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3. Text: To be Structured: Introduction, (Patients/ Subjects/ Materials) & Methods, Results, Discussions, Conclusions
4. References.
5. Tables, illustrations, and Figures; numbered single digit numbers and titles for tables placed (above) and for figures placed (underneath) with footnotes are within the results section in the sequence mentioned. **Note:** No more than **4** tables and **4** figures or illustrations.

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¬**Discussion:** Emphasize the new and important aspects of the study, conclude, and relate the observations to other relevant studies. Link the conclusions with the goals of the study.
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Conflicts of Interest

Funding

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Two authors

(10) **Al-qazzaz A, Altaie AF.** The Role of Omentin-1 and Fibroblast Growth Factor-23 in Iraqi Patients with Prostate Cancer during Chemotherapy. J Fac Med Baghdad. 2024; 66 (3): 254-259. https://doi.org/10.32007/jfacmedbaghdad.6632256.

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Table of Contents

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Research Article

Serum CXCL 9 as a Potential Biomarker for Patients with Ulcerative Colitis

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Abstract:

Background: Ulcerative colitis (UC) is an inflammatory bowel disease restricted to the large intestine, characterized by superficial ulceration. It is a progressive and chronic disease requiring long-term treatment. Although its etiology remains unknown, it is suggested that environmental factors influence genetically susceptible individuals, leading to the onset of the disease. (C-X-C) ligand 9 is a chemokine that belongs to the CXC chemokine family, it plays a role in the differentiation of immune cells such as cytotoxic lymphocytes, natural killer T cells, and macrophages. Its interaction with its corresponding receptor CXCR3 which is expressed by a variety of cells such as effector T cells, CD8+ cytotoxic T cells, and macrophage, leads to stimulation of the production of IFN-γ and TNF-α and in turn, stimulates the production of Th1 chemokines which results in promoting the inflammation.

Objectives: To assess the significance of serum chemokine (C-X-C) ligand 9 as a potential marker for identifying ulcerative colitis in adults with inflammatory bowel disease.

Patients and Methods: This is a case-control study that included 50 patients diagnosed with UC, aged between 18 and 75 years, compared to 50 apparently healthy controls, aged between 18 and 60 years. The study was conducted between November 2022 and March 2023, at the Gastroenterology and Hepatology Teaching Hospital at the Medical City Complex in Baghdad. The serum samples were analyzed using the Enzyme-Linked Immunosorbent Assay (ELISA) technique.

Results: The mean ± SD in pg/ml of serum CXCL9 in patient group was 26.9 ± 9.05 and in control group was 6.4 ± 2.37 ($p < 0.0001$) which indicates a highly significant difference.

Conclusion: CXCL 9 may be employed as a biomarker for identifying ulcerative colitis and it can be used as a tool for measuring disease activity, in addition to the possibility of being a potential therapeutic target.

Keywords: Inflammatory bowel disease (IBD), Ulcerative colitis (UC), T-Lymphocytes, Chronic inflammation, CXCL 9.

Introduction:

Ulcerative colitis (UC) is a chronic and recurrent inflammatory bowel disease (IBD) (1), restricted to the large intestine. It starts in the rectum and spreads all over the colon. The primary clinical manifestations of this condition include diarrhea, mucopurulent stools, and the presence of blood in the stool. Additionally, there may be systemic symptoms associated with the condition (2).

The precise etiology of IBD remains elusive despite ongoing research efforts (3–6). Symptoms vary from mild to severe during a relapse; however, they may decrease or disappear during disease remission (3).

The inflammation is limited to the epithelial layer, continuous in the colonic mucosa and not interrupted by healthy areas (7,8).

Cytokines are soluble glycoproteins with low molecular weight in which they act in an endocrine, paracrine, or autocrine manner. The cytokine system is crucial in the body's immunological response to infection and inflammation. Immune cells produce different types of cytokines. These cytokines include chemokines, interleukins (ILs), adipokines, interferons, colony-stimulating factors (CSFs), and tumor necrosis factor (TNF) (9). More than 200 Cytokines have been identified (10), with their physiological functions including division, apoptosis (programmed cell death), and tissue repair. However, their overproduction leads to unregulated inflammation that harms healthy cells (11). They modulate both the adaptive and innate immune responses to infections and antigens (12). The development and progression of inflammatory bowel disease are mediated by cytokines (13). Chemokine (C-X-C motif) ligand 9 or CXCL 9 is also known as monokine induced by gamma interferon (MIG). As other chemotactic chemokines, it acts to attract immune cells that have CXCR3+ (C-X-C motif chemokine receptor 3), such as effector T cells, regulatory T cells, CD8+ cytotoxic T cells, and macrophages (14).

T helper 1 cells express CXCR3 on their surfaces and thus they stimulate the secretion of interferon-γ (IFN-γ) locally in the inflamed tissues (15). IFN-γ and Tumor Necrosis Factor-α (TNF-α) secretion is enhanced by the recruited Th1 which in turn stimulates Th1 chemokines secretion by a variety of

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cells (16). CXCL9 is increased in many inflammatory diseases due to binding with its CXCR3 G protein-coupled receptor as it is highly expressed on different T cell subsets, which is mediated by IFN-γ (17).

Material and Methods:

This is a case-control study that was conducted between November 2022 and March 2023, with the aim of investigating the potential association between UC and a serum CXCL 9. Participants were recruited from the Gastroenterology and Hepatology Teaching Hospital at the Medical City Complex in Baghdad.

A total of 100 participants were included in the study, consisting of 50 individuals with UC, along with 50 apparently healthy controls. The two study groups were age-matched, apart from one case who fell at the extreme age value of 75 years, for which no control of a similar age during the time of the study.

UC patients were evaluated under the supervision of a gastroenterologist. They were asked about the severity of their conditions using the disease activity index of UC (Truelove and Witts Severity Index) (18). No sub-groupings were made according to disease severity due to the small numbers in the severe subgroup, making meaningful comparisons difficult. Participants' consent was obtained, and participants provided information about their symptoms and complications, outlining risks and general information.

Apparently healthy controls were selected from the blood bank of the Gastroenterology and Hepatology Teaching Hospital, following a comprehensive medical history assessment.

Inclusion criteria for patients

• Males and females with UC,
• Age from 18 years to 75 years,
• Patients diagnosed with only UC who don't have other autoimmune disorders.

Exclusion criteria

• Those who refused to participate in this study,
• UC patients with other autoimmune disorders,
• Patients younger than 18 or older than 75 years.

Kits utilized in this study

The serum marker CXCL 9 was analyzed using human CXC-chemokine ligand 9 (CXCL 9) ELISA kits from Sunlong Biotech Co., LTD at the International Center for Training and Development, utilizing the Enzyme-Linked Immunosorbent Assay (ELISA) technique.

Statistical Analysis

Statistical analysis was performed using the IBM SPSS 27 (Statistical Package for the Social Sciences, version 27) for demographic parameters, the GraphPad Prism 9 was used to draw figures, in addition to the Receiver-operating characteristic (ROC) curve.

The data in both groups exhibited a normal distribution. The researcher employed the parametric Welch's t-test due to unequal variances between the two groups. For qualitative variables, the Chi square was used. Statistical significance was determined at P value < 0.05.

