2. No significant correlation could be found between E-selectin Leu^{554}Phe polymorphism and atherosclerosis markers in the studied group of ESRD patients on HD.

3. E-selectin Leu^{554}Phe polymorphism may require an underlying risk factor that predisposes to endothelial dysfunction to exert its effect and be expressed.

4. The genotype distribution of the Leu554Phe polymorphism of the E-selectin gene was identical among ESRD patients on HD and control subjects of the same population.

REFERENCES

1. **Carrero JJ, Witasp A, Stenvinkel P, Qureshi AR, Heimbürger O, Bárány P, Suliman ME, Anderstam B, Lindholm B, Nordfors L, Schalling M, Axelsson J.** Visfatin is increased in chronic kidney disease patients with poor appetite and correlates negatively with fasting serum amino acids and triglyceride levels. Nephrol Dial Transplant 2010;25(3):901-906.
2. **Hegele RA.** The genetic basis of atherosclerosis. Int J Clin Lab Res 1997;27:2-13.
3. **Parekh RS, Carroll CE, Wolfe RA, Port FK.** Cardiovascular mortality in children and young adults with end-stage kidney disease. J Pediatr 2002;141:191-197.
4. **Wright RS, Reeder GS, Herzog CA, Albright RC, Williams BA, Dvorak DL, Miller WL, Murphy JG, Kopecky SL, Jaffe AS.** Acute myocardial infarction and renal dysfunction: a high-risk combination. Ann Intern Med 2002;137:563-570.
5. **Falk E, Fuster V.** Atherogenesis and its determinants. In: Fuster V, Alexander RW, O'Rourke RA, Roberts R (eds) Hurst's The Heart, 10th edn. McGraw-Hill, New York, 2011; pp 1065-1093.
6. **Schachinger V, Zeiher AM.** Atherogenesis-recent insights into basic mechanisms and their clinical impact. Nephrol Dial Transplant 2002;17:2055-2064.
7. **Ross R.** The pathogenesis of atherosclerosis: A perspective for the 1990. Nature 1993;362:801-809.
8. **Iiyama K, Hajra L, Iiyama M, Li H, DiChiara M, Medoff BD, Cybulsky MI.** Patterns of vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 expression in rabbit and mouse atherosclerotic lesions and at sites predisposed to lesion formation. Circ Res 1999;85:199-207.
9. **Luster AD.** Chemokines-chemotactic cytokines that mediate inflammation. N Engl J Med 1998;338(7):436-445.
10. **Fan J, Watanabe T.** Inflamatory reactions in the pathogenesis of atherosclerosis. J Atheroscler Thromb 2003;10:63-71
11. **Badimon JJ, Lettino M, Toschi V, Fuster V, Berrozpe M, Chesebro JH, Badimon L.** Local inhibition of tissue factor reduces the thrombogenicity of disrupted human atherosclerotic plaques. Effects of tissue factor pathway inhibitor on plaque thrombogenicity under flow conditions. Circulation 1999;99:1780-1787.
12. **Santoro A, Mancini E.** Cardiac effects of chronic inflammation in dialysis patients. Nephrol Dial Transplant 2002;17(8):10-15.
13. **Pecoits-Filho R, Stenvinkel P, Wang AY, Heimburger O, Lindholm B.** Chronic inflammation in peritoneal dialysis: The search for the holy grail? Perit Dial Int 2004;24(4):327-339.
14. **Madore F.** Cardiac risk factors in renal disease. Med Sci 2004;20(12):1100-1103.

15. **Rattazzi M, Puato M, Faggin E, Bertipaglia B, Grego F, Pauletto P.** New markers of accelerated atherosclerosis in end-stage renal disease. J Nephrol 2003;16(1):11-20.

16. **Stenvinkel P.** Anaemia and inflammation: What are the implications for the nephrologist? Nephrol Dial Transplant 2003;18(8):17-22.

17. **Locatelli F, Covic A, Chazot C, Leunissen K, Luno J, Yaqoob M.** Hypertension and cardiovascular risk assessment in dialysis patients. Nephrol Dial Transplant 2004;19:1058-1068.

18. **Locatelli F, Marcelli D, Conte F, D'Amico M, Del Vecchio L, Limido A, Malberti F, Spotti D.** Cardiovascular disease in chronic renal failure: The challenge continues. Nephrol Dial Transplant 2000;15(5):69-80.

19. **Stampfer MJ, Hennekens CH, Manson JE, Coldilz GA, Rosner B, Willett WC.** Vitamin E consumption and the risk of coronary disease in women. N Engl J Med 1993;328:1444-1449.

20. **Krieter DH, Canaud B.** High permeability of dialysis membranes: what is the limit of albumin loss? Nephrol Dial Transplant 2003;18:651-654.

21. **Locatelli F, Pozzoni P, Tentori F, Delvecchio L.** Epidemiology of cardiovascular risk in patients with chronic kidney disease. Nephrol Dial Transplant 2003;18(7):2-9.

22. **Kalantar-Zadeh K, Stenvinkel P, Pillon L, Kopple J.** Inflammation and nutrition in renal insufficiency. Adv Ren Replace Ther 2003;10(3):155-169.

23. **Tsuchiya K, Kirima K, Yoshizumi M, Tamaki T.** New methods to evaluate endothelial function. Evaluation of endothelial function: Evaluation of endothelial function by hemoglobin-nitric oxide complex using Electron Paramagnetic Resonance Spectroscopy. J Pharmacol Sci 2003;93:417-422.

24. **Bolton CH, Downs LG, Victory JG, Dwight JF, Tomson CR, Mackness MI, Pinkney JH.** Endothelial dysfunction in chronic renal failure: roles of lipoprotein oxidation and pro-inflammatory cytokines. Nephrol Dial Transplant 2001;16:1189-1197.

25. **Nasuno A, Matsubara T, Hori T, Higuchi K, Imai S, Nakagawa I, Tsuchida K, Ozaki K, Mezaki T, Tanaka T, Fuse I, Aizawa Y.** Levels of soluble E-selectin and ICAM-1 in the coronary circulation of patients with stable coronary artery disease. Association with the severity of coronary atherosclerosis. JPn Heart J 2002;43(2):93-101.

26. **Imhof BA, Dunon D.** Leukocyte migration and adhesion. Adv Immunol 1995;58:345-416.

27. **Carlos TM, Harlan JM.** Leukocyte-endothelial adhesion molecule. Blood 1994;84(7):2068-2101.

28. **Murray RK.** Glycoproteins. In: Murray RK, Granner DK, Mayes PA, Rodwell VW (eds) Harper's Biochemistry, 24th edn. John Wiley & Sons, Hoboken, New Jersey, 1996; pp 648-666.

29. **Springer TA** . Adhesion receptors of the immune system. Nature 1990;346:425-435.

30. **Schwizer D, Patton JT, Cutting B, Smieško M, Wagner B, Kato A, Weckerle C, Binder FP, Rabbani S, Schwardt O, Magnani JL, Ernst B.** Pre-organization of the core structure of E-selectin antagonists. Chemistry 2012;18:1342-1351.

31. **Hackman A, Abe Y, Insull W Jr, Pownall H, Smith L, Dunn K, Gotto AM Jr, Ballantyne CM.** Levels of soluble cell adhesion molecules in patients with dyslipidemia.Circulation 1996;93:1334-1338.

32. **DeGraba TJ.** Immunogenetic susceptibility of atherosclerotic stroke: implications on current and future treatment of vascular inflammation. Stroke 35:2712-2719.

33. **Zoccali C, Mallamaci F, Tripepi G.** Inflammation and atherosclerosis in end-stage renal disease. Blood Purif 2003;21:29-36.

34. **Poredos P.** Endothelial dysfunction and cardiovascular disease. Pathophysiol Haemost Thromb 2002;32:274-277.

35. **Burtis CA, Ashwood ER, Bruns DE.** Tietz Textbook of Clinical Chemistry and Molecular Diagnostics, 4th edn. Elsevier Saunders Company, St Louis, 2006; 797-802.

36. **Bain BJ, Lewis SM, Bates I.** Basic haematological techniques. In: Lewis SM, Bain BJ, Bates I (eds) Dacie and Lewis Practical Haematology, 10th ed. Churchill Livingstone, Philadelphia, 2006;25-54.

37. **Rifai N, Warnick GR.** Laboratory Measurement of Lipids, Lipoproteins, and Apolipoproteins. Amer Assn for Clinical Chemistry, Washington DC.1994.

38. **Weast RC, Astle MJ, Beyer WH.** CRC handbook of chemistry and physics : a ready-reference book of chemical and physical data, 65th ed. Chemical Rubber Company Publishing, Florida, 1984;110.

39. **Zoccali C, Benedetto FA, Mallamaci F.** Inflammation is associated with carotid atherosclerosis in dialysis patients. Creed Investigators. Cardiovascular risk extended evaluation in dialysis patients. J Hypertens 2000;18:1207-1213.

40. **Miller SA, Dykes DD, Polesky HF.** A simple salting outprocedure for extracting DNA from human nucleated cells. Nucleic Acids Res 1988;16:1215.

41. **Balbarini A, Buttitta F, Limbruno U, Petronio AS, Baglini R, Strata G, Mariotti R, Ciccone M, Mariani M.** Usefulness of carotid intima-media thickness measurement and peripheral B-mode ultrasound scan in the clinical screening of patients with coronary artery disease. Angiology 2000;51(4):269-279.

42. **Von Reutern GM.** Measuring the degree of internal carotid artery stenosis. Perspect Med 2012;1(12):104-107.

43. **Eigenbrodt ML, Bursac Z, Tracy RE, Mehta JL, Rose KM, Couper DJ.** B-mode ultrasound common carotid artery intima-media thickness and external diameter: Cross-sectional and longitudinal associations with carotid atherosclerosis in a large population sample. Cardiovasc Ultrasound 2008;6:10.

44. **Kotz S, Balakrishnan N, Read CB, Vidakovic B.** Encyclopedia of statistical sciences, 2nd edn. Wiley-Interscience, Hoboken. 2006.

45. **Balakrishnan VS, Rao M, Jaber BL.** Genomic medicine, gene polymorphisms, and human biological diversity. Semin Dial 2005;18:37-40.

46. **Issac MS, Afif A, Gohar NA, Fayek NA, Zayed B, Sedrak H, Salah El Din LA.** Association of E-selectin gene polymorphism and serum PAPP-A with carotid atherosclerosis in end stage renal disease. Mol Diagn Ther 2014;18:243-252.

47. **Testa A, Benedetto FA, Spoto B, Pisano A, Tripepi G, Mallamaci F, Malatino LS, Zoccali C.** The E selectin gene polymorphism and carotid atherosclerosis in end stage renal disease. Nephrol Dial Transpl 2006;21:1921-1926.

48. **Hajilooi M, Alizadeh HM. Ranjbar M, Fallahian F, Alavian S.** E-selectin gene polymorphism in Iranian chronic hepatitis B patients. Hepat Mon 2007;7(4):211-216.

49. **Zoccali C.** Cardiorenal risk as a new frontier of nephrology: research needs and areas for intervention. Nephrol Dial Transplant 2002;17(Suppl 11):50-54.

50. **Von Reutern GM.** Measuring the degree of internal carotid artery stenosis. Perspect Med 2012;1(12):104-107.

51. **Bernat R, Szavits-Nossan J, Trbović A, Kapov-Svilicić K, Sesto I, Sipić T.** Relationship of genetic markers for atherosclerosis and long-term outcome after percutaneous coronary intervention with stenting. Coll Antropol 2012;36(4):1385-1390.

52. **Srivastava K, Chandra S, Narang R, Bhatia J, Saluja D.** E-selectin gene in essential hypertension. Eur J Clin Invest 2018;48:e12868.

53. **Marteau JB, Sass C, Pfister M, Lambert D, Noyer-Weidner M, Visvikis S.** The Leu554Phe polymorphism in the E-selectin gene is associated with blood pressure in overweight people. J Hypertens 2004;22:305-311.

Original Article

Prevalence of Subclinical Hypothyroidism Among Hemodialysis Patients in Egypt: Two Centers Study

Nagwa F. Abdelsalam[1], Eman Ahmed Hamdy [2]

[1] Department of Internal Medicine (Nephrology Unit), Helwan University, Egypt., [2] Department of Clinical and Chemical Pathology, Helwan University, Egypt.

Abstract:

Background:

Thyroid dysfunction is commonly observed in chronic kidney disease patients. Both subclinical hypothyroidism (SCH) and end stage kidney disease are independent risk factors for cardiovascular mortality. **Aim of the Work:** Our aim has been to study the prevalence of subclinical hypothyroidism in hemodialysis patients and associated risk factors. **Patients & Methods:** The study was conducted at hemodialysis units of two tertiary care centers in Egypt. The study group included 92 end stage renal disease patients on regular hemodialysis 3 times/ week. Demographic data, current smoking, underlying kidney disease, duration of dialysis were obtained from each patient. Also, history taking, clinical examination and laboratory investigations (hemoglobin, serum level of urea before and urea after dialysis, urea reduction ratio, creatinine, calcium, phosphorus, albumin, C. reactive protein, thyroid stimulating hormone TSH and free thyroxine T4) were done for each patient. **Results:** Prevalence of SCH among our 92 patients was 13.1%. Cases of renal failure due to lupus nephritis were significantly higher in SCH group compared to normal TSH group (p=0.028). Patients with SCH had significantly higher duration of dialysis compared to the normal TSH group (p=0.019) and higher level of CRP compared to the normal TSH group (P<0.001). Multivariate analysis (both adjusted and unadjusted) showed that CRP was the most independent variable affecting SCH followed by duration of dialysis. **Conclusion:** The prevalence rate of SCH among HD patients in Egypt is 13.1% and we suggest the presence of an association between inflammatory markers and SCH. We suggest also that SCH is associated with longer HD duration.

Key words: hemodialysis patients, subclinical hypothyroidism, prevalence.