Results

Demographic parameters: In the UC group 23 (46%) were males and 27 (54%) were females Compared to 24 (48%) males and 26 (52%) females in the control group. The age range for UC cases was 18 - 75 years, compared to 18 - 60 years in the controls. The mean age was 36.4 ± 10.1 years for the UC cases and 36.4 ± 10.6 years for the controls. Nearly two thirds of the UC cases had a disease duration of five years or less, table 1.

Table 1: Demographic variables distribution between patient and control groups

Variables	Categories	Study Groups – No (%)		P-value
		Ulcerative Colitis	Controls	
Gender	Males	23 (46)	24 (48)	> 0.05
	females	27 (54)	26 (52)	
Age (Years)	/ Mean ± SD	36.4 ± 10.1	36.4 ± 10.6	> 0.05
Age groups	≤ 40 years	35 (70)	26 (52)	> 0.05
	> 40 years	15 (30)	24 (48)	
Disease Duration	≤ 5 years	31 (62)	N/A	
	> 5 years	19 (38)	N/A	

NA= not applicable / SD= standard deviation

Data Analysis: Welch's t-test results for the CXCL 9 show that the mean ± SD of CXCL9 was 26.9 ± 9.05 pg/ml in UC patients compared to 6.4 ± 2.37 pg/ml in controls, (p-value < 0.0001).

Table 2: CXCL9 serum concentrations in study groups

CXCL9	Ulcerative Colitis (50)	Controls (50)
mean ± SD (pg/ml)	26.9 ± 9.05	6.4 ± 2.37
Min – Max (pg/ml)	8.60 - 39.64	3.11 - 10.97
Welch's T-test		
P value <0.0001		

UC = Ulcerative colitis / CO = Control Validity of the test

The CXCL 9 demonstrates excellent validity in the diagnosis of patients with UC, with a cut-off point of ≥ 10.9 pg/ml, and an AUC of 99.2, signifying excellent discriminatory ability of the test. Sensitivity is 96%, specificity is 98%, PPV is 97.9%, NPV is 96%, and accuracy is 97% (P < 0.0001).

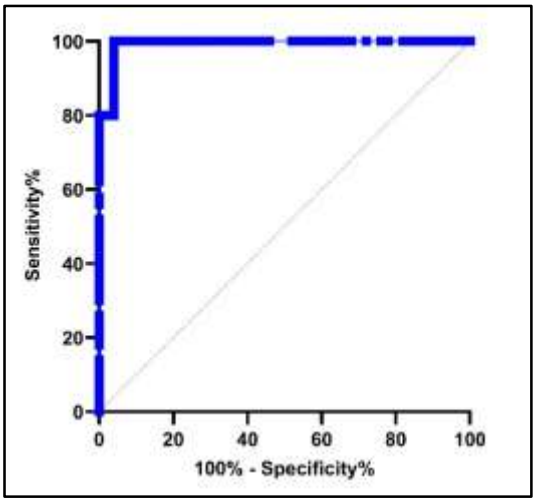

Figure 1: Receiver Operating Characteristic (ROC) Curve Analysis of CXCL 9 for Ulcerative Colitis Diagnosis

Discussion

This study found slightly more females than males within the the study group which is consistent findings of other studies from Iraq on the sex distribution of UC (19–21). However, these results are in contrast with the study from Saudi Arabia made by Alharbi, *et al.,* (22), as well as other studies from Iraq made by Abdul-Hussein, *et al.,* (23,24).

The reported difference in some studies between males and females in the occurrence of UC was explained by X-linked genetic factors and sex hormone signaling which may act together to trigger the sex-specific development of autoimmune disease (25). A study that is specifically designed to investigate this issue in depth is needed.

The majority of UC cases occur in the age group of 17-40 years (22), which is consistent with the results of the current study. the predominance of disease duration is less than 5 years among our cases, This result is consistent with that reported by Al-Khazraji, *et al.*, (26), where the majority of patients had a disease duration of less than 5 years.

It is possible that age-related changes in the immune system, diet, family history, genetic factors, or other physiological factors, in addition to the disease phenotype, may contribute to the differences in disease extent observed in patients of different age groups with UC as well as the disease duration. It is important to note that the Montreal classification of UC does not include age at diagnosis as a criterion, nor does the Truelove and Witts Severity Index.

Many studies indicated that CXCL9 and its receptor are highly expressed in the tissues of Crohn's disease (CD) patients in addition to the finding that same the elevation in the expression of CXCL9 and its receptor in IBD patients (27). CXCL9 and its receptor are highly expressed in lymphocytes, macrophages, and epithelial cells in patients with UC due to the overexpression of IFN-γ, as the first one plays a crucial role in the recruitment of mononuclear cells and granulocytes to the site of inflammation in UC (28). CXCR3 is expressed by epithelial, endothelial, and lymphoid cells, and its ligands CXCL9, induced by IFN- and attract Th1 cells expressing high levels of CXCR3. Increased expression of CXCR3, especially in CD4 T lymphocytes, has been observed in the mucosa of IBD patients compared to controls (29). Serum levels of CXCL9 are elevated in CD and UC patients (30), which is in agreement with the results of the current study and this can be attributed to an overactive immune response that targets the colon tissues with overwhelming inflammation.

In response to inflammation, the IFN-γ stimulates the production of CXCL9, a chemokine that attracts immune cells like CD4+, CD8+, and natural killer cells to the inflamed site. This leads to increased CXCL9 levels in the blood.

The researcher suggests that CXCR3 and its ligand may be a therapeutic target for IBDs in general and for UC in particular which may result in reducing the activity of the disease due to the expression of CXCR3 by Th1, as it stimulates the secretion of IFN-γ which is a pro-inflammatory cytokine. IFN-γ and TNF-α secretion is enhanced by the recruited Th1, which enhances in synergism between IFN-γ and TNF-α and stimulates Th1 chemokines secretion by a variety of cells. Studies on a larger sample size may provide a clearer picture of this marker in UC and its association with disease severity.

Conclusion

CXCL 9 may be employed as a biomarker for identifying ulcerative colitis. It can be used as a tool for measuring disease activity, in addition to the possibility of being a potential therapeutic target.

Authors declaration:

We confirm that all figures and tables presented in the manuscript are our original work.

Ethical Approval: Ethical approval for this study was obtained from the University of Baghdad, College of Medicine. All participants provided written informed consent before sample collection. The study protocol, subject information sheet, and consent form were reviewed and approved by a local ethics committee according to document number 0230, dated November 7, 2023.

Conflict of interest: None

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Author Contributions:

Study conception & design: (*Mohammed A. Qusay, Sarmad M.H. Zeiny, Haider J.M. Al-Maraashi*). Literature search: (*Mohammed A. Qusay*). Data acquisition: (*Mohammed A. Qusay*). Data analysis & interpretation: (*Mohammed A. Qusay*). Manuscript preparation: (*Mohammed A. Qusay*). Manuscript editing & review: (*Mohammed A. Qusay, Sarmad M.H. Zeiny, Haider J.M. Al-Maraashi*).

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CXCL9 في المصل كعلامة بيولوجية محتملة لمرضى التهاب القولون التقرحي

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الخلاصة:

الخلفية: التهاب القولون التقرحي هو مرض التهابي معوي يقتصر على الأمعاء الغليظة، ويتميز بتقرح سطحي. إنه مرض متفاقم ومزمن يتطلب علاجا طويل الأمد. على الرغم من أن سببه لا يزال مجهولا، يقترح أن العوامل البيئية تؤثر على الأفراد ذوي القابلية الوراثية، مما يؤدي إلى ظهور المرض.

عامل الجذب 9(C-X-C) هو عامل جذب ينتمي إلى عائلة عوامل الجذب CXC، ويلعب دورا في تمايز خلايا المناعة مثل الخلايا الليمفاوية السامة وغيرها، ويتفاعل مع مستقبله المناظر CXCR3 الموجود على خلايا مختلفة مثل الخلايا التائية الفعالة والبلعمة، مما يحفز إنتاج إنترفيرون غاما وعامل نخر الورم ألفا، اللذان بدورهما يحفزان إنتاج عوامل جذب الخلايا التائية المساعده 1 التي تؤدي إلى تعزيز الالتهاب.

الأهداف: الهدف الرئيسي لهذه الدراسة هو تقييم أهمية مصل عامل الجذب 9(C-X-C) كعلامة محتملة لتحديد التهاب القولون التقرحي عند البالغين الذين يعانون من مرض التهاب الأمعاء.

المرضى والطرق: هذه دراسة الحالات والشواهد، شملت 50 مريضا تم تشخيص إصابتهم بالتهاب القولون التقرحي، تتراوح أعمارهم بين 18 و75 عاما، مقارنة بـ 50 فردا سليما ، تتراوح أعمارهم بين 18 و60 عاما. أجريت الدراسة بين نوفمبر 2022 ومارس 2023، في المستشفى التعليمي لأمراض الجهاز الهضمي والكبد في مجمع مدينة الطب في بغداد. تم تحليل عينات المصل باستخدام تقنية المقايسة المناعية المرتبطة بالإنزيم (ELISA).

النتائج: أظهرت الدراسة اختلافا كبيرا في الأهمية الإحصائية بين مستوى عامل الجذب 9 (C-X-C) في مجموعتي الحالات والشواهد (بمتوسط ± انحراف معياري 9.05 ± 26.9 مقابل2.37 ± 6.4، وقيمة p أقل من (0.0001) مما يشير إلى قيمة تشخيصية عالية للكشف عن التهاب القولون التقرحي.

الخاتمة: تشير نتائج هذه الدراسة إلى إمكانية استخدام عامل الجذب 9 (C-X-C) كعلامة حيوية لتشخيص التهاب القولون التقرحي وكمقياس لنشاط المرض، بالإضافة إلى إمكانية استخدامه كهدف علاجي محتمل.

الكلمات المفتاحية: مرض التهاب الأمعاء، التهاب القولون التقرحي، الخلايا التائية اللمفاوية، التهاب مزمن، CXCL 9.

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Research Article

Immunohistochemical Expression and Histopathological Role of CD47 in Colorectal Cancer in Iraqi Patients

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Abstract

Background: Bowel cancer is the most prevalent digestive system cancer and is the 4th largest cause of cancer-related death worldwide. In Iraq, colon and rectal cancer (CRC) is the 6th most common malignancy in males and the 5th in females. This cancer is sluggish in growth, which gives a window of opportunity to screen for both precursor lesions and early cancer. The Cluster of Differentiation 47 (CD47) protein is a type of transmembrane glycoproteins found on nearly all human cells, including non-hematopoietic and hematopoietic cells. CD47 promotes CRC growth by triggering angiogenesis and apoptosis of tumor cell.

Objectives: To evaluate the immunohistochemical expression of (CD47) in various colorectal samples from Iraqi patients with CRC by immunohistochemistry (IHC) assay.

Methods: A total of 45 paraffin-embedded CRC tissue specimens and clinical data were obtained from the Medical City Teaching Hospital in Baghdad and a number of private laboratories in Baghdad, Iraq. In addition, 30 control colon and rectum tissues with no significant pathology were collected from the Forensic Medicine Department for comparison purposes after taking the official approvals.

Results: The results revealed a high expression of CD47 in CRC cases, but with no significant correlation with clinicopathological features. Also, the result of figures in this study revealed negative membranous expression of CD47 (score 0), strong membranous expression of CD47 (score 8), moderately membranous expression of CD47 (score 7), and weak membranous expression of CD47 (score 4)

Conclusion: Patients with CRC had high CD47 expression, allowing tumor cells to modulate CD47SIRP inhibitory signaling and prevent immune cell attack.

Keywords: Colorectal cancer; Cluster of Differentiation 47; Immunohistochemistry; Integrin-associated protein.

Introduction

Colon and rectal cancer (CRC), also known as bowel cancer, is the 4th most prevalent malignant disease worldwide, characterized by irregular growth of the colon epithelium which occupies the entire wall thickness of the colon, with metastasis to the surrounding lymph nodes and neighboring tissues, and finally distant metastatic spread (1). Most CRCs are slow-growing lesions that develop from polyps that are adenomatous or sessile serrated lesions (2). The probability of developing CRC is influenced by genetic and/or environmental factors (3).

CRC begins with vague signs and symptoms such as altered bowel habits, including constipation or diarrhea, changes in stool consistency, bleeding from the rectum, persistent gastrointestinal discomfort, e.g., cramps, pain or gases, tenesmus, fatigue, and unexplained weight loss. Screening for CRC can be done by measuring serum levels of various tumor markers, such as CA19-9, CEA, CCSA-3, and CCSA-4 (4). In accordance with the American Cancer Society, approximately 1 in 21 males and 1 in 23 females in the United States will develop CRC over their lifetime (5). CRC can be classified as proximal or right side if it develops from proximal portions to the splenic flexure (cecum, transverse colon and ascending colon), distal or left side if it originates distally to the descending colon or sigmoid, and rectal if it develops within 15 cm of the anal sphincter. (6).

CRC become more prevalent with increasing age, especially after the age of 55 years, with roughly 60% of the cases affecting those 70 years or older. Iraq has a low incidence of CRC, although it has been steadily increasing over time (7).

Prediction models based on tumor markers, as well as clinicopathological and demographic factors, have recently been broadly developed for the prognosis of CRC (8).

Cluster of differentiation 47 (CD47) (also referred as OA3 and IAP) a transmembrane glycoprotein that is present on nearly all cells in the human body. CD47 was found independently in numerous cell types, resulting in a wide range of nomenclature. Because it was found to be associated with integrins, such as v3, on various types of cells, it was first referred as Integrin-Associated Protein (IAP). Furthermore,

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being highly expressed on ovarian malignant cells, this antigen was also identified as OA3 (9). CD47 is the signaling receptor with high affinity for the secretory protein thrombospondin-1 (THBS1) and a counter-receptor for the signal regulatory protein alpha (SIRPα) (10). The binding of SIRPα to CD47 triggers SIRPα phosphorylation of ITIMs (immunoreceptor tyrosine-based inhibitory motifs) resulting in deactivation of myosin IIA, which is a critical step to block phagocytosis (11).

Specimens and Methods

From October 2022 to January 2023, 75 cases of colonic biopsies (excisional biopsy and total colectomy) were collected. The tissue samples were collected in the form of formalin-fixed paraffin-embedded (FFPE) blocks of tissue from the archive files of the Pathology Department/ Teaching Laboratories/ Baghdad Medical City Teaching Hospital and a number of private laboratories in Baghdad, Iraq. These samples belong to the years 2019, 2020, 2021, 2022, and 2023. The samples were further classified into 45 cases of malignant tumors from CRC cases and 30 colon biopsies from cadavers in the Forensic Medicine Department (not CRC cases) for comparison purposes after obtaining the official approvals. They were preserved in 10% formalin before being subjected to the standard tissue processing sequence and being turned into paraffin blocks.