Introduction

Changes in thyroid hormones are commonly observed in chronic kidney disease (CKD) patients and thyroid dysfunction increases as CKD progresses.[1] End stage renal disease (ESRD) affects thyroid function and now formally listed as an established cause of chronic non-thyroidal illness.[2]

Alterations in thyroid hormones level and / or metabolism have been described in ESRD patients.[3] Also, acute and chronic renal disease has its effects on the hypothalamus-pituitary-thyroid axis. Thyroid stimulating hormone (TSH) levels may be normal or increased in CKD but with reduced response to thyrotropin releasing hormone (TRH).[4]

Subclinical hypothyroidism (SCH) defined as high serum TSH that is above the defined upper limit of the reference range and normal free T4 levels that is within the reference range, is frequently seen in CKD patients.[5] SCH is known to be a risk factor for atherosclerotic cardiovascular disease, hyperlipidemia and low grade inflammation.[6]

Both ESRD and SCH are independent risk factors for cardiovascular mortality. Previous studies have shown an increased prevalence of SCH in CKD patients but there is a little data with respect to prevalence of SCH in ESRD.[7] Factors associated with SCH in ESRD patients have not yet been clearly identified.[8] So, our aim in this study is to identify the prevalence of SCH in ESRD patients in two tertiary care centers in Egypt and associated risk factors.

Patients & Methods

Our study is a cross sectional study done in 2 tertiary care centers in Egypt over 2 years period. The first is the hemodialysis unit of Helwan University Hospital and the second is the hemodialysis unit of one of the Ministry of Health Hospitals in Alexandria. The total number of patients from both centers was 103 ESRD patients on regular hemodialysis (HD) 3 times / week. After exclusion of patients that met the exclusion criteria, those who consented were 92 adult ESRD patients on regular HD 3 times /week and those who formed our study cohort. Exclusion criteria included those who are previously diagnosed as overt hypothyroidism, SCH, overt hyperthyroidism, those who take medications affecting thyroid functions and pregnant females.

Demographic data, current smoking, underlying kidney disease, duration of dialysis in months were obtained from all the patients. Also, history taking, clinical examination, laboratory investigations (Hb, serum level of urea before dialysis, urea after dialysis, urea reduction ratio, creatinine, calcium, phosphorus, albumin and C reactive protein, TSH and FT4) were obtained from all study patients. TSH and FT4 were measured by chemiluminescence immunoassay using the Moldular Analytics E170 immunoassay analyzer (Roche Diagnostic, Basel, Switzerland). Diagnosis of SCH was defined as high TSH level but normal free T4 level. The reference range for free T4 was 0.93- 1.7 ng/dl and for TSH 0.27-4.2 µIU/ml. The intra-assay coefficient of variation for these hormones ranged from 1.4% to 2.0% for free T4 and 3.0% to 1.1% for TSH.

Informed consent was obtained from the entire study group and the study was approved by Faculty of Medicine, Helwan University Research Ethics Committee.

Statistical Analysis: Data were fed to the computer and analyzed using IBM SPSS software package version 20.0. **(**Armonk, NY: IBM Corp**).** The Kolmogorov- Smirnov test was used to verify the normality of distribution of variables. Comparisons between groups for categorical variables were assessed using Chi-square test (Fisher or Monte Carlo). Student t-test was used to compare two groups for normally distributed quantitative variables. Mann Whitney test was used to compare between two groups for not normally distributed quantitative variables, while Wilcoxon signed ranks test was assessed for comparison between different periods. Univariate and Multivariate logistic regression was assessed. Significance of the obtained results was judged at the 5% level.

Results: Of the 92 ESRD patients recruited, the mean age was 51.4± 12.8 years, 38 (41.3%) were males, 54 (58.7%) were

females, 19 (20.7%) were smokers and the mean dry weight was 72.2 ± 15.7 kilogram. The baseline characteristics and different laboratory parameters are listed in table II and III. Based on the TSH level, the studied group was divided into two groups; subclinical hypothyroidism group and normal TSH, normal FT4 group. 80 patients (86.9%) of the total studied group showed normal TSH and normal free T4. 12 patients (13.1%) showed high TSH > 4.2 µIU/ml and normal free T4. The prevalence of SCH was 13.1%. (Table I)

Table (I): Distribution of the studied cases according to TSH level

	Total (n = 92)	**Normal (≤4.2) n = 80 (86.9%)**	**Subclinical hypothyroidism (>4.2) n = 12 (13.1%)**
TSH			
Mean ± SD.	2.8±1.7	2.2±0.9	6.4±1.4
Median (Min. – Max.)	2.4(0.7–8.9)	2.3(0.7–4.1)	6.4(4.6–8.9)

(n : number)

Table (II): Baseline demographic and clinical characteristics of the patients

	Total (n=92)	TSH		Test of sig.	p
		Normal (n=80)	Subclinical hypothyroidism (n=12)		
Sex					
Male	38 (41.3%)	34 (42.5%)	4 (33.3%)	χ^2=	FEp=
Female	54 (58.7%)	46 (57.5%)	8 (66.7%)	0.362	0.755
Age (years)					
Median (Min.–Max.)	52.5 (21 – 77)	53.0 (21 – 77)	47.5 (22 – 68)	t=0.946	0.346
Mean ±SD.	51.4 ± 12.8	51.9 ± 12.7	48.2 ± 13.5		
Weight (kg)					
Median (Min.–Max.)	70.5(45 – 118)	70.0 (45 – 112.5)	74.5 (47 – 118)	t=0.891	0.390
Mean ±SD.	72.2 ± 15.7	71.4 ± 14.3	77.6 ± 23.3		
Current smoking					
Negative	73 (79.3%)	63 (78.8%)	10 (83.3%)	χ^2=	FEp=
Positive	19 (20.7%)	17 (21.3%)	2 (16.7%)	0.134	1.000
Underlying kidney disease					
Unknown	18(19.6%)	17 (21.3%)	1(8.3%)	χ^2=1.106	FEp=0.448
HTN	34 (37%)	27 (33.8%)	7 (58.3%)	χ^2=2.707	FEp=0.118
D.N	27 (29.3%)	25 (31.3%)	2 (16.7%)	χ^2=1.070	FEp=0.498
G.N	2 (2.2%)	2 (2.5%)	0 (0%)	χ^2=0.307	FEp=1.000
CH pyelonephritis	2 (2.2%)	2 (2.5%)	0 (0%)	χ^2=0.307	FEp=1.000
Lupus nephritis	6 (6.5%)	3 (3.8%)	3 (25%)	χ^2=7.729*	FEp=0.028*
Obstructive uroparthy	8 (8.7%)	8 (10%)	0 (0%)	χ^2=1.314	FEp=0.590
PCKD	2 (2.2%)	2 (2.5%)	0 (0%)	χ^2=0.307	FEp=1.000
Renal amyloidosis	2 (2.2%)	2 (2.5%)	0 (0%)	χ^2=0.307	FEp=1.000
Sepsis	1 (1.1%)	1 (1.3%)	0 (0%)	χ^2=0.152	FEp=1.000
Vasculitis	1 (1.1%)	1 (1.3%)	0 (0%)	χ^2=0.1s52	FEp=1.000
Duration of dialysis(month)					
Median (Min.–Max.)	13.0(1.0–156.0)	12.0(1.0–125.0)	48.5(1–156)	U= 278.5*	0.019*
Mean ±SD.	26.3 ± 31.2	21.9 ± 25.3	55.8 ± 48.6		

χ^2: Chi square test, FE**:** Fisher Exact, t: Student t. test, U**:** Mann Whitney test

p: p value for comparing between the two groups

*: Statistically significant at p ≤ 0.05

Normal: TSH **≤ 4.2** **, Subclinical hypothyroidism**: TSH > 4.2

Table (III): Blood pressure and different laboratory parameters for the total group and two studied groups

	TSH				
	Total (n=92)	**Normal (n=80)**	**Subclinical hypothyroidism (n=12)**	**Test of sig.**	**p**
Blood pressure					
Systolic					
Median (Min.–Max.)	120 (90 – 160)	120 (90 – 160)	130 (100 – 160)	t=0.894	0.374
Mean ±SD.	125.1 ± 20.3	124.4 ± 20.5	130 ± 19.1		
Diastolic					
Median (Min.–Max.)	80 (50 – 100)	80 (50 – 100)	80 (60 – 90)	t=0.394	0.694
Mean ±SD.	78.7 ± 11.2	78.9 ± 11.5	77.5 ± 9.7		
Hemoglobin					
Median (Min.–Max.)	9.1 (5.9 – 14.1)	9.2 (5.9 – 14.1)	9.1 (7.3 – 10.8)	t=0.698	0.487
Mean ±SD.	9.2 ± 1.8	9.3 ± 1.9	8.9 ± 1.2		
Urea					
Before					
Median (Min.–Max.)	154.5 (47–294)	160 (50 – 294)	131.5 (47 – 240)	U=384.0	0.266
Mean ±SD.	157.8 ± 49.4	160.6 ± 45.7	139.3 ± 68.7		
After					
Median (Min.–Max.)	39 (11 – 124)	39 (11 – 124)	43.5 (15 – 96)	U=457.50	0.794
Mean ±SD.	48.3 ± 24.8	47.7 ± 23.8	51.9 ± 31.9		
p_1	**<0.001***	**<0.001***	**0.002***		
URR%					
Median (Min.–Max.)	68.9 (6.6–89.9)	69.6 (6.6 – 89.9)	68.4 (49.7 – 78.8)	t=0.616	0.540
Mean ±SD.	68.9 ± 13	69.2 ± 13.6	66.7 ± 7.8		
Cr (mg/dl)					
Median (Min.–Max.)	9.9 (2.3 – 19.8)	10.1 (2.3 – 19.8)	9.2 (2.5 – 16.6)	t=1.524	0.131
Mean ±SD.	10.2 ± 3.3	10.4 ± 3.2	8.9 ± 3.9		
Ca (mg/dl)					
Median (Min.–Max.)	9.2 (6.2 – 12)	9.1 (6.2 – 12)	9.2 (7.3 – 12)	t=0.657	0.513
Mean ±SD.	9.1 ± 1.2	9.1 ± 1.2	9.3 ± 1.4		
Ph (mg/dl)					
Median (Min.–Max.)	5.7 (2.7 – 10.7)	5.8 (2.7 – 10.7)	4.8 (4.4 – 7)	t=1.707	0.091
Mean ±SD.	5.7 ± 1.4	5.8 ± 1.4	5.1 ± 0.8		
Albumin (g/dl)					
Median (Min.–Max.)	3.9 (2.5 – 5.2)	3.9 (2.5 – 5.2)	4.1 (3 – 5.2)	t=0.390	0.697
Mean ±SD.	3.9 ± 0.6	3.9 ± 0.5	4 ± 0.7		
Free T4					
Median (Min.–Max.)	1.2 (1 – 1.7)	1.2 (1 – 1.7)	1.3 (1 – 1.5)	t=0.130	0.897
Mean ±SD.	1.3 ± 0.1	1.3 ± 0.1	1.3 ± 0.2		
CRP					
Median (Min.–Max.)	5.0(3.0 – 15.0)	5.0(3.0 – 12.0)	9.0(8.0 – 15.0)	U= 41.50*	<0.001*
Mean ±SD.	6.1 ± 2.4	5.5 ± 1.7	10.3 ± 2.8		

t: Student t-test U: Mann Whitney test

p: p value for comparing between the two groups

p_1: p for Wilcoxon signed ranks test for comparing between before and after

*: Statistically significant at $p \leq 0.05$

Normal: TSH **≤ 4.2** **, Subclinical hypothyroidism**: TSH > 4.2

Patients with SCH had significantly higher duration of dialysis and higher level of CRP compared to patients with normal TSH. (p value = 0.019, <0.001 respectively). Also, the lupus nephritis as an underlying kidney disease was significantly higher in the SCH group compared to the normal TSH group (p= 0.028).

By studying the independent variables affecting SCH by assessing multivariate analysis (table IV), it was found that higher duration of dialysis and CRP were significantly associated with SCH in both unadjusted and adjusted multivariate analysis.

Table (IV): Univariate and multivariate analysis for the parameters affecting TSH (n = 92) for total group

TSH	Univariate		#Multivariate		#Multivariate (adjusted age and sex)	
	p	OR (95%C.I)	p	OR (95%C.I)	p	OR (95%C.I)
Lupus nephritis	0.016*	8.56(1.50 – 48.89)	0.563	2.04(0.18 – 22.98)	0.467	2.71(0.18 – 40.00)
Duration of dialysis (month)	0.003*	1.03(1.01 – 1.04)	0.029*	1.03(1.00 – 1.06)	0.020*	1.03(1.00 – 1.06)
CRP	<0.001*	2.57(1.56 – 4.25)	0.001*	2.56(1.48 – 4.41)	0.003*	2.90(1.45 – 5.79)

OR: Odd`s ratio, C.I: Confidence interval,
#: All variables with p < 0.05 were included in the multivariate
*: Statistically significant at p ≤ 0.05

Discussion:

The importance of understanding the thyroid functions in CKD & ESRD patients is highlighted by recent studies indicating that subclinical hypothyroidism is common in CKD patients.(9) Our results had shown a prevalence rate for SCH in ESRD equal to 13.1% which is concordant with Lim (10) who identified a prevalence rate 9.5 % for SCH in ESRD. Chonchol et al (11) also studied the prevalence of SCH in CKD patients but not requiring dialysis and they showed 18% prevalence rate.