Demographic and clinical data including age, gender, size and site of tumor, pathological stage, and grade were collected from the patients' records. All the preparations for slide sectioning, Hematoxylin/Eosin staining, and IHC staining were performed at a private laboratory.

Immunohistochemistry staining: The specific antibody/ antigen binding in tissue is the basic concept of this method. A variety of spotting techniques are then used to visualize the bound antigen-antibody complex. IHC detects the antigens of interest using a variety of enzymatic markers, including alkaline phosphatase and peroxidase (12). The IHC staining process was followed exactly as specified by the kit manufacturers' protocols (Abcam, anti-CD47 antibody, EPR21794, ab218810 and PolyExcel, HRP/DAB Detection System). The general protocol was adopted in accordance with Magaki *et al*., 2019 (13). First, the tissue sections were de-waxed by heating the slides in an oven at 60-65°C for 30 minutes, followed by rapid rinsing in xylene and re-heating in the oven for 5 minutes. The sections were hydrated again via immersing them in decreasing concentrations of alcohol. After dipping the slides in the solution of antigen retrieval, they were put in a water bath heated to (95 °C) and allowed to boil for 30 minutes. After that, the slides were treated for 10 minutes with a Peroxidase Quencher (H_2O_2) solution. (CD47) primary antibody diluted to (1:2000) was applied to the tissue sections, and incubated at room temperature in a humidity chamber for 30 minutes. After cleaning the tissue sections, the PolyExcel Target Binder (Cat# IPS006) was added to the slides which were then incubated for 15 minutes at room temperature in the humidity chamber. The slides were then coated with PolyExcel PolyHRP (Cat# IPS007) and incubated in the humidity chamber for 15 minutes at room temperature. Afterwards, PolyExcel StunnDAB with its substrate were applied to the slides and incubated in a humidity chamber at room temperature for 5 minutes until the brown stains appeared. The tissue sections were then washed two times with Tris-Buffer-Saline (TBS) for 5 minutes each. The slides were then counterstained with drops of Mayer' Hematoxylin for 1-2 minutes before being carefully cleaned in distilled water (DW) until clear. The slides were then dehydrated in increasing concentrations of alcohol and cleared in xylene for two times at two minutes each. In the end, by using the DPX mounting medium, the slides were mounted and coverslipped.

Scoring system of immunohistochemistry: CD47 scoring system was applied according to Allred score (14). The altered score was calculated using the formula clarified in Table 1. The findings were evaluated and analyzed by a pathologist.

Table 1: Formula and scores employed based on the Allred score system for immunohistochemistry

Intensity score (IS)	Proportion score (% Stained Cells) (PS)
0 (non-staining)	0 (no cells)
1 (stain is weak)	1 (<1%)
2 (stain is moderate)	2 (1-10%)
3 (stain is strong)	3 (11-33%)
	4 (34-66%)
	5 (67-100%)

Total score (TS)= PS+IS; total score ranges from (0-8), where (0-2) were considered negative and (3-8) was considered positive

Statistical analysis

For data analysis, the SPSS-28 (Statistical Packages for Social Sciences - Version 28) was used. The data were presented in simple frequencies, percentages, means, standard deviations, and ranges. To test for associations between variables, the Chi-square test was performed (15), and a P value of <0.05) was considered significant.

Results

Immunohistochemical expression of CD47 in CRC: CD47 expression is observed in tumor cells as brown membranous staining (Figures: 1, 2, 3, and 4).

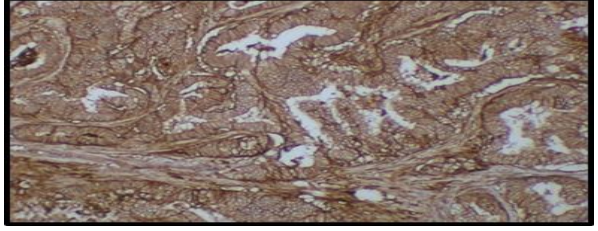

Figure 1: Strong membranous expression of CD47 in CRC (score 8) (4X)

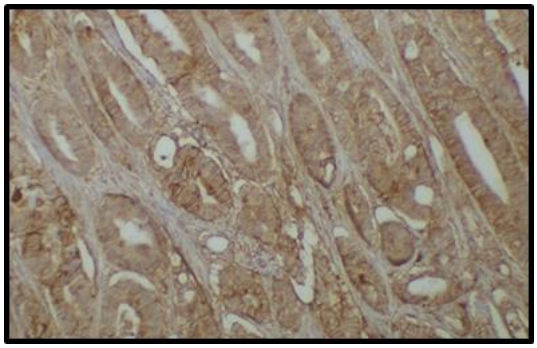

Figure 2: Moderate membranous expression of CD47 in CRC (score 7) (4x)

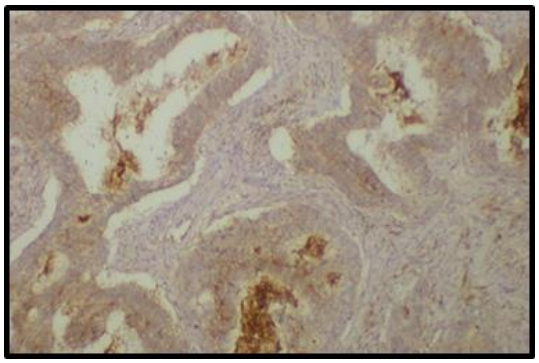

Figure 3: Weak membranous expression of CD47 in CRC (score 4) (4x)

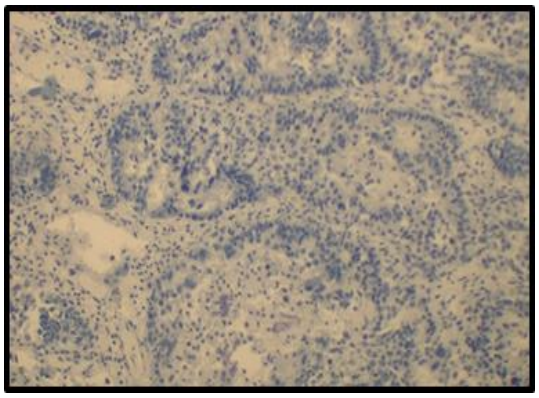

Figure 4: Negative membranous expression of CD47 in CRC (score 0) (4x)

CD47 expression was positive in 91.1% of malignant cases, while it was negative in 100% of normal colorectal tissues, with a significant association, (Table 2).

Table 2: Expression of CD47 malignant colon tissue from CRC patients and controls

Expression	CD47			
	Malignant		Control	
	No.	(%)	No.	(%)
Positive	41	91.1	0	0
Negative	4	8.9	45	100
P value	(<0.001) *			

Significant association between CD47 expression in CRC cases and controls using Chi-square test.

Association of CD47 with demographic and clinicopathological features: The results showed no significant associations between CD47 expression and age, gender, tumor site and diameter, tumor stage and grade, histological type, and lymph node metastasis (Table 3).

Table 3: Associations of CD47 with demographic and clinical variables in Iraqi CRC patients

Clinicopathological features		CD47 +	CD47 -	*p-value*
Age	<40	6	0	0.754 NS
	40-49	7	0	
	50-59	10	3	
	>60	18	1	
Gender	Male	19	2	0.165 NS
	Female	22	2	
Tumor Size	<5 cm	29	1	0.213 NS
	>5 cm	12	3	
Tumor Site	Left	26	4	0.574 NS
	Right	15	0	
Stage	IIA	13	3	0.463 NS
	IIC	2	0	
	IIIA	3	0	
	IIIB	18	1	
	IIIC	5	0	
Grade	I	1	1	0.092 NS
	II	38	4	
	II	1	0	
Histological Type	Adenocarcinoma	35	4	0.756 NS
	Mucinous	4	0	
	Signet ring cell	2	0	
Lymph Node Metastasis	Positive	19	0	0.307 NS
	Negative	22	4	

No significant associations were found using the Chi-square test.

Discussion

In the recent years, extensive research has been carried out in an effort to predict illness progression and discover novel treatments for CRC. Immunotherapy has been a significant advance in cancer treatment. This immunotherapy works through blocking checkpoints, one of which is CD47 (16). CD47, an important molecule for the macrophage checkpoint, was discovered to be excessively expressed in the hematological and solid cancers with poor prognosis. CD47 tumor expression is associated with immune evasion via malignant cells through the CD47-SIRP alpha pathway (17). CD47 was first identified as a tumor antigen on human ovarian cancer in the 1980s, and its overexpression is associated with a poor prognosis in ovarian cancer (18).

Most normal cells have the CD47 integrin, which binds to the extracellular domain of SIRP on macrophages and inhibits their phagocytic activity. When mature blood cells, such as RBCs lose their CD47 molecules, they become the target for macrophage-mediated removal from the circulation (19). Cancer cells overexpress CD47 in order to avoid the innate immune response of M1 macrophages by attaching to their SIRP receptor.

The host's adaptive and innate immune systems play essential roles in eliminating malignant cells and preventing progression of tumors, while cancer cells display immunological escape by expressing immune checkpoint proteins such as CD47 (20). When activated by CD47 binding, the regions of

immunoreceptor tyrosine-based inhibitory motif (ITIM) turn into phosphorylated, resulting in activation and recruitment of the protein tyrosine phosphatases, which results in the de-phosphorylation of next molecules, like myosin IIA, and the suppression of phagocytosis activity (21). Many studies reported that expression of CD47 is a prognostic marker in many different types of malignancy (22).

In the current study, 45 cases of CRC were immunohistochemically examined for CD47, and the association of their CD47 expression with demographic and clinicopathological characteristics was investigated.

The result demonstrated that CD47 was positively expressed in (91.1%) of all malignant cases. This result is in corresponds to those reported by Sugimura-Nagata et al who found a higher expression of CD47 in malignant than in normal cells. This suggests that the limitedly-expressed CD47-dependent activation of the CD47–SIRPA signaling in the CRC microenvironment has a significant impact on the clinical outcome of CRC, as similarly observed for other malignancies (17).

Another aspect of the current study is the association between CD47 expression and different demographic and clinicopathological features, especially age. The lack of association between the expression of CD47 and age groups is in agreement with the results of Fujiwara-Tani et al (23) who reported no such association between the expression of CD47 and age groups in CRC cases. A study on stomach cancer also found no such association (24).

In the current study, there was no association between tumor site and size of CRC with CD47 expression. These findings correspond with those reported by Hu et al (25). A study on gastric carcinoma (26) reported no association between expression of CD47 and tumor diameter and location. Moreover, there was no association between CD47 expression in CRC cases and gender in the current study, and in that conducted by Ji et al on CRC cases (27) and that conducted by Peng et al on lung squamous cell cancer (28).

The current study found no association between CD47 expression and the histological type of the malignant tumor. Similarly, Bang found no association between CD47 expression and histological type of non-melanoma skin cancers (29), while Sugimura-Nagata et al (17) reported that CD47 positivity was associated with histological differentiation in CRC. The current investigation found no association between CD47 and the stage or grade of CRC, whereas Yuan et al in their breast cancer study, found a statistically significant association between CD47 expression levels and the stage and grade of breast cancer (30).

Immune avoidance is critical for progressing to the developed stage of colorectal adenoma (CRA), and some researchers have found that a higher concentration of tumor-associated macrophage (TAM) associate with more developed CRC stages. This might indicate that, through the "don't eat me signal", the CD47 expression, which inhibits phagocytic activity, rises in more developed stage as well (31). The current study found no association between CD47 expression and lymph node involvement. In CRC patients, CD47 was found to be strongly associated with recurrence, American Joint Committee on Cancer (AJCC) stage, and metastasis. A study on CD47 expression in triple negative breast cancer found no significant association with lymph node metastasis (32). These findings strongly suggest that CD47 expression may play an important role in controlling human CRA progression. Our findings show that CD47 overexpression enhances CRC metastasis and cell proliferation.

The differences between our results and those of other studies may due to differences in histological specimens, race, genetic factors, methodology and kits used. This is the first study in Iraq on CD47 in CRC patients.

Conclusions

The study suggests that increased CD47 expression in CRC patients, allows tumor cells to control CD47/SIRP inhibitory signals and prevent immune cell attack. There was no association between CD47 expression and demographic and clinicopathological features of CRC patients.

Authors' declaration:

We confirm that all the Figures and Tables in the manuscript are mine/ ours. Besides, the authors have signed an ethical consideration's approval-Ethical Clearance. The project was approved by the local ethical committee in the College of Science, according to the guidelines on biomedical research, the license has the code number CSEC/0922/0109 and is dated September 28, 2022.

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Author contributions:

Study conception & design: (Fatima O. Abd alkareem) and Dr. Ban J. Mohamad), Literature search: (Fatima O. Abd alkareem), Data acquisition: (Fatima O. Abd alkareem), Data analysis & interpretation, Manuscript preparation (Fatima O. Abd alkareem and Dr. Ban J. Mohamad).

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التعبير النسيجي الكيميائي المناعي لبروتين CD47 في سرطان القولون في المرضى العراقيين

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الخلاصة:
خلفية البحث: سرطان الأمعاء هو أحد أكثر الأورام الخبيثة شيوعا في الجهاز الهضمي ويعتبر السبب الرابع للوفيات بسبب السرطان في العالم. في العراق، يعتبر سرطان القولون والمستقيم أحد الأورام الخبيثة والسادس الأكثر شيوعا في الرجال والخامس في النساء. بسبب النمو البطيء لهذا النوع من السرطان، هناك فرصة لفحص كل من السرطان المبكر والآفات المسبقة. مجموعة التمايز 47 (CD47) هو أحد أنواع البروتينات الغشائية السكرية ويوجد تقريبا في جميع خلايا جسم الانسان، والتي تشمل الخلايا المكونة للدم وغير المكونة للدم. CD47 يحفز نمو سرطان القولون عن طريق تكوين الاوعية الدموية والموت المبرمج للخلايا السرطانية.
الاهداف: تقييم التعبير الكيميائي النسيجي المناعي ل(CD47) في عينات مختلفة لسرطان القولون في المرضى العراقيين عن طريق الفحص الكيميائي المناعي النسيجي.
المنهجية: تم جمع 45 عينة من سرطان القولون والمستقيم المدمجة في شمع البارافين والمعلومات السريرية من مستشفى مدينة الطب التعليمية المختبرات الخاصة في بغداد، العراق. بالإضافة الى ذلك تم جمع 30 عينة من القولون والمستقيم التي لا تعاني حالة مرضية من قسم الطب الشرعي لأغراض المقارنة في هذه الدراسة بعد اخذ الموافقات الرسمية.
النتائج: اظهرت النتائج وجود تعبير عالي ل CD47 في سرطان القولون والمستقيم، ولم يظهر أي ارتباط معنوي بين السمات السريرية والمرضية. أيضًا، اظهرت نتائج الصور في هذه الدراسة عن تعبير غشائي سلبي لـ CD47 (النتيجة 0)، وتعبير غشائي قوي لـ CD47 (النتيجة 8)، وتعبير غشائي معتدل لـ CD47 (النتيجة 7)، وتعبير غشائي ضعيف لـ CD47 (النتيجة 4). **الاستنتاجات:** أظهرت الدراسة الحالية تعبيرًا عاليًا لـ CD47 في المرضى الذين يعانون من سرطان القولون والمستقيم، مما يسمح للخلايا السرطانية بالتحكم الإشارات المثبطة لـ CD47-SIRPα وتجنب هجوم الخلايا المناعية.
مفتاح الكلمات: سرطان القولون والمستقيم، مجموعة التمايز 47، الكيمياء المناعية النسيجية، البروتين المرتبط بالإنتغرين