Eun et al (5) found that unresolved SCH was significantly associated with a more rapid decline in renal function and unresolved SCH was an independent factor contributing to the rate of estimated glomerular filtration rate (e GFR) decline and that these individuals had a higher risk of progression to ESRD. Similarly, Khatiwada et al found abnormal thyroid functions in CKD patients and SCH to be associated with CKD progression.(12)

It is known that there is alteration in thyroid hormone physiology in CKD. Baseline TSH may be elevated, there is blunted response to exogenous TRH, disturbed diurnal rhythm of TRH and sometimes there is an observed reduction in serum T4 level.(13)

Inflammation is known to be more prevalent in SCH than in euthyroidism. (14, 15) In our study, we identified that CRP was significantly high in SCH group and this is supported by what is mentioned by Zoccali et al who found that biomarkers of inflammation as CRP, IL6 are consistently associated with low T3 levels in ESRD patients on HD. (16) Also, experimental and clinical studies indicate that inflammatory cytokines play a central role in low T3 syndrome.(17)

What was also noticed that duration of dialysis was longer in SCH patients in comparison to normal TSH and T4 group. In 2011, Yee et al (18) found that in peritoneal dialysis (PD) duration was a significant risk factor for SCH and it was associated with longer PD duration and this may be due to retention of excess iodide and uremic toxins. (19,20)

There have been controversies about thyroxine supplementation for SCH in ESRD. Decreased thyroid function can be considered as an adaptation to decrease protein

catabolism in ESRD. Hence, thyroxine replacement in ESRD patients may lead to a negative nitrogen balance. [21]

Conclusions: From previous data, it was found that the prevalence rate of SCH among HD patients in Egypt is 13.1% and we suggest that there is an association between inflammatory markers and SCH. We suggest also that SCH is associated with longer HD duration.

Recommendations: Large trials and follow up may be needed to solve the controversies regarding thyroxin replacement in case of SCH in ESRD. Also, the relation of lupus nephritis as a cause of ESRD and SCH in HD patients needs further research.

References:

1. **Asvold BO, Bjøro T, Vatten LJ**. Association of thyroid function with estimated glomerular filtration rate in a population-based study: the HUNT study. Eur J Endocrinol 2011;164: 101-5.
2. **Wartofsky L, Burman KD**. Alterations in thyroid function in patients with systemic illness: the ‘‘euthyroid sick syndrome’’. Endocr Rev 1982; 3: 164
3. **Lo JC, Chertow GM**, Go AS, Hsu CY. Increased prevalence of subclinical and clinical hypothyroidism in persons with chronic kidney disease. Kidney Int 2005; 67: 1047–1052
4. **Iglesias P**, Díez JJ. Thyroid dysfunction and kidney disease. Eur J Endocrinol 2009; 160:503–15
5. **Eun Oh Kim**, Ihn Suk Lee, Yoo A Choi, Sang Ju Lee, Yoon Kyung Chang, Hye Eun Yoon et al. Unresolved subclinical hypothyroidism is independently associated with progression of chronic kidney disease. International Journal of Medical Sciences 2014; 11(1): 52-9
6. **Baskin HJ, Cobin RH, Duick DS, Gharib H, Guttler RB, Kaplan MM et al.** American Association of Clinical Endocrinologists medical guidelines for clinical practice for the evaluation and treatment of hyperthyroidism and hypothyroidism. **Endocr Pract 2002**; 8: 457–69
7. Serter R, Demirbas B, Korukluoglu B, Culha C, Cakal E, Aral Y: The effect of L - thyroxine replacement therapy on lipid based cardiovascular risk in subclinical hypothyroidism. J Endocrinol Invest 2004; 27: 897–903
8. **Ghanshyam Palamaner Subash Shantha, Anita Ashok Kumar, Viraj Bhise, Rohit Khanna, Kamesh Sivagnanam, Kuyilan Karai Subramanian.** Prevalence of subclinical hypothyroidism in patients with end stage renal disease and the role of serum albumin: a cross sectional study from south India. Cardiorenal 2011;1: 255-60
9. **Chonchol M, Lippi G, Salvagno G, Zoppini G, Muggeo M, Targher G.** Prevalence of subclinical hypothyroidism in patients with chronic kidney disease. Clin J Am Soc Nephrol 2008; 3: 1296-300.
10. **Lim VS: Thyroid function in patients with chronic renal failure.** Am J Kidney Dis 2001; 38(suppl 1):S80–S84.
11. **Chonchol M, Lippi G, Salvagno G, Zoppini G, Muggeo M, Targher G:** Prevalence of subclinical hypothyroidism in patients with chronic kidney disease. Clin J Am Soc Nephrol 2008; 5: 1296–300.
12. **Khatiwada S, Rajendra KC, Gautam S, Lamsal M, Baral N.** Thyroid dysfunction and dyslipidemia in chronic kidney disease patients. BMC Endocr Disord 2015;15:65
13. **Kaptein EM**: Thyroid hormone metabolism and thyroid diseases in chronic renal failure. Endocr Rev 1996; 17: 45–63.
14. **Chen HS, Wu TE, Jap TS, Lu RA, Wang ML, Chen RL, et al.** Subclinical hypothyroidism is a risk factor for nephropathy and cardiovascular diseases in type 2 diabetic patients. Diabet Med 2007; 24: 1336-44.
15. **Cikim AS, Oflaz H, Ozbey N, Cikim K, Umman S, Meric M et al.** Evaluation of endothelial function in subclinical hypothyroidism and subclinical hyperthyroidism. Thyroid 2004;14: 605-9.
16. **Zoccali C, Tripepi G, Cutrupi S, Pizzini P, Mallamaci F**. Low triiodothyronine: A new facet of inflammation in end stage renal disease. J Am Soc Nephrol 2005;16:2789–95
17. **Docter R, Krenning EP, Jong M, Hennemann G**. The sick euthyroid syndrome: changes in thyroid hormone serum parameters and hormone metabolism. Clin Endocrinol 1993;39: 499–518
18. **Yee Yung Ng, Shiao Chi Wu, Hong Da Lin, Fen Hsiang Hu, Chun Cheng Hou, Yea Yun Chou,et al.** Prevalence of clinical and subclinical thyroid disease in a

peritoneal dialysis population. Peritoneal dialysis international 2012;Vol.32, 86-93

19. **Lim VS.** Thyroid function in patients with chronic renal failure. Am J Kidney Dis 2001; 38(Suppl 1):S80–4.

20. **Sanai T, Inoue T, Okamura K, Sato K, Yamamoto K, Abe T et al.** Reversible primary hypothyroidism in Japanese patients undergoing maintenance hemodialysis. Clin Nephrol 2008; 69:107–13

21. **Lim VS, Flanigan MJ, Zavala DC, Freeman RM.** Protective adaptation of low serum triiodothyronine in patients with chronic renal failure. Kidney Int 1985; 28: 541-9

Original Article

Study of Prevalence and Effects of Multiple Drug Intake in Elderly Type 2 Diabetes Patients with Associated Comorbidities

Mohammed Badr, Abdelrahman Kamel , Mai Badrah, Reem Fathalla

Department of Internal Medicine, Faculty of Medicine, Alexandria University, Egypt.

Abstract

Background: Elderly patients with diabetes are a vulnerable group especially for associated co-morbidities. The physicians should be aware of the pitfalls of caring for these patients and prioritize an individualized treatment plan to ensure an optimal glycemic control, without placing the patient at unnecessary risk. **Objective:** Assess the effects of multiple drug intakes in elderly type 2 diabetes patients with associated comorbidities. **Patients & Methods**: This study included 100 patients with type 2 diabetes and other associated comorbidities recruited from the outpatient clinic of Internal Medicine Department, Alexandria Main University Hospital. The studied patients were divided in to two groups: Group I**;** 50 patients aged 55-64 years and group II: 50 patients ≥65 years. Each group was further re-divided to (A) subdivision: patients who were receiving <5drugs and (B) subdivision: pients who were receiving ≥5 drugs. The effects of polypharmacy in elderly type 2 diabetes patients with comorbidities especially on drug adherence and the observed adverse drug events were assessed. **Results:** Our results demonstrated that non adherence to drugs was significantly higher in patients who received ≥5 drugs in group I compared to those who received <5 drugs. In comparison between the polymedicated groups (IB and IIB), non adherence was significantly higher in IIB group. Among the studied adverse drug events, arrhythmia, repeated hypoglycemia, orthostatic hypotension, GIT upsets and bleeding events were significantly higher in group (IIB) than group (IB). **Conclusion:** Prescription in elderly diabetics must balance between the need for drugs and hazards of polypharmacy. Medication non-adherence and adverse drug events are major consequences of polypharmacy.

Corresponding Author: Mai Hesham Badrah, E-mail: drmaibadrah@gmail.com

Key Words: Polypharmacy, Type 2 DM, Elderly population.

INTRODUCTION

Recent advances in pharmacological therapy, although offer hope for patients, its inappropriate use carry some risks. [(1)] Prescription for the elderly is particularly challenging owing to the high prevalence of comorbidities in these population. [(2)] In general, polypharmacy means the use of multiple drugs without clear cutoff point. [(3)] A more practical definition for polypharmacy entails the use of multiple drugs that are not obviously needed. [(4)]

Diabetes is a highly prevalent systemic disease with lots of complications including microvascular complications (retinopathy, nephropathy, neuropathy and dermopathy) and macrovascular ones (cerebrovascular diseases, peripheral vascular diseases and coronary heart diseases).[(5)] Many comorbid conditions are frequently encountered in elderly patients with diabetes like hypertension and

dyslipidemia in addition to the high incidence of geriatric syndromes like fall, depression and cognitive dysfunction. [6] That's why management of diabetes with its complications and associated comorbidities make these patients prone to hazards of polypharmacy.

PATIENTS & METHODS

This study included 100 patients with type 2 diabetes associated with other comorbidities recruited from the Outpatient Clinic of the Department of Internal Medicine of the Main University Hospital of Alexandria during the period of 2017-2018. The patients were classified into 2 groups each of 50 patients; group I included patients aged between 55 and 64 years and group II included patients aged ≥65 years. Each group was further subdivided in relation to total number of drug intake in to (A) subdivision including patients receiving <5 drugs and (B) subdivision including patients receiving ≥5 drugs (polymedicated).

The study protocol was approved by the Ethics Committee of Alexandria University. The study was conducted according to the criteria set by the Declaration of Helsinki and each subject signed an informed consent before participating in the study.

All cases were subjected to complete history taking with emphasis on drug history. Data were obtained about the presence of new symptoms due to drug intake with differentiation whether they were adverse drug events or drug-drug interaction. An Adverse Drug Event (ADE) is defined as 'any physical or mental harm resulting from medication use be it misuse, under-dosing or overdosing' **(11)**. A drug interaction is defined either as increase or decrease of a medical diagnostic or therapeutic effect of a specific drug caused by another substance, which may be another drug, plant or a dietary supplement **(12)**. Full clinical examination was done to all included patients. In addition, glycated hemoglobin (HbA1C %) was done to assess glycemic control.

Statistical analysis

Data were analyzed using Statistical Package for the Social Sciences (SPSS 21.0, IBM/SPSS Inc., Chicago, IL) software. Baseline characteristics of the study population were presented as frequencies and percentages (%) or mean values and standard deviations (SD) and median and range (after testing of normality by Kolmogorov-Smirnov and Shapiro-Wilk's tests).

For comparison of data, Chi-Square test (or Fisher's exact test) was used to compare two independent groups of qualitative data. For quantitative data, independent-Samples t-test and Mann-Whitney U test were used to compare two groups of parametric and non-parametric quantitative data respectively. For all tests, P values <0.05 are considered significant.

RESULTS:

Table I shows the baseline characteristics of the studied population. There was no significant difference in terms of male/female ratio. Body mass index (BMI) was significantly higher in group I. The mean diabetes duration was 7.04 ± 4.97 and 12.14 ±6.53 in group I and II respectively.

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

	Total (n=100)		Age (years)				Test of Sig.	P
			Group I (n = 50)		Group II (n = 50)			
	No.	%	No.	%	No.	%		
Sex								
Male	52	52.0	28	56.0	24	48.0	χ^2= 0.641	0.423
Female	48	48.0	22	44.0	26	52.0		
BMI (kg/m^2)								
Min. – Max.	23.0 –36.0		26.0 –34.0		23.0 –36.0		t= 2.315*	0.023*
Mean ± SD.	29.80 ±2.21		30.30 ±1.92		29.30 ±2.38			
Median (IQR)	30.0 (28.0 –31.0)		30.0 (29.0 – 32.0)		29.0 (32.0 – 31.0)			
Diabetes duration								
Min. – Max.	1.0 –31.0		1.0 –25.0		31.0		U= 642.0*	<0.001*
Mean ± SD.	9.59 ±6.32		7.04 ±4.97		12.14 ±6.53			
Median (IQR)	9.0 (4.0 –14.0)		7.0 (3.0 – 10.0)		12.50 (7.0 – 15.0)			

χ^2: **Chi square test** **t: Student t-test**
p: p value for comparing between the studied groups
*: Statistically significant at p ≤ 0.05

The detected comorbidities in the included patients were: hypertension (HTN), dyslipidemia, arthropathies, gout, ischemic heart disease (IHD), stroke, cognitive problems, urinary incontinence and hepatitis C virus (HCV). Among them, arthropathies, IHD and stroke were significantly higher in group II (p=0.003, p=<0.001, p=0.006) respectively.

As shown in table II 80% of the studied population were receiveing ≥5 drugs. The number of patients who were receiving ≥5 drugs (polymedicated patients) were significantly higher in group II.

Table II: Comparison between the two studied groups according to total number of drugs

Total number of drugs	Total (n=100)		Age (years)				Test of sig.	P
			Group I (n = 50)		Group II (n = 50)			
	No.	%	No.	%	No.	%		
<5 (A)	20	20.0	16	32.0	4	8.0	χ^2=9.0*	0.003*
≥5 (polymedicated (B))	80	80.0	34	68.0	46	92.0		
Min. – Max.	3.0–14.0		4.0–14.0		3.0–13.0		U= 749.0*	<0.001*
Mean ± SD.	6.98±2.58		6.18±2.46		7.78±2.47			
Median (IQR)	7.0(5.0–9.0)		5.0(4.0–8.0)		7.0(6.0–10.0)			

U: Mann Whitney test
p: p value for comparing between the studied groups
*: Statistically significant at p ≤ 0.05

The relation between the total number of drugs and drug adherence is demonstrated in Table III. Non adherence to drugs was significantly higher in patients who received ≥5 drugs in group I compared to those who received <5 drugs. In comparison between the polymedicated groups (IB and IIB), non-adherence was significantly higher in IIB group.