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Research Article

Nitric Oxide, Procalcitonin and Oxidative Stress Index Levels in Acute Bronchitis Patients

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Abstract:

Background: Acute bronchitis, an inflammation of the lower respiratory tract characterized by an acute cough, is a prevalent clinical illness that leads patients to seek out primary healthcare services. About 5 percent of adults in the United States report having acute bronchitis annually, with 90 percent of those affected seeking medical attention.

Objectives: The study aimed to determine Nitric oxide, Procalcitonin (PCT), WBCs, neutrophils, lymphocytes, and Oxidative stress index (OSI) levels in acute bronchitis patients.

Methods: The study involved 120 volunteers aged 20–50 years old in Al-Zahra Teaching Hospital, Wasit City. 80 patients with acute bronchitis were conducted between (10 November 2022 to 20 March 2023). 40 people were used as a control group. Blood samples were collected from patients and controls. Complete blood account CBC was calculated using a blood sample with EDTA. Serum was used to calculate NO, PCT, and OSI. Blood counts were performed using the SYSMEX XP-300. Nitric oxide and Procalcitonin levels were measured using an ELISA kit. OSI was calculated using the equation OSI = Total oxidant status /Total antioxidant status x100.

Results: The current research presents the results of the Procalcitonin, nitric oxide, oxidative stress index, neutrophils, and lymphocytes. Age, BMI, and WBCs in acute bronchitis did not show any significant variances when compared between the two groups. In contrast, nitric oxide, Procalcitonin, oxidative stress, and Neutrophil levels showed a highly significant change among the acute bronchitis patient group compared to the control group.

Conclusion: Procalcitonin and nitric oxide may have a role in the diagnosis of acute bronchitis, in addition to lymphocytes and neutrophils.

Keywords: Acute bronchitis; lymphocytes; Neutrophils; Nitric oxide; Procalcitonin.

Introduction

Acute bronchitis is a common clinical illness resulting in visits to primary care physicians because it causes inflammation of the lower respiratory tract and, consequently, an acute cough. Around five percent of adults in the United States report having acute bronchitis annually, with ninety percent of those affected seeking medical attention. Antibiotics are not effective in treating acute bronchitis since the condition typically gets better on its own within a week or two of its beginning and is caused by a virus in at least 90 percent of the cases (1,2). Nitric oxide (NO) is a crucial signaling molecule and a free radical gas. The Nitric Oxide Synthase (NOS) enzymes, which catalyze the conversion of L-arginine to NO and L-Citrulline, are ubiquitously expressed and their expression is controlled in a cell-type-specific manner, Vasodilation, systemic circulation, hemodynamics, neuronal functions such as neurotransmission, neuroprotection, or memory, and immune response activities such as innate immunity or inflammation are just a few of the many physiological processes in which NO plays a part, immune system macrophages produce NO locally to eliminate dangerous bacteria (3,4). NO is regarded as a pro-inflammatory mediator that induces inflammation in abnormal situations due to excessive production (5). Viral infections, especially respiratory disorders, can cause oxidative stress through a variety of mechanisms, and many studies have proven this phenomenon (6). Chronic oxidative stress impairs immunological function, apoptosis, inflammatory response, and may induce organ and tissue failure in almost all viral infection patients (7). Oxidative stress arises from a state of disequilibrium between the endogenous generation of oxidative stress, antioxidant defenses, and reactive oxygen species (8,9). Procalcitonin is a gene product that is closely linked to calcitonin. It is produced by human epithelial cells in response to bacterial infections, but its expression is lowered during viral infections (10). The production of the biomarker procalcitonin (PCT) occurs in the parenchymal tissues through the mediation of cytokines IL-6, TNF-α, and IL-β. The extent to which PCT levels increase is directly associated with the severity of the infection. PCT synthesis, on the other hand, is inhibited by

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interferon-γ, a cytokine that is mainly secreted in response to viral infection (11,12). White blood cells WBCs play a significant role in the immune response against the invasion of pathogens. Neutrophils, which are the predominant WBCs in the human body, assume a pivotal function in the initiation of acute inflammation caused by pathogens (13). Lymphocytes are integral components of the immune system, providing crucial assistance in combating malignancies, as well as external pathogens such as viruses, bacteria, and parasites (14). The study aims to evaluate the relationship between nitric oxide and procalcitonin levels and oxidative stress, in addition to knowing the change in white blood cells in patients with acute bronchitis.

Patients and methods

The current research was conducted in Iraq to determine NO, PCT, and OSI levels in acute bronchitis patients, This research included 120 volunteers aged 20–50 years old in Al-Zahra Teaching Hospital, Wasit City. Eighty patients with acute bronchitis were conducted between(10 November 2022 to 20 March 2023). Forty people were used as a control group

Blood sample collection: Five ml of blood was taken from every patient and controlled through venipuncture using a 5 ml syringe. One ml of blood was placed into a tube containing ethylene diamine tetra etic acid (EDTA), and this blood was isolated and used to use to calculate the CBC. Serum was used to calculate Nitric oxide (NO), Procalcitonin (PCT), and oxidative stress index (OSI).

Acute bronchitis-related parameters determination: For a complete blood count, the SYSMEX XP-300 from Sysmex Corporation, Japan, was used. An ELISA kit was used to measure nitric oxide (NO) and procalcitonin (PCT) levels according to the manufacturer's instructions (MyBioSource, America). While OSI was calculated using the equation OSI = Total oxidant status (mM H2O2/L) /Total antioxidant status (mM vit.C/L) x 100.

Statistical analysis:

Statistical analysis software (SPSS 25) was used to analyze the findings. The General descriptive statistic was used to explain the primary findings, and an independent *t*-test was used to compare groups. The cutoff values for the parameters were determined using the receiver operating characteristic curve (ROC).

Results:

According to the data in Table 1, acute bronchitis patients were compared to the controls, the levels showed non-significant differences in age and body mass index.