Table III: Relation between total number of drugs and adherence

	Total number of drugs							
	Group I (n = 50)				**Group II (n = 50)**			
	<5 (IA) (n = 16)		**≥5 (1B) (n = 34)**		**<5(IIA) (n = 4)**		**≥5 (IIB) (n = 46)**	
	No.	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**
Adherence								
Yes	16	100.0	15	44.1	2	50.0	8	17.4
Non adherence	0	0.0	19	55.9	2	50.0	38	82.6
$\chi^2(p_1)$	14.421*(<0.001*)				2.446 (^{FE}p=0.174)			
$\chi^2(p_2)$	6.817* (0.009*)							

χ^2: **Chi square test**, FE**: Fisher Exact**
p_1: p value for comparing between <5 and ≥ 5 in each group
p_2: p value for comparing between group I and II for ≥5 group
*: Statistically significant at $p \leq 0.05$

Regarding the adverse drug events there was significant difference in the incidence of arrhythmia, repeated hypoglycemia, orthostatic hypotension, gastrointestinal (GIT) upsets and bleeding tendency between the patients in the two studied groups being higher in the older patients (group II) (p= 0.007, 0.012, 0.007, 0.001, 0.008) respectively. Other adverse events did not reveal significant difference between the two groups. Table (IV) shows the relation between the total number of drugs and the detected drug adverse events in the studied groups. Arrhythmia, repeated hypoglycemia, orthostatic hypotension, GIT upsets and bleeding events were significantly higher in elderly polymedicated group (IIB) than polymedicated patients less than 65 year old (IB) group.

Table IV: Relation between total number of drugs and adverse drug events

Adverse drug effect	Total number of drugs							
	Group I (n = 50)				Group II (n = 50)			
	<5 (IA) (n = 16)		≥5 (IB) (n = 34)		<5 (IIA) (n = 4)		≥5 (IIB) (n = 46)	
	No.	%	No.	%	No.	%	No.	%
Arrhythmia	0	0.0	2	5.9	0	0.0	11	23.9
$\chi^2(p_1)$	0.980 (^{FE}p=1.000)				1.226 (^{FE}p=0.563)			
$\chi^2(p_2)$	4.670*(0.031*)							
Repeated hypoglycemia	0	0.0	0	0.0	0	0.0	7	15.2
$\chi^2(p_1)$	-				0.708 (^{FE}p=1.000)			
$\chi^2(p_2)$	5.670*(^{FE}p=0.019*)							
Orthostatic Hypotension	0	0.0	2	5.9	0	0.0	11	23.9
$\chi^2(p_1)$	0.980 (1.000)				1.226 (^{FE}p=0.563)			
$\chi^2(p_2)$	4.670*(0.031*)							
GIT upsets	6	37.5	13	38.2	2	50.0	34	73.9
$\chi^2(p_1)$	0.002 (0.960)				1.044 (0.310)			
$\chi^2(p_2)$	10.269*(0.001*)							
Bleeding tendency	0	0.0	1	2.9	0	0.0	9	19.6
$\chi^2(p_1)$	0.480 (^{FE}p=1.000)				0.954 (1.000)			
$\chi^2(p_2)$	4.940*(0.038*)							
Hyperuricemia	0	0.0	4	11.8	0	0.0	4	8.7
$\chi^2(p_1)$	2.046 (^{FE}p=0.292)				0.378 (^{FE}p=1.000)			
$\chi^2(p_2)$	0.205(^{FE}p=0.717)							
Dry cough	0	0.0	0	0.0	0	0.0	4	8.7
$\chi^2(p_1)$	-				0.378 (^{FE}p=1.000)			
$\chi^2(p_2)$	3.112 (^{FE}p=0.133)							
Lower limb edema	0	0.0	0	0.0	0	0.0	5	10.9
$\chi^2(p_1)$	-				0.483(^{FE}p=1.000)			
$\chi^2(p_2)$	3.942(^{FE}p=0.069)							
Dry mouth	0	0.0	0	0.0	0	0.0	1	2.2
$\chi^2(p_1)$	-				0.089 (^{FE}p=1.000)			
$\chi^2(p_2)$	0.784 (^{FE}p=1.000)							
Headache	0	0.0	6	17.6	0	0.0	4	8.7
$\chi^2(p_1)$	3.209 (^{FE}p=0.159)				0.378(^{FE}p=1.000)			
$\chi^2(p_2)$	1.432(^{FE}p=0.310)							

χ^2: **Chi square test**, FE**: Fisher Exact**
p_1: p value for comparing between <5 and ≥ 5 in each group
p_2: p value for comparing between group I and II for ≥5 group
*: Statistically significant at $p \leq 0.05$

Regarding glycemic control, the mean glycated hemoglobin (HbA1c) were significantly higher in IB (8.26 ± 0.83) than IA subdivision (7.46 ± 0.55) of group I (P=0.001), while there was no significant difference between IIA and IIB subdivisions. On comparing the polymedicated subdivisions (IB, IIB) in both groups, no significant difference was detected regarding the mean HbA1c (p=0.290).

DISCUSSION:

Diabetes Mellitus (DM) is an important health issue for the elderly population. [7] More than half of patients with diabetes mellitus have two or more associated diseases.[8,9] Multimorbidity, commonly characterized as the co-existence of two or more chronic health conditions, is common within the elderly.[10] Polypharmacy is defined as "the administration of many drugs at the same time or the administration of an excessive number of drugs".[11] But unfortunately, there's no standard cut point with respect to the number of drugs that's

agreed upon for the definition. The foremost commonly utilized term was polypharmacy which was characterized as five or more drugs. [12] Comprehensive medication review and hazard evaluation ought to be carried out by interdisciplinary team to distinguish the polypharmacy and its unfavorable impacts.[13]

Medication nonadherence may be a major cause of morbidity, particularly in elderly patients. Roughly 10% of hospitalizations may be a result of medicine nonadherence.[14] Our results demonstrated that non adherence to drugs was significantly higher in patients who received ≥5 drugs in group I compared to those who received <5 drugs. In comparison between the polymedicated groups (IB and IIB), non-adherence was significantly higher in IIB group.

Pasina L. et al. [15] reported that low medicine adherence may be a real, complex issue for geriatric patients receiving polypharmacy. They found that the increasing number of drugs endorsed at hospital discharge is related to non-adherence and a high rate of patients did not understand the reason of their medicines. Simplification of drug regimens and decrease of pill burdens as well as way better clarifications of the reason for the medicines ought to be targets for intervention. Delamater [16] reported that among the factors associated with medication adherence, adherence with a simple prescription is higher than that for a more complex prescription.

On the contrary, Grant RW et al. [17] which demonstrated that in their study sample, patients reported very high medicine adherence rates regardless of number of medications endorsed. Among patients on numerous drugs, most patients with suboptimal adherence were perfectly adherent to all but one medication. Unreported side effects and a lack of confidence in immediate or future benefits were significant indicators of suboptimal adherence. Doctors ought to not feel hindered from endorsing multiple agents in arrange to achieve satisfactory control of hyperglycemia, hypertension, and hyperlipidemia.

Moreover, Cárdenas-Valladolid et al [18] study revealed that poor therapeutic adherence in homebound elderly patients receiving polypharmacy may be a serious issue influencing one of each three individuals concerned, and is directly related to caregiver burden, regardless of age, gender, cognitive status or number of drugs administered.

The variation in results were due to problems with tools for the evaluation of adherence as different studies used different tools such as questionnaires, tests, pill count or judgment by research nurse. [18-21] The same variability was in defining polypharmacy, where the researchers used different definitions. [20,22]

Adverse drug reactions happen nearly day by day in health care institutions and can unfavorably influence a patient's quality of life, often causing significant morbidity and mortality. [23]. Regarding the adverse drug events, our results showed that there was significant difference in the incidence of the following ADEs; arrhythmia, repeated hypoglycemia, orthostatic hypotension, GIT upsets and bleeding tendency between the patients in the two studied groups being higher in the older patients (group II) (p= 0.007, 0.012, p=0.007, 0.001, 0.008) respectively. Moreover, these ADEs were significantly higher in elderly polymedicated group (IIB) than (IB) group.

These results go hand in hand with the findings of various studies carried out by Rajesh et al. and Pirmohamed et al who reported that the percentage of ADRs found was higher with age and geriatric population. [24,25] Akhideno P et al. [26] showed that the elderly age group, presence of polypharmacy and the presence of multiple comorbidities found to be associated with and predisposed to ADRs in this study. Moreover, Shareef J et al. [27] revealed that the geriatric patients with diabetes had more

associated comorbidities which forces them to get multiple medications leading to more ADRs. In addition, Subeesh VK et al. [28] revealed that the prevalence of polypharmacy is very high among geriatric population in the study site. The study supported the consequences of polypharmacy and was closely related with multiple comorbidity and advanced age.

On the contrary, Min Zhang et al. [29] in their study of variables that anticipate repeat admission for ADRs in geriatric population uncovered that comorbidity, but not advancing age, predicts repeat admission for ADRs.

Khan LM et al. [30] in his retrospective and prospective studies detailed that the highest incidence of ADR (retrospective 15% and prospective 14.5%) was watched in both groups in patients getting more than 10 medications. The frequency of ADR in relation to age in both groups was highest in patients of age >60 years; it was 52.7% in retrospective study and 54.5% in prospective study. The system most commonly included in ADR was gastrointestinal tract 47.4% in retrospective study and 57.6% in prospective study. This studies recognized gastrointestinal system as the foremost habitually affected system by ADRs in both studies.

Conflict of Interest:

We confirm that there are no known conflicts of interest associated with this publication

REFERENCES:

1. **Topinková E, Baeyens JP, Michel JP, et al.** Evidence-based strategies for the optimization of pharmacotherapy in older people. Drugs Aging 2012;29:477-94
2. **Martin D, Tony A, Rupert P.** Polypharmacy and medicines optimisation. Making it safe and sound. London, UK: Kings Fund; 2013.
3. **Maher RL, Hanlon J, Hajjar ER**. "Clinical consequences of polypharmacy in elderly." Expert opinion on drug safety Expert Opin Drug Saf 2014; 13: 57-65
4. **Tjia J, Velten SJ, Parsons C, et al**. Studies to reduce unnecessary medication use in frail older adults: a systematic review. Drugs Aging 2013; 30: 285-307.
5. **Gregg EW, Beckles GL, Williamson DF, et al**. Diabetes and physical disability among older U.S. adults. Diabetes Care 2000; 23: 1272-7.
6. **Araki A, Ito H.** Diabetes mellitus and geriatric syndromes. Geriatr Gerontol Int 2009; 9: 105-14.
7. **Centers for Disease Control and Prevention**. National diabetes statistics report 2014.
8. **Dawes M.** Co-morbidity: We need a guide line for each patient not a guide line for each disease. Fam Pract 2010; 27:1-2.
9. **Griffin SJ.** The management of diabetes. BMJ 2001;323:946-947.
10. **Salive ME.** Multimorbidity in older adults. Epidemiol Rev. 2013:1–9.
11. **WHO Centre for Health Development.** A glossary of terms for community health care and services for older persons. World Health Organization, editor. WHO/WKC/Tech.Ser./04.2. 2004. Geneva, Switzerland. Ageing and Health Technical Report.
12. **Tjia J, Velten SJ, Parsons C, et al.** Studies to reduce unnecessary medication use in frail older adults: a systematic review. Drugs Aging. 2013;30:285–307.
13. **Hajjar ER, Hanlon JT, Artz MB, et al.** Adverse drug reaction risk factors in older outpatients. Am J Geriatr Pharmacother. 2003;1(2):82-9.
14. **Iuga AO, McGuire MJ**. Adherence and health care costs. *Risk Manag Healthc Policy*. 2014; 7:35-44.

15. **Pasina L, Brucato AL, Falcone C, et al**. Medication Non-Adherence Among Elderly Patients Newly Discharged and Receiving Polypharmacy. Drugs & Aging. 2014 2014/04/01;31(4):283-9.
16. **Delamater AM**. Improving patient adherence. Clinical diabetes. 2006;24(2):71-7.
17. **Grant RW, Devita NG, Singer DE, et al.** Polypharmacy and Medication Adherence in Patients With Type 2 Diabetes. Diabetes Care. 2003;26(5):1408-12.
18. **Cárdenas-Valladolid J, Martín-Madrazo C, Salinero-Fort MA, et al.** Prevalence of Adherence to Treatment in Homebound Elderly People in Primary Health Care. Drugs & Aging. 2010 2010/08/01;27(8):641-51.
19. **Stoehr GP, Lu SY, Lavery L, et al.** Factors associated with adherence to medication regimens in older primary care patients: the Steel Valley Seniors Survey. Am J Geriatr Pharmacother. 2008;6(5):255–63.
20. **Olesen C, Harbig P, Barat I, et al.** Generic substitution does not seem to affect adherence negatively in elderly polypharmacy pactients. Pharmacoepidemiol Drug Saf. 2013;22(10):1093–8.
21. **Moral RR, Torres LA, Ortega LP, et al.** Effectiveness of motivational interviewing to improve therapeutic adherence in patients over 65 years old with chronic diseases: A cluster randomized clinical trial in primary care. Patient Educ Couns. 2015;98(8):977–83.
22. **Gray SL, Mahoney JE, Blough DK.** Medication Adherence in Elderly Patients Receiving Home Health Services following Hospital Discharge. Annals of Pharmacotherapy. 2001;35(5):539-45.
23. **Schatz S, Weber R.** Adverse drug reactions. Pharmacy Practice. 2015;1:1.
24. **Rajesh R, Ramesh M, Parthasarathi G**. A study on adverse drug reactions related hospital admission and their management. Indian J Hosp Pharm 2008;45:143-8.
25. **Pirmohamed M, James S, Meakin S, et al.** Adverse drug reactions as cause of admission to hospital. BMJ 2004;329(456):15-9
26. **Akhideno P, Fasipe O, Isah A, et al.** Pattern of medications causing adverse drug reactions and the predisposing risk factors among medical in-patients in clinical practice: A prospective study. Journal of Medical Sciences 2019;39(1):18-27.
27. **Shareef J, Fernandes J, Samaga L, et al.** A study on adverse drug reactions in hospitalized patients with diabetes mellitus in a multi-specialty teaching hospital. Asian Journal of Pharmaceutical and Clinical Research. 2016;9:114-7.
28. **Subeesh VK, Gouri N, Beulah ET, et al**. A Prospective Observational Study on Polypharmacy in Geriatrics at A Private Corporate Hospital. Journal of Applied Pharmaceutical Science. 2017;7(10):162-7.
29. **Zhang M, Holman CDAJ, Price SD, et al.** Comorbidity and repeat admission to hospital for adverse drug reactions in older adults: retrospective cohort study. BMJ. 2009;338:a2752.
30. **Khan LM, Al-Harthi SE, Saadah OI, et al**. Impact of pharmacovigilance on adverse drug reactions reporting in hospitalized internal medicine patients at Saudi Arabian teaching hospital. Saudi Med J. 2012;33(8):863-8.

Original Article

The Effect of 1, 25 Dihydroxy-Vitamin D3 on Insulin Sensitivity and Expression of JNK in Diabetes – Induced Liver Complications in Rats

Kawkab Elsabah Mohamed Ragab, Hala Salah Ibrahim, Gehan Yassin Soliman, Rasha Mohamed Adel Nassra, Aml Gomaa Ali Kobbas

Department of Medical Physiology, Faculty of Medicine, University of Alexandria

Abstract

Introduction: NAFLD one of most common complication of diabetes mellitus. *Several studies tried to understand its mechanism or to prevent it. The current study aim* is to study the possible effect of treatment of vitamin D on the expression of inflammatory cytokine C-Jun N-terminal kinase (JNK) in rat models of diabetic liver. **Methods:** *thirty male albino rats were assigned into two groups group I (healthy control) that were fed normal diet (n= 10), group II (n=20) received HFD for 8 week and then injected with STZ 30* mg/kg. *After detection of hyperglyceamia this group was randomly divided into subgroups, group IIA received vitamin D for 8 weeks in a dose of* (0.03 µg/kg/day), *and group IIB was kept without treatment.* **Results & Conclusion:** vitamin D significantly improves glucose intolerance, insulin sensitivity, decreased HOMA-IR, decreased serum ALT and decreased AST, increased SOD and decreased liver JNK expression.

Key Words: diabetic liver, NAFLD, JNK, 1,25dihydroxy vitamin D3, insulin sensitivity, SOD, Interleukine-1β **.**

Introduction

Diabetes mellitus affects people worldwide and poses major public health and socio-economic challenges.[(1)] Monitoring of blood glucose is essential in optimizing long-term outcomes towards reducing the co-morbid conditions that may arise in patients with T2DM[(2)]. High Fat Diet (HFD) has also been utilized to model chronic inflammation, which is an important pathogenic mechanism of T2DM. The HFD - mediated chronic inflammation is marked by increased TNF-α, IL-1β, and IL-6 in the circulation. [(3)]

T2DM is strongly associated with obesity and insulin resistance [(4)] as well as defects in pancreatic β-cell function and mass. Insulin is important in carbohydrate, fat and protein metabolism[(5)].

Insulin resistance acts as a key link between obesity and T2DM , It is likely that the development of obesity-induced insulin resistance involves a complex interplay of genetic and environmental factors.[(6)] The Insulin Receptor (IR) signaling pathway is triggered by its binding of the IR a receptor that belongs to tyrosine kinase receptor family. Hormone engagement stimulates the intrinsic tyrosine kinase activity of the IR resulting in the auto phosphorylation on the β subunit cytoplasmic tails. [(7)]

JNK: JNKs are serine threonine protein kinases that phosphorylate Jun family members, components of activator protein 1 (AP-1) transcription factors JNK also known as stress-activated Mitogen Protein Kinase (AMPK).[(8)]

Complication of diabetes: Chronic complications of diabetes which involve coronary artery disease, renal, liver and ophthalmologic diseases are the primary cause of disability and mortality in DM patients. It is reported that the standardized mortality rate from end-stage liver disease (i.e. cirrhosis) is higher than that for cardiovascular disease among patients with diabetes.[9]

Liver complications: NAFLD the most common chronic hepatopathy worldwide, reaching a prevalence of above 70% in patients with T2DM. [10] Inflammation is a crucial part of almost all acute and chronic liver disorders including (NAFLD).[11]

Interleukine-1β: IL-1β is a Pro-inflammatory cytokine not expressed in healthy liver. It acts via specific receptors involved in all inflammatory processes in the liver including regulation of insulin resistance and fibrosis. [12]

Oxidative stress is defined as an imbalance in the oxidant-to-antioxidant ratio, causing the generation of free radicals [13]. **Super oxide-dismutase (SOD):** SOD is a key antioxidant enzyme that dismutates superoxide anion (O_2-) to continuously form H_2O_2 and O_2 .[14]

Vitamin D: Vitamin D is a lipophilic molecule essential to maintain calcium and phosphate balance and osteometabolic system regulation. The steroid hormone Vitamin D (Vit D) can be produced by the body or obtained through the diet.[15] Evidence from various directions, including observational and experimental studies, indicates the protective capacity of 1,25-dihydroxyvitamin D3 (active vitamin D) in chronic low-intensity inflammation and IR in T2DM and its complications; although the underlying mechanism is yet to be clarified[16].

Aim of the Work

The purpose of the present study is to develop an appropriate stable animal model which is analogous to the human T2DM through a combination of high-fat diet with e low-dose STZ injection to test the hypothesis that vitamin D may improve the diabetes-induced liver complications by suppressing the expression of inflammatory cytokine and C-Jun N-terminal kinase (JNK) in the liver of this rat models of diabetes**.**

Materials & Methods

The study was conducted on thirty healthy male albino rats with body weight (100–150 g). Rats were housed in standard polypropylene cages (three rats/cage).[17] The period of experiment was 16 weeks. The Experiment was conducted in accordance with the guidelines set by the Research Ethics Committee of the Faculty of Medicine Alexandria University. After a week of acclimatization, rats were randomly divided into two groups:

Group (I): [(Control):(n=10)] rats in this group were fed a regular standard chow diet (60% carbohydrates,5% fat and 18% protein).[18]

Group(II): (n=20) rats of this group were fed a high fat diet HFD (22% fat, 48% carbohydrate, 20% protein) with a total caloric value of 44.3 kcal /kg. [17]:

After 8 weeks from the start of the diet regimen, all rats of group II received a single low dose of STZ 30 mg/kg (dissolved in 0.1 ml sodium citrate buffer at pH (4.4) intra peritoneal injection (IP) .[17]

Rats of the Control group (Group I) received a single IP injection of ml of sodium citrate buffer only. One week later, random blood glucose level of all rats was measured in blood samples collected from tail vein, using a glucometer.[19] Diabetes was confirmed by the detection of hyperglycemia (random non-fasting glucose level, >300mg/dl),[20] T2DM model rats (n=10) were randomly divided equally into 2 groups IIA and IIB each of 10 **.**

Group IIA (Vit D treated group):(n=10)

Ten diabetic rats received vitamin D (1,25-dihydroxyvitamin D_3) with a dose of (0.03 µg/kg/day) dissolved in corn oil, administrated orally using a gavage needle. The dose was 1 µg =40 IU and this treatment was continued **for 8 weeks.**[21]

Group IIB (untreated group): (n=10)

These ten diabetic rats received the vehicle oral gavage per day for (8 weeks) .

Both control and diabetic untreated rats received an equivalent administration of corn oil for 8 weeks.[21]

Body Weight of rats was measured at the beginning of the experiment and at week 8 and then after 16 weeks at the end of the experiment.

At the end of the experiment, rats were fasted overnight and measuring the weight plexus of the rat by capillary under light ether anesthesia. [22] The serum was separated by centrifugation at 3000 rpm for 15 minutes and was stored at -20^{0}C until assayed for measuring the following parameters:

1. Fasting blood glucose level by colorimetric method. [19]
2. Serum insulin level by enzyme linked immunosorbent assay (ELISA).[23]
3. Insulin resistance via the (HOMA-IR).[23]
4. Serum Liver enzymes; alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity by colorimetric method.[24]
5. Serum super oxide dismutase (SOD) activity by colorimetric method.[25]
6. Serum IL1β level by ELISA.[26]

Liver tissue preparation

The rats were sacrificed with decapitation, the anterior abdomen was incised to expose the liver. The liver from each rat was excised immediately after perfusion and rinsed with ice-cold saline solution. The ventral median lobe of the liver was fixed in 10% neutral buffered formalin for histopathological study. The remaining portion was homogenized in 0.9% saline, centrifuged at 400×g for 15 minutes and the supernatant was decanted and stored at 70^{0}C until used for 7.Reverse transcription real time quantitative-polymerase chain reaction of (RT-qPCR) was performed on liver tissue to quantify gene expression level of JNK.[27, 28]

Results

Table (I): Comparison between the three studied groups regarding body weight (g) .

Body weight	Group I (n = 10)	Group IIA (n = 10)	Group IIB (n = 10)	Test of sig.	P
At the beginning of the experiment1	**Group I (n = 10)**	**Group II (n = 20)**		t= 0.231	0.819
Min. – Max.	130.0 – 150.0	130.0 – 150.0			
Mean ± SD.	139.7 ± 5.93	139.15 ± 6.23			
Median	140.5	139.0			
At 8 weeks					
Min. – Max.	138.0 – 155.0	202.0 – 339.0	200.0 – 230.0	F= 40.869*	<0.001*
Mean ± SD.	147.5 ± 5.87	268.6[a] ± 50.66	218.2[ab] ± 10.76		
Median	147.0	270.0	220.5		
Sig. bet. grps	$p_1<0.001^*, p_2<0.001^*, p_3=0.002^*$				
At the end of the experiment					
Min. – Max.	165.0 – 238.0	177.0 – 299.0	171.0 – 218.0	F= 10.485*	<0.001*
Mean ± SD.	189.8 ± 20.95	246.5[a] ± 46.32	193.4[b] ± 17.29		
Median	186.5	244.5	191.0		
Sig. bet. grps	$p_1=0.001^*, p_2=0.964, p_3=0.002^*$				

t: Student t-test

F: F for ANOVA test, Pairwise comparison bet. each 2 groups was done using Post Hoc Test (Tukey)

p: p value for comparing between the three studied groups

p_1: p value for comparing between group I and group IIA

p_2: p value for comparing between group I and group IIB

p_3: p value for comparing between group IIA and group IIB

*: Statistically significant at $p \leq 0.05$

GroupI: Control

Group IIA: Vitamin D treated

Group IIB: Untreated

a: significant with Group I

b: significant with Group IIA

Table (II): Comparison between the three studied groups regarding fasting blood glucose levels (mg/dl).

Fasting blood glucose(mg/dl)	Group I (n = 10)	Group IIA (n = 10)	Group IIB (n = 10)	F	P
At 9 weeks					
Min. – Max.	68.0 – 95.0	311.0 – 365.0	310.0 – 370.0		
Mean ± SD.	77.80 ± 9.27	336.80[a] ± 22.49	333.3[a] ± 20.32	658.84*	<0.001*
Median	74.50	341.50	328.0		
Sig. bet. grps	$p_1<0.001^*, p_2<0.001^*, p_3=0.904$				
At the end of the study					
Min. – Max.	63.0 – 97.0	63.0 – 100.0	130.0 – 185.0		
Mean ± SD.	74.80±10.14	86.90±12.80	151.1[ab]±17.93	85.774*	<0.001*
Median	72.0	93.50	148.5		
Sig. bet. grps	$p_1=0.149, p_2<0.001^*, p_3<0.001^*$				
p_4	**0.558**	**<0.001***	**<0.001***		

F: F for ANOVA test, Pairwise comparison bet. each 2 groups was done using Post Hoc Test (Tukey)

p: p value for comparing between the three studied groups

p_1: p value for comparing between group 1 and group 2A

p_2: p value for comparing between group 1 and group 2B

p_3: p value for comparing between group 2A and group 2B

p_4: p value for comparing between the same groups at the 9 weeks and at the end of the experiment

*: Statistically significant at $p \leq 0.05$

Group 1: Control

Group 2A: Vitamin D treated

Group 2B: Untreated

a: significant groups 2A , 2B versus group1

b: significant with Group 2A

Table (III): Comparison between the three studied groups regarding Serum Insulin and HOMA-IR

Sugar picture	Group I (n = 10)	Group IIA (n = 10)	Group IIB (n = 10)	F	p
Serum Insulin (Pg/ml)					
Min. – Max.	1857.0 – 2354.0	2203.0 – 2927.0	2315.0 – 3560.0		
Mean ± SD.	2053.6 ± 181.8	2551.3[a]± 220.1	2900.0[ab]±440.8	19.658*	<0.001*
Median	1973.0	2557.0	3003.0		
Sig. bet. grps	p_1=0.003*,p_2<0.001*,p_3=0.041*				
HOMA-IR					
Min. – Max.	7.98–11.25	8.51–17.04	19.51–33.69		
Mean ± SD.	9.37±1.09	13.66[a]±2.61	26.76[ab]±4.38	90.431*	<0.001*
Median	9.03	14.09	27.64		
Sig. bet. grps	p_1=0.010*,p_2<0.001*,p_3<0.001*				

F: F for ANOVA test, Pairwise comparison bet. each 2 groups was done using Post Hoc Test (Tukey)

p: p value for comparing between the three studied groups

p_1: p value for comparing between group I and group IIA

p_2: p value for comparing between group I and group IIB

p_3: p value for comparing between group IIA and group IIB

*: Statistically significant at $p \leq 0.05$

Group I: Control

Group IIA: Vitamin D treated

Group IIB: Untreated

a: significant with Group I

b: significant with Group IIA

Table (IV): Comparison between the three studied groups regarding serum ALT (U/ml) ,serum AST(U/ml) .