Table 1: The mean ±SD for the age and BMI of patients with acute bronchitis and healthy subjects

Parameter	Mean ±SD		*P-value*
	Control Group (n= 40)	Group1 Acute bronchitis (n=80)	
Age (Yr).	37 ± 8	31 ± 10	0.1
BMI (Kg/m^2)	26.1± 2	25.7 ± 3.8	0.3

According to Table 2, the results of the WBC revealed a mean ±SD for acute bronchitis patients and controls (8.1 ±0.6), (6.9 ± 0.2), respectively. The results indicate a nonsignificant change in the white blood cells in the two groups (P>0.05). The mean and SD of neutrophils compared to control is (66.5 ± 2.3) (60.2 ± 0.8), respectively. The results show a significant change among the two groups in Neutrophils (P<0.05). Lymphocyte results revealed a mean SD for acute bronchitis patients and controls (23.8 ± 2.1) and (30.8 ± 0.8) which shows a significant difference between the two groups regarding lymphocyte number (P< 0.05).

Table2. Distribution of WBC, Lymphocytes, and Neutrophils for patient

parameter	Mean ±Sd		p-value
	Control Group (n= 40)	Group1 Acute bronchitis (n=80)	
WBCs (k/ul)	6.9 ± 0.2	8.1 ±0.6	0.18
Lymphocytes (%)	30.8 ± 0.8	23.8 ± 2.1a	0.007
Neutrophils (%)	62.2 ± 0.8	66.5 ± 2.3a	0.008

*Significant using ONEWAY-ANOVA and at 0.05 level.

a) Indicate a significant difference between control and Group1

Oxidative stress index results show a statistically significant difference between the control and patient groups, as shown in Table 3 patient groups (P<0.05). The results show that there is a clear significant difference in the concentrations of nitric oxide in patients with acute bronchitis compared to the control group (P<0.05), as shown in Table 3. It was found that there are significant differences between patients with acute bronchitis and the controls regarding NO and PCT (P<0.05; Table 3).

Table3. Distribution of Oxidative stress index, Nitric oxide and Procalcitonin for patients and control groups

parameter	Mean ±Sd		p-value
	Control Group (n= 40)	Group1 Acute bronchitis (n=80)	
Oxidative stress index	1.41±0.44	0.59±0.09a	0.02
Nitric oxide (pg/ml)	429.7±30.7	748.3±36.4a	0.00
Procalcitonin (pg/ml)	172.6±4.7	366.3±16.6a	0.00

*Significant using ONEWAY-ANOVA and at 0.05 level.

a) Indicate a significant difference between control and Group1

Receiver Operating Characteristic (ROC)

According to the Receiver Operating Characteristic (ROC) curve for patients with acute bronchitis, the results show sensitivity (86%) for Nitric oxide and specificity of (99%) with a cutoff value (of 605), and procalcitonin shows a sensitivity of (100) and specificity of (100) with a cutoff value (254) as shown in Figure (1)

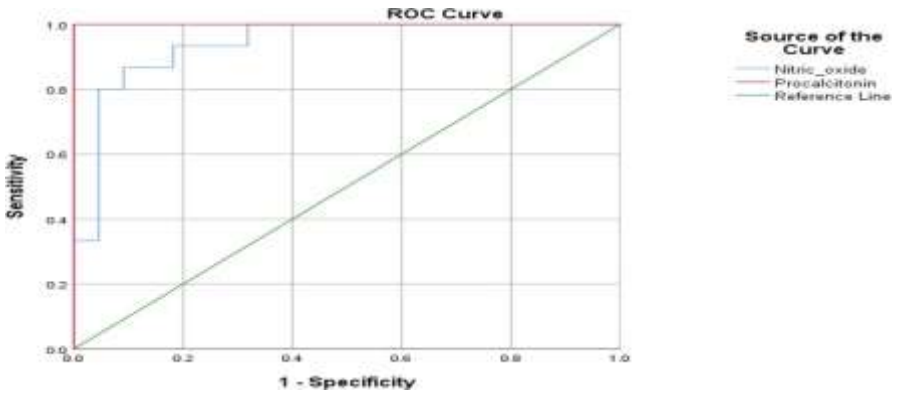

Figure 1. ROC curve for Nitric Oxide and Procalcitonin in acute bronchitis patients.

Discussion:

In this study, it was found that age does not have a significant effect, since (P> 0.05). While in a previous study, there were a total of 99 males (or 77.95%) and 28 females (22.05%). The incidence of acute bronchitis was highest in people between the ages of one and sixty years old, a group of people aged 11-20 years old (20.47%), than those aged 21-30 years old (27.56%) (15). Likewise, for the body mass index (BMI), It was found that it had no significant effect. While a previous study indicated that bronchitis incidence is more likely to occur in adolescents whose body mass index is in the higher percentiles, who are overweight, or who are obese (16), obesity and underweight have been observed in multiple studies to increase the risk of infection in adults in a U-shaped pattern, suggesting that normal weight is associated with a lower risk of infection in the majority of participants (17).

The clinical diagnosis of infection frequently involves a routine blood WBC count (18). Our study indicated there is no change in the white blood cell count (WBC) in acute bronchitis was found to be similar in smokers and non-smokers, but higher in people with a history of bronchitis (19). The research results showed a decrease in the percentage of lymphocytes in patients with acute bronchitis. A 2020 meta-analysis showed that lymphopenia was associated with worse outcomes in individuals infected with COVID-19 (20). Lymphocytes are the primary effector cells of the immune system. Lymphocytes count is inversely related to inflammation and positively related to immunity and defense against harmful germs (21). The results indicated a high percentage of neutrophils in patients with acute bronchitis. As previous studies indicated, the inflammatory response in both the upper and lower airways during viral-induced respiratory disease is characterized and dominated by airway neutrophilia (22). The body's usual response to infection or inflammation is a slight or temporary increase in neutrophils (23). Immune cells tend to react rapidly near the site of infection when harmful microorganisms penetrate the body. These immune cells serve the role of host defense as well as immune control (24). The results show a clear decrease in the levels of OSI in the patients compared to the control group. Many studies have been published demonstrating a relationship between oxidative stress and human health and disease. Oxidative stress can induce inflammation, mucus hypersecretion, airway remodeling, and fibrosis in the bronchial tubes, leading to chronic obstructive pulmonary disease (COPD) (25). OSI studies have proven to be dependable, practical, and clinically helpful (26). Oxidative stress is generated by a variety of viral diseases and is associated with the severity of infections and their ability to predict, including HIV-1, viral hepatitis B, C, and D viruses, herpesviruses, and respiratory viruses such as coronaviruses (27,28). Nitric oxide (NO) exhibits various antiviral mechanisms in host defense. These mechanisms include the nitrosylation of cysteine residues, resulting in the deactivation of viral enzymes. Additionally, NO contributes to the generation of reactive nitrogen species, such as peroxynitrite, which induces breaks in DNA strands. Furthermore, NO suppresses viral transcription factors, thereby inhibiting viral replication and the propagation of disease states (29,30). Nitric oxide (NO) is recognized as a pro-inflammatory mediator that can induce inflammation when produced excessively in abnormal circumstances (31). To the best of our knowledge, this study is the first of its kind, linking acute bronchitis and procalcitonin The results of our

research showed a direct relationship between acute bronchitis and high procalcitonin levels. Procalcitonin exhibits a direct correlation with the severity of sickness in cases of pure viral infection, and its levels remain unaffected by interferon signaling. This proposition posits that procalcitonin has superior efficacy as an indication of illness severity in comparison to bacterial coinfection in the context of viral respiratory infections (32.33)

Conclusions:
procalcitonin and nitric oxide may have a role in the diagnosis of acute bronchitis, in addition to lymphocytes and neutrophils

Authors' declaration:-
We hereby affirm that all the Figures and Tables included in the manuscript are the original work of the authors. Authors sign on ethical consideration's approval-Ethical Clearance: This research was approved by the Committee of the University of Baghdad, College of Science for Women, Department of Chemistry with session 10, number 6364/22 on 5/12/2022.
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Study conception & design: (Ekhlass M. Taha). Literature search: (Huda A. Abdulsada). Data acquisition: (Huda A. Abdulsada). Data analysis & interpretation: (Huda A. Abdulsada). Manuscript preparation: (Huda A. Abdulsada). Manuscript editing & review: (Ekhlass M. Taha).