Investigation laboratory	Group I (n = 10)	Group IIA (n = 10)	Group IIB (n = 10)	F	p
Serum ALT (U/ml)					
Min. – Max.	15.0 – 37.0	11.0 – 28.0	22.0 – 28.0		
Mean ± SD.	21.0 ± 8.64	17.60 ± 6.13	25.0[b]± 2.16	3.520*	0.044*
Median	19.0	16.0	24.50		
Sig. bet. grps	p_1=0.453,p_2=0.339,p_3=0.034*				
Serum AST (U/ml)					
Min. – Max.	32.0 – 70.0	32.0 – 49.0	43.0 – 98.0		
Mean ± SD.	45.20 ± 14.32	41.10 ± 7.28	61.70[b]± 20.33	5.315*	0.011*
Median	43.0	43.0	56.0		
Sig. bet. grps	p_1=0.814,p_2=0.051,,p_3=0.013**				

F: F for ANOVA test, Pairwise comparison bet. each 2 groups was done using Post Hoc Test (Tukey)

p: p value for comparing between the three studied groups

p_1: p value for comparing between group I and group IIA

p_2: p value for comparing between group I and group IIB

p_3: p value for comparing between group IIA and group IIB

*: Statistically significant at $p \leq 0.05$

Group IControl

Group IIA:Vitamin D treated

Group IIB:Untreated

a:significant with Group I

b:significant with Group IIA

Table (V): Comparison between the three studied groups regarding Serum SOD (U/ml) and IL-1β Eliza (Pg/ml)

	Group I (n = 10)	Group IIA (n = 10)	Group IIB (n = 10)	F	p
Serum SOD (U/ml)					
Min. – Max.	210.0 – 233.0	152.0 – 273.0	142.0 – 203.0		
Mean ± SD.	222.4 ± 8.40	219.5 ± 38.13	178.4[ab]± 23.51	8.744*	0.001*
Median	223.0	226.5	177.0		
Sig. bet. grps	p_1=0.967,p_2=0.002*,p_3=0.005*				
IL-1β Eliza (Pg/ml)					
Min. – Max.	513.7 – 618.4	410.5 – 844.7	571.8 – 923.4		
Mean ± SD.	563.5 ±41.27	678.9 ± 128.2	761.7[a]± 121.4	9.041*	0.001*
Median	560.0	716.9	804.2		
Sig. bet. grps	p_1=0.052,p_2=0.001*,p_3=0.199				

F: F for ANOVA test, Pairwise comparison bet. each 2 groups was done using Post Hoc Test (Tukey)
p: p value for comparing between the three studied groups
p_1: p value for comparing between group I and group IIA
p_2: p value for comparing between group I and group IIB
p_3: p value for comparing between group IIA and group IIB
*: Statistically significant at $p \leq 0.05$
GroupI: Control
Group IIA: Vitamin D treated
Group IIB: Untreated
a: significant with Group I
b: significant with Group IIA

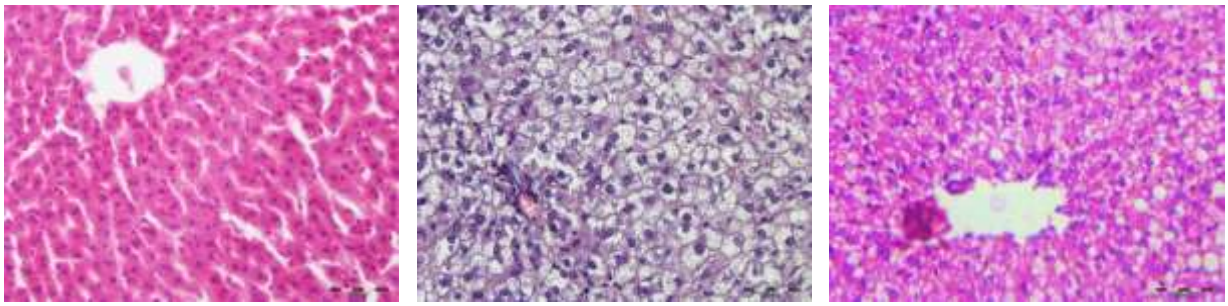
Table (VI): Comparison between the three studied groups regarding ΔΔCT (unit ?)

	Group I (n = 10)	Group IIA (n = 10)	Group IIB (n = 10)	F	P
ΔΔCT(unit ?)					
Min. – Max.	0.02 – 0.10	0.04 – 1.05	1.0 – 1.0		
Mean ± SD.	0.07 ± 0.03	0.25 ± 0.31	1.0[ab]± 0.0	76.407*	<0.001*
Median	0.09	0.12	1.0		
Sig. bet. grps	p_1=0.091,p_2<0.001*,p_3<0.001*				

F: F for ANOVA test, Pairwise comparison bet. each 2 groups was done using Post Hoc Test (Tukey)
p: p value for comparing between the three studied groups
p_1: p value for comparing between group I and group IIA
p_2: p value for comparing between group 1 and group IIB
p_3: p value for comparing between group 2A and group IIB
*: Statistically significant at $p \leq 0.05$
Group I: Control
Group IIA: Vitamin D treated
Group IIB: Untreated
a: significant with Group I
b: significant with Group IIA

Effects of vitamin D on liver histopathology

In the control group, the structure of liver is normal and clear. Various pathological changes appeared in the untreated group, including narrow liver sinusoid, distortion of liver architecture, hepatocyte swelling, fatty degeneration, cytoplasm rarefaction, spotty necrosis scattering in the hepatic lobule as well as inflammatory cell infiltration. However, these changes described above were markedly improved in the VD treated group



Control group Untreated group Vit D treated group

Figure (1): Changes in liver histology (magnification, × 400; stain, hematoxylin and eosin). In the control group, liver cell alignment is normal. In the untreated group, liver cells appear swelled with fatty degeneration and inflammatory cell infiltration. In the vit D treated group, fatty degeneration and inflammatory lesions are alleviated, in comparison with untreated group.

Discussion

It has been demonstrated that inflammation and IR play an initial role in the onset of T2DM and the progression of its complications. Vitamin D is a well-known steroid hormone that has been identified as a regulator of inflammation and IR. Based on available clinical and epidemiological data, the positive effects of vitamin D appear to be primarily related to its action on inflammation and secondary to its action on insulin sensitivity and secretion.

In our study, at the end of the experiment there was significant decrease in body weight in Vit D treated group and untreated group compared by their weight at the 8 weeks. But the BWt was significantly increased in Vit D treated group in relation to untreated group. Most probably the weight loss in untreated group occurred as a result of increased muscle wasting owing to loss of tissue proteins instead of using glucose in aerobic glycolysis. The improvement in BWt in Vit D treated group is suggested to be due to its effect on insulin sensitivity and glucose metabolism.[29, 30] Vitamin D influences β-cell insulin secretion through a rise in intracellular calcium concentration via non-selective voltage-dependent calcium channels. [31, 32]Similar results were found by Rossmeisl et al., 2003. [33]

Moreover, at the end of the experiment, there was a statistically significant decrease in fasting blood glucose level in group IIB (untreated) and group IIA (Vit D treated group) in relation to control, yet still significantly higher in group IIB than group IIA. Blood glucose return to normal in group IIA may be a result of Vit D improved insulin resistance and improved insulin signaling pathway, [34] Benetti et al., 2018 [35] demonstrated that animals treated with vitamin D showed a significant improvement of glucose tolerance.

In the present study, at the end of the experiment, serum insulin in the untreated group was significantly increased in comparison to control and Vit D treated group. This decrease in serum insulin level in Vit D treated group, supports the evidence that the beneficial effects of vitamin D were mediated through the modulation of VDR a receptor of vitamin D thereby improving signal transduction in the treatment of diabetes-induced liver complications.[36, 37] Benetti, et al[35] demonstrated similar results .

In the present work, HOMA-IR in un treated group was significantly increased in relation to control and Vit D treated group. It has been well documented that HFD causes insulin resistance and also reductions in GLUT4 protein levels which are usually associated with increased insulin resistance.[36, 38] Also significant decreased of HOMA-IR in Vit D treated group suggested that Vit D improves insulin resistance; which was also found by Zhuo et al[39]

In the present study, serum ALT was significantly increased in the untreated group compared with Vit D treated group. Serum AST showed the same pattern, due to the role of 1, 25 $(OH)_2D_3$ in regulating pro inflammatory cytokines and lipid metabolism in a diabetic liver model. [40] [41][41][41]Ning et al[42] demonstrated similar results

In present work serum SOD between significantly increased in the Vit D treated group and significantly decreased in untreated group, suggesting that vitamin D played a vital role in the protection of tissues from damage by free radicals as a non-enzymatic antioxidant compound. [43, 44] Claro da Silva Tet al work is supporting our results. [45].

In the present study, there was a significantly increased Serum interleukin 1β in untreated group compared to Vit D treated one and in the control group, supporting the role of 1,25-dihydroxy vitamin D as an anti-inflammatory agent .1,25-(OH)2D3 inhibits the release of the pro-inflammatory cytokine [46, 47] , Similar result shown by Liu et al[20] .

In this study, RT-qPCR analysis showed that the expression level of JNK which are implicated in inflammation and insulin resistance, increased in untreated and decreased following treatment with vitamin D. Based on these results, we speculate that vitamin D exerts therapeutic effects on diabetes-induced liver complications, possibly by downregulating the expression of JNK. This modulation may be in part due to the anti-inflammatory effect of vitamin D; however. the underlying mechanism remains to be elucidated[20]. Our findings are supported by Liu et al. [20]

The histopathological study of liver was performed to assess the direct effects of vitamin D on diabetes-induced liver complications. The results showed that the administration of vitamin D significantly alleviated certain pathological changes, particularly steatosis and inflammatory cell infiltration.

References

1. **Saleh F, Mumu SJ, Ara F, Begum HA, Ali L.** Knowledge and self-care practices regarding diabetes among newly diagnosed type 2 diabetics in Bangladesh: a cross-sectional study. BMC Public Health 2012;12:1112.
2. **Amod A, Berg G.** The 2012 SEMDSA guidelines for the management of type 2 diabetes. J Endocrinol Metab Diabetes S Afr 2012;17(2):S4.
3. **Gregor MF, Hotamisligil GS.** Inflammatory mechanisms in obesity. Annu Rev Immunol 2011;29:415-45.

4. **Shaw JE, Sicree RA, Zimmet PZ.** Global estimates of the prevalence of diabetes for 2010 and 2030. Diabetes Res Clin Pract 2010;87(1):4-14.
5. **Guo XX, Wang Y, Wang K, Ji BP, Zhou F**. Stability of a type 2 diabetes rat model induced by high-fat diet feeding with low-dose streptozotocin injection. J Zhejiang Univ Sci B 2018;19(7):559-69.
6. **Samuel VT, Shulman GI.** Mechanisms for insulin resistance: common threads and missing links. Cell 2012;148(5):852-71.
7. **Copps KD, White MF.** Regulation of insulin sensitivity by serine/threonine phosphorylation of insulin receptor substrate proteins IRS1 and IRS2. Diabetologia 2012;55(10):2565-82.
8. **Nakagawa H, Maeda S.** Molecular mechanisms of liver injury and hepatocarcinogenesis: focusing on the role of stress-activated MAPK. Patholog Res Int 2012;2012:172894.
9. **Manna P, Das J, Ghosh J, Sil PC.** Contribution of type 1 diabetes to rat liver dysfunction and cellular damage via activation of NOS, PARP, IkappaBalpha/NF-kappaB, MAPKs, and mitochondria-dependent pathways: Prophylactic role of arjunolic acid. Free Radic Biol Med 2010;48(11):1465-84.
10. **Chalasani N, Younossi Z, Lavine JE, Diehl AM, Brunt EM, Cusi K, et al.** The diagnosis and management of non-alcoholic fatty liver disease: practice guideline by the American Gastroenterological Association, American Association for the Study of Liver Diseases, and American College of Gastroenterology. Gastroenterology 2012;142(7):1592-609.
11. **Netea MG, Balkwill F, Chonchol M, Cominelli F, Donath MY, Giamarellos-Bourboulis EJ.** A guiding map for inflammation. 2017;18(8):826-31.
12. **Petrasek J, Bala S, Csak T, Lippai D, Kodys K, Menashy V, et al.** IL-1 receptor antagonist ameliorates inflammasome-dependent alcoholic steatohepatitis in mice. J Clin Invest 2012;122(10):3476-89.
13. **Leung TM, Nieto N**. CYP2E1 and oxidant stress in alcoholic and non-alcoholic fatty liver disease. J Hepatol 2013;58(2):395-8.
14. **Trivedi S, Lal N, Mahdi AA, Mittal M, Singh B, Pandey S.** Evaluation of antioxidant enzymes activity and malondialdehyde levels in patients with chronic periodontitis and diabetes mellitus. J Periodontol 2014;85(5):713-20.
15. **Wintermeyer E, Ihle C, Ehnert S, Stöckle U, Ochs G, de Zwart P, et al.** Crucial Role of Vitamin D in the Musculoskeletal System. Nutrients 2016;8(6).
16. **Pittas AG, Lau J, Hu FB, Dawson-Hughes B.** The role of vitamin D and calcium in type 2 diabetes. A systematic review and meta-analysis. J Clin Endocrinol Metab 2007;92(6):2017-29.
17. **Zhang M, Lv XY, Li J, Xu ZG, Chen L.** The characterization of high-fat diet and multiple low-dose streptozotocin induced type 2 diabetes rat model. Exp Diabetes Res 2008;2008:704045.
18. **Tzeng TF, Liou SS, Liu IM**. Myricetin Ameliorates Defective Post-Receptor Insulin Signaling via beta-Endorphin Signaling in the Skeletal Muscles of Fructose-Fed Rats. Evid Based Complement Alternat Med 2011;2011:150752.
19. **Akude E, Zherebitskaya E, Chowdhury SK, Smith DR, Dobrowsky RT, Fernyhough P.** Diminished superoxide generation is associated with respiratory chain dysfunction and changes in the mitochondrial proteome of sensory neurons from diabetic rats. Diabetes 2011;60(1):288-97.

20. **Liu L, Lv G, Ning C, Yang YE, Zhu J.** Therapeutic effects of 1,25-dihydroxyvitamin D3 on diabetes-induced liver complications in a rat model. Exp Ther Med 2016;11(6):2284-92.

21. **Tsuruoka S, Nishiki K, Sugimoto K, Fujimura A.** Time of day improves efficacy and reduces adverse reactions of vitamin D3 in 5/6 nephrectomized rat. Life Sci 2002;71(15):1809-20.

22. **Shin MH, Ryu JS, Min DY.** Serum IgE levels in rats infected with Paragonimus westermani. Kisaengchunghak Chapchi 1991;29(4):397-401.

23. **Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC.** Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia 1985;28(7):412-9.

24. **Serrao EM, Kettunen MI, Rodrigues TB, Dzien P, Wright AJ, Gopinathan A, et al.** MRI with hyperpolarised [1-13C]pyruvate detects advanced pancreatic preneoplasia prior to invasive disease in a mouse model. Gut 2016;65(3):465-75.

25. **Nishikimi M, Appaji N, Yagi K.** The occurrence of superoxide anion in the reaction of reduced phenazine methosulfate and molecular oxygen. Biochem Biophys Res Commun 1972;46(2):849-54.

26. **Sajid F, Bano S.** Pro inflammatory interleukins and thyroid function in Naswar (dipping tobacco) users: a case control study. BMC Endocr Disord 2016;16(1):47.

27. **Mi S, Lee X, Shao Z, Thill G, Ji B, Relton J, et al.** LINGO-1 is a component of the Nogo-66 receptor/p75 signaling complex. Nat Neurosci 2004;7(3):221-8.

28. **Cao J, Wang J, Dwyer JB, Gautier NM, Wang S, Leslie FM, et al.** Gestational nicotine exposure modifies myelin gene expression in the brains of adolescent rats with sex differences. Transl Psychiatry 2013;3:e247.

29. **Wu C, Qiu S, Zhu X, Li L.** Vitamin D supplementation and glycemic control in type 2 diabetes patients: A systematic review and meta-analysis. Metabolism 2017;73:67-76.

30. **Mirhosseini N, Vatanparast H, Mazidi M, Kimball SM.** The Effect of Improved Serum 25-Hydroxyvitamin D Status on Glycemic Control in Diabetic Patients: A Meta-Analysis. J Clin Endocrinol Metab 2017;102(9):3097-110.

31. **Alvarez JA, Ashraf A.** Role of vitamin d in insulin secretion and insulin sensitivity for glucose homeostasis. Int J Endocrinol 2010;2010:351385.

32. **Röder PV, Wu B, Liu Y, Han W.** Pancreatic regulation of glucose homeostasis. Exp Mol Med 2016;48(3):e219.

33. **Rossmeisl M, Rim JS, Koza RA, Kozak LP.** Variation in type 2 diabetes--related traits in mouse strains susceptible to diet-induced obesity. Diabetes 2003;52(8):1958-66.

34. **Chen S, Villalta SA, Agrawal DK.** FOXO1 Mediates Vitamin D Deficiency-Induced Insulin Resistance in Skeletal Muscle. J Bone Miner Res 2016;31(3):585-95.

35. **Benetti E, Mastrocola R, Chiazza F, Nigro D, D'Antona G, Bordano V, et al.** Effects of vitamin D on insulin resistance and myosteatosis in diet-induced obese mice. PLoS One 2018;13(1):e0189707.

36. **Shen X, Zhou N, Mi L, Hu Z, Wang L, Liu X, et al.** Phloretin exerts hypoglycemic effect in streptozotocin-induced diabetic rats and improves insulin resistance in vitro. Drug Des Devel Ther 2017;11:313-24.

37. **Targher G, Bertolini L, Scala L, Cigolini M, Zenari L, Falezza G, et al.** Associations between serum 25-hydroxyvitamin D3 concentrations and liver histology in patients with non-alcoholic fatty liver disease. Nutr Metab Cardiovasc Dis 2007;17(7):517-24.

38. **Miana M, Galan M, Martinez-Martinez E, Varona S, Jurado-Lopez R, Bausa-Miranda B, et al.** The lysyl oxidase inhibitor beta-aminopropionitrile reduces body weight gain and improves the metabolic profile in diet-induced obesity in rats. Dis Model Mech 2015;8(6):543-51.

39. **Zhuo J, Zeng Q, Cai D, Zeng X, Chen Y, Gan H, et al.** Evaluation of type 2 diabetic mellitus animal models via interactions between insulin and mitogenactivated protein kinase signaling pathways induced by a high fat and sugar diet and streptozotocin. Mol Med Rep 2018;17(4):5132-42.

40. **Salum E, Kampus P, Zilmer M, Eha J, Butlin M, Avolio AP, et al.** Effect of vitamin D on aortic remodeling in streptozotocin-induced diabetes. Cardiovasc Diabetol 2012;11:58.

41. **Fram K, Tamim F, Takruri H, Thikrallahb Fa, Khadra M, Basha A.** Effect of Vitamin D Supplementation for Obese Pregnant Women on Gestational Diabetes and Diabetes Biomarkers. J Food Nut Dis 2017;1:7.

42. **Ning C, Liu L, Lv G, Yang Y, Zhang Y, Yu R, et al.** Lipid metabolism and inflammation modulated by Vitamin D in liver of diabetic rats. Lipids Health Dis 2015;14:31.

43. **Bhat M, Ismail A.** Vitamin D treatment protects against and reverses oxidative stress induced muscle proteolysis. J Steroid Biochem Mol Biol 2015;152:171-9.

44. **Lima LAR, Lopes MJP, Costa RO, Lima FAV, Neves KRT, Calou IBF, et al.** Vitamin D protects dopaminergic neurons against neuroinflammation and oxidative stress in hemiparkinsonian rats. J Neuroinflammation 2018;15(1):249.

45. **Claro da Silva T**, Hiller C, Gai Z, Kullak-Ublick GA. Vitamin D3 transactivates the zinc and manganese transporter SLC30A10 via the Vitamin D receptor. J Steroid Biochem Mol Biol 2016;163:77-87.

46. **Hewison M, Gacad MA, Lemire J, Adams JS.** Vitamin D as a cytokine and hematopoetic factor. Rev Endocr Metab Disord 2001;2(2):217-27.

47. **Kolb H, Mandrup-Poulsen T.** An immune origin of type 2 diabetes? Diabetologia 2005;48(6):1038-50.

Original Article

Prevalence of Serum Cryoglobulins in Patients with Proliferative Lupus Nephritis and its Relation to Srological Markers of Disease Activity

Hesham El Ghoneimy [1], Hanaa Donia [2], Tasnim Kamel [1], Amr Ebeid [1]

(1) Departments of Internal Medicine Faculty of Medicine, Alexandria University; Alexandria Egypt.

(2) Clinical & Chemical Pathology Faculty of Medicine, Alexandria University; Alexandria Egypt.

Abstract

Background: Cryoglobulins are found in patients with systemic lupus erythematosus (SLE) with a variable percentage but their relation to disease activity in patients with proliferative lupus nephritis is not determined yet. **Objectives:** To determine the prevalence of cryoglobulinemia in patients with proliferative lupus nephritis and its relation to disease activity. **Methods:** In a comparative cross-sectional study we investigated 60 candidates, 30 patients with renal biopsy proven proliferative Lupus nephritis (LN), 15 patients with SLE without nephritis and 15 healthy subjects. Serum samples were obtained at 37°C, and cryoglobulinemia was estimated by centrifugation at 4°C after incubation for 7 days in all the studied groups. We also compared between cryo-positive patients and cryo-negative patients according to serological markers of disease activity and activity and chronicity indices in renal biopsy. **Results:** Cryoglobulins were detected in the sera of 2 patients in the LN group (6.7%) , 1 patient in the SLE without nephritis group (6.7%) and non of the healthy subjects. Rheumatoid factor (RF) titre was significantly higher in cryo-positive patients (p=0.006). Complement levels; C3 and C4 were significantly lower in cryo-positive patients (p=0.006 and 0.011 respectively). There was no statistically significant difference between cryo-postive and cryo-negative patients as regard Anti-double stranded (ds DNA) titre, activity or chronicity indices in renal biopsy. **Conclusion:** Cyoglobulins were positive in 6.7% of cases in our study with no difference between patients with or without nephritis and their presence were not related to pathological markers of disease activity in patients with proliferative LN.

Corresponding Author: Hesham Abdallah El Ghoneimy. Department of Internal Medicine, Nephrology Unit, Alexandria University Faculty of Medicine

Hesham_elghoneimy@yahoo.com

Key Words: Cryoglobulinemia, Lupus nephritis, Lupus activity.

INTRODUCTION

Among patients with lupus, LN affects both sexes equally, is more severe in children and men, and is less so in older adults. The incidence of LN is about 30% in White, 60% in Black and Hispanic, and 40% to 80% in Asian patients with SLE. Nephritis is more common in African Americans and Hispanics than in whites, and is also more in men than in women. Renal damage is more likely to develop in nonwhite groups.[1-4]

Patients with International Society of Nephrology (ISN) class I biopsies usually have no evidence of clinical renal disease. Similarly, patients with ISN class II may have elevated anti-dsDNA or low complement levels, dut usually they do not have active urinary sediment, hypertension is uncommon, the GFR is normal, and proteinuria is rarely above 1 gram/24 hours. Patients with class I and class II LN in renal biopsy have a good renal prognosis unless they transform to another class.[5]

Patients with active ISN class IIIA or IIIA/C usually have microscopic hematuria, hypertension, low complement levels, and proteinuria.[5] Patients with mild proliferation affecting a few glomeruli usually have good response to treatment, with less than 5% progressing to renal failure during 5 years of follow-up. Others with more glomerular affection or with necrotizing lesions and crescent formation have a prognosis similar to that of class IVA patients.[6]

Patients with ISN class IVA usually have high serologic activity (low serum complement and high anti-dsDNA–binding activity). Clinically, they have active urinary sediment, hypertension, heavy proteinuria, and reduced GFR. Class IV diffuse proliferative disease has the worst renal prognosis in all studies. [7]

Cryoglobulinemia is a clinical disorder characterized by the presence of cryoglobulins in the serum. Cryoglobulins are immunoglobulins that have a specific physical feature of being able to precipitate at cold temperatures and re-dissolve when rewarmed. Cryoglobulinemia may be asymptomatic without end-organ damage and is sometimes accidentally discovered. In type I cryoglobulinemia, the cryoglobulins are monoclonal immunoglobulins (Ig's), usually of the IgG or IgM isotypes and rarely IgA or free immunoglobulin light chains.[8] Type I cryoglobulinemia develops in the case of protein-secreting monoclonal gammopathies.[9,10]
In type II cryoglobulinemia, the cryoglobulins are a mix of monoclonal IgM with rheumatoid factor (RF) activity and polyclonal IgG. This type is usually associated with hepatitis C virus (HCV) infection in up to 90% of patients.[11] Other causes of type II cryoglobulinemia include other infections (mainly HIV and hepatitis B virus [HBV]), connective tissue diseases (CTDs) like SLE, and lymphoproliferative disorders. Approximately 10% of patients have unknown cause (termed essential mixed cryoglobulinemia). Type III cryoglobulins are by polyclonal IgM with RF activity and polyclonal IgG. This type is seen in CTDs or secondary to infection (mainly HCV). Generally, cryoglobulins in mixed cryoglobulinemia result from a B-cell proliferation in the setting of chronic immune activation induced by chronic infection, autoimmune disease, or an unknown cause.[12] However, its role in the pathogenesis and activity of lupus nephritis hasn't been established yet.

PATIENTS & METHODS

We conducted a comparative cross sectional study in which patients were divided into three groups: Group (I): Thirty patients with biopsy proven proliferative lupus nephritis, group (II): Fifteen patients with SLE without lupus nephritis and group (III): Fifteen age and sex matched healthy subjects. We excluded patients with positive hepatitis c virus (HCV) antibodies and antiphospholipid antibodies.

All patients in the study were subjected to thorough history taking with emphasis on symptoms suggestive of cryoglobulinemia as hyperviscosity symptoms (like headache, blurring of vision), skin rash, neuropathy, (as burning sensation and numbness), urinary symptoms (as red or frothy urine) or previously diagnosed thrombotic episodes. They were also well examined for signs of cryoglobulinemia like vasculitic rash and neuropathy.

Ultrasound guided percutaneous renal biopsy with light microscopic examination of obtained specimen and estimation of activity and chronicity index was done.

Complete blood count (CBC), Serum urea & creatinine, complete urine analysis, Urinary protein/creatinine ratio, Anti-nuclear antibody (ANA), Anti dsDNA, Complement factors C3 & C4, Erythrocyte sedimentation rate (ESR) & C-reactive protein (CRP), Rheumatoid factor (RF) and serum cryoglobulins were done.

Blood samples were obtained and kept at 37°C for 30 minutes before separation. Serum was prepared by centrifuging at 37°C for 10 minutes at 2,500 rpm. Fresh centrifugated serum was incubated at 4°C for 7 days after collection and examined for cryoprecipitation.

Statistical Analysis:

We used conventional chi-square and Fisher's exact tests to analyze qualitative differences. For comparison of quantitative parameters, Student t test was used for normally distributed quantitative variables to compare between two studied groups, and the nonparametric Mann-Whitney U test was used for abnormally distributed quantitative variables to compare between two studied groups. F-test (ANOVA) was used for normally distributed

quantitative variables to compare between more than two groups and Kruskal Wallis test for abnormally distributed quantitative variables to compare between more than two studied groups. Post Hoc (Dunn's multiple comparisons test) was used for pairwise comparisons.

A value of P 0.05 indicated statistical significance. This statistical analysis was performed by the SPSS program (SPSS Inc, Chicago, IL) with the information stored in the database program.

RESULTS:

Cryoglobulins were detected in the sera of 3 patients (6.7%), of whom 1 was a female with SLE without nephritis and 2 were females with proliferative LN.

The duration of SLE ranged between 0.0 – 9.0 years with a median of 2.0 years in group I and 0.20 – 17.0 years with a median of 2.0 years in group II with no statistically significant difference (p=0.608). At the study initiation, the mean age of patients was 31.93 ± 8.68 in group I, 32.60 ± 10.08 in group II and 30.60 ± 8.25 in group III with no statistically significant difference between the three groups (p=0.821).

Table I shows the affected body systems by SLE in the three studied groups.

Table I: Comparison between the three studied groups according to the affected systems

Systems affected	Group I (n = 30)		Group II (n = 15)		Group III (n = 15)		χ^2	P
	No.	%	No.	%	No.	%		
Kidney	30	100.0	0	0.0	0	0.0	–	–
Skin	7	23.3	6	40.0	0	0.0	1.352	^{FE}p=0.304
CNS	2	6.7	2	13.3	0	0.0	0.549	^{FE}p=0.591
Blood	6	20.0	3	20.0	0	0.0	0.00	^{FE}p=1.000
Heart	0	0.0	2	13.3	0	0.0	4.186	^{FE}p=0.106
Joints	10	33.3	8	53.3	0	0.0	1.667	0.197
Serosa	2	6.7	2	13.3	0	0.0	0.549	^{FE}p=0.591
Lung	1	3.3	0	0.0	0	0.0	–	–

χ^2: **Chi square test** FE**: Fisher Exact**
p: p value for comparing between the studied groups
Group I: LN
Group II: SLE without nephritis

As shown in table II on comparing between cryo-positive and cryo-negative patients according to serological and pathological markers of the disease activity, we found that C3 had a median of 70.5 mg/dl in cryo negative patients and 26.0 mg/dl in cryo positive patients (p=0.006). C4 had a median of 10.95 mg/dl in cryo negative patients and 4.0 mg/dl in cryo positive patients (p=0.011). Anti-Ds DNA titre had a median of 134.5 IU/mL in cryo negative patients and 130.0 IU/mL in cryo positive patients (p=0.909). RF titre had a median of 8.0 IU/mL in cryo negative patients and 16.50 IU/mL in cryo positive patients (p=0.006).

Table II: relation between cryoglobulinemia and C3, C4, RF and anti-ds DNA titre

	CRYO		U	P
	Negative (n=42)	Positive (n=3)		
C3				
Min. – Max.	12.0 – 176.0	25.0 – 27.0		
Mean ± SD.	71.05 ± 31.81	26.0 ± 1.0	3.0*	0.006*
Median	70.50	26.0		
C4				
Min. – Max.	3.0 – 47.0	2.10 – 5.0		
Mean ± SD.	14.13 ± 10.60	3.70 ± 1.47	7.0*	0.011*
Median	10.95	4.0		
Anti-Ds DNA				
Min. – Max.	15.0 – 1000.0	32.0 – 173.0		
Mean ± SD.	183.26 ± 210.41	111.67 ± 72.27	60.50	0.909
Median	134.50	130.0		
RF				
Min. – Max.	3.90 – 13.30	11.50 – 24.70		
Mean ± SD.	7.66 ± 2.32	17.57 ± 6.66	2.50*	0.006*
Median	8.0	16.50		

U: Mann Whitney test
p: p value for comparing between **Negative** and **Positive**
*: Statistically significant at $p \leq 0.05$

The activity index (AI) ranged between 1.0 – 12.0/24 with a median of 7.0/24 in cryo negative patients and 6.0 – 10.0/24 with a median of 8.0/24 in cryo positive patients with no significant statistical difference between the two groups (p=0.901).

The chronicity index (CI) ranged between 0.0 – 10.0/12 with a median of 3.0/12 in cryo negative patients and 0.0 – 0.0/12 with a median of 0.0/12 in cryo-positive patients with no significant statistical difference between the two groups (p=0.074) as shown in table III.

Table III: relation between cryoglobulinemia and activity and chronicity indices in the renal bioosy

	CRYO		U	P
	Negative (n=28)	Positive (n=2)		
AI/24 (n=27)				
Min. – Max.	1.0 – 12.0	6.0 – 10.0		
Mean ± SD.	7.57 ±2.66	8.0 ±2.83	26.50	0.901
Median	7.0	8.0		
CI/12				
Min. – Max.	0.0 – 10.0	0.0 – 0.0		
Mean ± SD.	3.54 ±2.89	0.0 ±0.0	6.0	0.074
Median	3.0	0.0		

U: Mann Whitney test
p: p value for comparing between **Negative** and **Positive**
*: Statistically significant at $p \leq 0.05$

C3 and C4 were significantly lower in cryo positive patients. RF titre was significantly higher in cryo positive patients. But there was no statistically significant difference between cryo positive and cryo negative patients as regard Anti-DsDNA titre, serum creatinine, urinary protein/ creatinine ratio or activity and chronicity indices in the renal biopsy.

DISCUSSION

In this study, we have investigated 60 subjects, 30 patients with proliferative lupus nephritis, 15 patients with SLE without renal affection and 15 healthy control subjects. Females represented 95% of all cases forming 93% of the LN group and 100% of the group without nephritis. This is consistent with the female predominance shown in previous studies.

The mean age of the three study groups was around 30 years. This is consistent with the literature data that SLE occurs more commonly between 15and 45 years of age. Group I and group II had a comparable duration of the disease with a mean of 2 years in group I and 3 years in group II.

As regards the affected systems, apart from the kidney that was affected in 100% of group I and not affected at all in group II, the most common presentation of SLE was musculoskeletal affection (40% of cases), followed by mucocutaneous affection (28 % of cases),other affected systems included : blood, lungs, CNS, the heart and serosal surfaces.

In this study, we compared between patients of SLE without nephritis, patients with proliferative LN and healthy controls as regards their routine laboratory investigations, serological investigations for SLE activity and cryogolbulinemia. As ruled in the inclusion and exclusion criteria of each group in the study, all patients in group II had normal renal function tests, normal urinay protein creatinine ratio and normal urine analysis findings.

In group I, they all had renal biopsy proven proliferative LN. The most commonly found pathology is the diffuse proliferative form with or without superadded membranous affection accounting for 63% of cases.

As regards cryoglobulinemia, cryoglobulins were found in 6.7% of SLE patients either with or without LN. There was no relation between the presence of cryoglobulins and the occurrence of LN. Also there was no relation between the presence of cryoglobulins and the activity or chronicity indices in renal biopsy.

Previous studies done to investigate the prevalence of cryoglobilinemia in SLE showed different results ranging from 16% to 83% in small series of cases. A study done in Barcelona in 2001 on a large series of 122 SLE patients found that cryoglobulins were positive in 25% of cases.

In this study neither HCV positive nor antiphospholipid positive patients were excluded. They found that a cryocrit greater than 1% was more frequent in those SLE patients with HCV infection. As our study showed they found a higher frequency of some immunologic markers in cryoglobulinemic SLE patients (RF and hypocomplementemia).

The association between hypocomplementemia and cryoglobulins is well known. Adu and Williams [13] described the ability of SLE cryoglobulins to fixate complement in vitro and suggested that these immune complexes can fixate complement in vivo and so, they cause tissue damage in this disease.

Roberts et al., [14] suggested that, in SLE patients with diffuse proliferative glomerulonephritis, cryoglobulins and glomerular immune deposits can fixate complement via the classic and alternative pathways. They found a higher prevalence of RF in cryoglobulinemic SLE patients, may be due to the RF activity of some cryoglobulinemic component. RF can be used as an immunologic marker that points to the presence of cryoglobulinemia in SLE patients.[15]

A cross-sectional study in Isfahan, Iran and was conducted from September 2010 to May 2011. They studied 80 women with SLE, they found a prevalence of 48.8% of cryoglobulins in a large number of SLE patients, all of them showed high titre of circulating cryoglobulins (cryocrit >5%). There was a correlation between Anti-dsDNA, ANA, CRP, and hypocomplementemia and cryoglobulinemia in the studied SLE patients. These finding may identify the SLE patients with cryoglobulinemia. That study helped in the recognition of immunological characteristics of SLE which are involved in systemic inflammation.

In 2016 a case-control study was done. All patients with a cryoglobulinemia between January 2005 and December 2016 in a third level referral centre in Mexico City were included. Cryoglobulinemic SLE patients (cryocrit 1%) were included in the case group, whereas non cryoglobulinemic SLE patients were considered controls.

The investigators studied the demographic, clinical and immunological characteristics at the time of the positive cryoglobulin result, as well as three months earlier, and 6 and 12 months later. Thirty-six SLE patients had a positive test for cryoglobulins throughout the study period. Ten patients had cryocrit of 1% and were included in the case group, whereas 26 patients with a negative test were included as controls. Mean age was 37.7±18.3 in cases and 41.7±19.3 in controls. Women represented 70% of cases and 88.5% of controls.

Among the case group, the cryocrit was 1% in 9 patients, and 3% in one. Regarding clinical and immunological features, a positive lupus anticoagulant and vasculitis were more common in cryoglobulinemic patients (p=0.004 and 0.04, respectively). At the time of the cryoglobulin detection, patients in the case group had lower levels of C3 and C4 (p=0.026 and p=0.003, respectively), and serum albumin (p=0.028). They also had a higher frequency of serositis (p=0.021), peripheral oedema (p=0.034) and SLICC Damage Index score (p=0.014) than controls.

On follow-up, the cryoglobulinemic patients had a higher SLEDAI score after six and twelve months (p=0.009 and 0.034, respectively). After 12 months they had a higher frequency of renal activity (p=0.004) and lower C4 levels (p=0.001). Among patients with renal activity, 20% of cases and 55% of controls had achieved complete remission after 12 months. So according to that study serum cryoglobulins in SLE patients were relates to positive lupus anticoagulant and hypocomplementemia. At follow-up, patients with cryoglobulinemia had a higher frequency of renal activity, as well as an increased disease activity overall.[16]

Comparing our study to the previous studies, the prevalence of cryoglobulinemia found in our study was lower with no difference between LN and non LN patients. Cryoglobulinemia was related to disease activity in SLE represented by hypocomplementemia. There was no relation between the presence of cryoglobulins and activity or chronicity indices in the renal biopsies of LN patients.

These differences between our results and the results of the previous studies may be attributed to exclusion of HCV positive patients who represented some percentage of positive cryoglobulins in SLE patients in the previous studies.

The same also applies to exclusion of patients with positive antiphospholipid antibodies in whom the presence of cryoglobulins are more likely. Other probable explanation includes the use of immunosuppressive therapy in most of our studied patients which may have its impact on the occurrence of seronegativity. Lastly, cryoglobulins may be falsely negative in some patients due to the strict precautions needed for sample collection and analysis.

CONCLUSION

Cryoglobulinemia is not related to pathological markers of disease activity in the renal biopsies of patients with proliferative LN.

Conflict of Interest: Authors declare no conflicts of interest.

REFERENCES:

1. **Dooley MA, Aranow C, Ginzler EM.** Review of ACR renal criteria in systemic lupus erythematosus. Lupus 2004; 13:857-60.

2. **Kasitanon N, Magder LS, Petri M.** Predictors of survival in systemic lupus erythematosus. Medicine 2006; 85:147-56.

3. **Ward MM, Pyun E, Studenski S.** Mortality risks associated with specific clinical manifestations of systemic lupus erythematosus. Arch Intern Med 1996; 156:1337-44.

4. **Alarcon GS, McGwin G Jr, Petri M, et al.** Baseline characteristics of a multiethnic lupus cohort: PROFILE. Lupus 2002; 11:95-101.

5. **Gutiérrez S, Petiti JP, De Paul AL, et al.** Lupusrelated podocytopathy: Could it be a new entity within the spectrum of lupus nephritis. Nefrologia 2012; 32:245-6.

6. **Najafi CC, Korbet SM, Lewis EJ, et al.** Significance of histologic patterns of glomerular injury upon long-term prognosis in severe lupus glomerulonephritis. Kidney Int 2001; 59:2156-63.

7. **Markowitz GS, D'Agati VD.** The ISN/RPS 2003 classification of lupus nephritis: An assessment at 3 years. Kidney Int 2007; 71:491-5.

8. **Dammacco F, Sansonno D, Piccoli C, et al.** The cryoglobulins: an overview. Eur J Clin Invest 2001; 31:628-38.

9. **Harel S, Mohr M, Jahn I, et al.** Clinico-biological characteristics and treatment of type I monoclonal cryoglobulinaemia: a study of 64 cases. Br J Haematol 2015; 168:671-8.

10. **Terrier B, Karras A, Kahn JE, et al.** The spectrum of type I cryoglobulinemia vasculitis: new insights based on 64 cases. Medicine 2013; 92:61-8.

11. **Saadoun D, Sellam J, Ghillani-Dalbin P, et al.** Increased risks of lymphoma and death among patients with non-hepatitis C virus-related mixed cryoglobulinemia. Arch Intern Med 2006; 166:2101-8.

12. **Trejo O, Ramos-Casals M, García-Carrasco M, et al.** Cryoglobulinemia: study of etiologic factors and clinical and immunologic features in 443 patients from a single center. Medicine 2001; 80:252-62.

13. **Adu D, Williams DG.** Complement activating cryoglobulins in the nephritis of systemic lupus erythematosus. Clin Exp Immunol 1984; 55:495-501.

14. **Roberts JL, Wyatt RJ, Schwartz MM, et al.** Differential characteristics of immune-bound antibodies in diffuse proliferative and membranous forms of lupus glomerulonephritis. Clin Immunol Immunopathol 1983; 29:223-41.

15. **García-Carrasco M, Ramos-Casals M, Cervera R, et al.** Cryoglobulinemia in systemic lupus erythematosus: prevalence and clinical characteristics in a series of 122 patients. Semin Arthritis Rheum 2001; 30(5):366-73.

16. **Barrera-Vargas A, Antiga-López FJ, Rangel-Patiño J, et al.** AB0546 Cryoglobulinemia in systemic lupus erythematosus: clinical and immunological features. Ann Rheum Dis 2018; 77(2):1429.