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مستويات أكسيد النيتريك و بروكالسيتونين مؤشر الإجهاد التأكسدي في مرضى التهاب القصبات الهوائية الحاد

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الخلاصة:
خلفية البحث: التهاب الشعب الهوائية الحاد، وهو التهاب في الجهاز التنفسي السفلي يتسم بسعال حاد، مرض سريري شائع يدفع المرضى إلى البحث عن خدمات الرعاية الصحية الأولية. تقريبا 5 في المئة من البالغين في الولايات المتحدة يبلغون عن تعرضهم لالتهاب الشعب الهوائية الحاد سنويا، ومعظمهم (90 في المئة) يلجأون إلى الرعاية الطبية.
الاهداف : هدفت الدراسة إلى تحديد مستويات أكسيد النيتريك (NO) وبروكالسيتونين (PCT) وخلايا الدم البيضاء (WBCs) وخلايا العدلات والخلايا اللمفاوية ومؤشر الاجهاد التأكسدي (OSI) في مرضى التهاب الشعب الهوائية الحاد.
المواد وطرق العمل: شملت الدراسة 120 متطوعا تتراوح أعمارهم بين 20-50 سنة في مستشفى الزهراء التعليمي في مدينة واسط. أجريت الدراسة على 80 مريضا مصابا بالتهاب الشعب الهوائية الحاد في الفترة ما بين (10 نوفمبر 2022 إلى 20 مارس 2023). تم استخدام 40 شخصا كمجموعة سيطرة(ضابطة). تم جمع عينات الدم من المرضى ومجموعة السيطرة. تم حساب العد الكامل للدم (CBC) باستخدام عينة دم تحتوي على EDTA. تم استخدام المصل لحساب NO و PCT و OSI. تم إجراء عد الدم باستخدام جهاز SYSMEX XP-300. تم قياس مستويات أكسيد النيتريك وبروكالسيتونين باستخدام مجموعة اختبار ELISA. تم حساب مؤشر الاجهاد التأكسدي (OSI) باستخدام معادلة OSI = إجمالي حالة الأكسدة / إجمالي حالة مضادات الأكسدة x 100.
النتائج: تقدم الدراسة نتائج مستوى البروكالسيتونين وأكسيد النيتريك ومؤشر التوتر الأكسدي ونسبة العدلات و الخلايا اللمفاوية. لم يظهر العمر ومؤشر كتلة الجسم (BMI) وخلايا الدم البيضاء أي اختلاف ذو دلالة إحصائية عند المقارنة بين المجموعتين. على النقيض من ذلك، أظهر أكسيد النيتريك وبروكالسيتونين ومستويات التوتر الأكسدي والعدلات تغييرا ذو دلالة عالية بين مجموعة مرضى التهاب الشعب الهوائية الحاد مقارنة بالمجموعة السيطرة.
الاستنتاجات: يمكن أن يكون للبروكالسيتونين وأكسيد النيتريك دور في تشخيص التهاب الشعب الهوائية الحاد، بالإضافة إلى الخلايا اللمفاوية والعدلات.
الكلمات المفتاحية: التهاب الشعب الهوائية الحاد، بروكالسيتونين، أكسيد النيتريك، العدلات، خلايا لمفاوية.

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Research Article

Study of ATPase and GTPase levels in Fibrotic Lung Disease with and without COVID-19 Vaccination

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Abstract:

Background: In eukaryotic cells, the acidification of intracellular compartments is the responsibility of vacuolar H+ ATPase, a family of proton pumps, sometimes known as V-ATPases. Small GTPases are signaling molecules that regulate important cellular processes as well as subcellular activities making the essential players, particularly in a wide variety of coronavirus infection processes.

Objectives: The purpose of this research was to assess the levels of ATPase and GTPase in fibrotic lung disease patients who had received or had not received the COVID-19 vaccination, and then to compare these levels with those of the control group.

Methods: A total of 150 individuals took part in this study, which were divided into three groups. The first group was the control group (N=50). In the second group (N=50) patients with fibrotic lung disease did not get the COVID-19 vaccination. A total of fifty patients who had received the COVID-19 vaccination made up the third group(N=50). Enzyme-linked immunosorbent assay was the method that was used to determine the amounts of ATPase and GTPase. The P- P-value of 0.05 or less is considered statistically significant. ROC tests were examined for ATPase and GTPase.

Results: The data analysis reported that there was a significant rise in alkaline phosphatase, Alanine aminotransferase, and Aspartate-aminotransferase () among the three groups. Both ATPase and GTPase levels were shown to have significantly increased in Groups 3 and 2 as compared to Group 1 levels. Moreover, a substantial rise was discovered in Group 3 in comparison to Group 2 which was detected.

Conclusion: ATPase and GTPase levels are increased in patients with fibrotic lung disease regardless of the COVID-19 vaccination state.

Keywords: ATPase; GTPase**;** COVID-19 Vaccination; Fibrotic Lung Disease**.**

Introduction

Fibrotic lung disease (FLD) is a chronic lung ailment that is characterized by an inflammation that interferes with the lung's ability to receive sufficient oxygen (1). SARS-COV-2 is a virus that is transmitted to humans. It has been shown that, even within the same SAAS-COV-2 strain, several genomic alterations have been found which suggests that mutations were acquired by a process of consensus (2,3). Research demonstrated that lung dysfunction is a consequence of COVID-19 caused by prolonged ventilation acute respiratory distress syndrome or direct harm from the virus (4,5). vacuolar H+ ATPase, the proton pumps are reliant on ATP. They are important for several cellular functions that require vacuolar compartment acidification. These processes include the trafficking of membranes, proteolysis of lysosomes, the limination of invading pathogens by phagosomes, and the secondary transport of metabolites (6). Two subunits make up the V-ATPase: a V0 complex that is trans-membrane and a V1 complex that is cytosolic (7). This V-ATPase subunit was examined in the lung damage and fibrosis model in a recent work, which showed a function that had not been known about before. Within the tiny airway, the concentration of proton is responsible for regulating location (8).

The GTPases, also known as Rho, are enzymes that play the role of a molecular switch. They are regulated by guanine nucleotide exchange agents and are responsible for catalyzing the conversion of guanidine diphosphate to guanosine triphosphate (GTP). The GTPase-activating proteins, which are responsible for stimulating GTP hydrolysis, are responsible for inactivating the enzyme when it is in its active state, which occurs when the enzyme is phosphorylated (9).

The Rho/ROCK signaling system is implicated in smooth muscle contraction sensitization via its contribution. This is a significant aspect of the system, by demonstrating that the Rho/ROCK signaling system is accountable for airway smooth muscle contraction. Myosin phosphatase, which is an essential part of the process, is inhibited to achieve this goal (10).

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The purpose of this research was to assess the levels of ATPase and GTPase in FLD patients who had, or had not, received the COVID-19 vaccination, and then to compare these levels with those of the control group Additionally, ROC studies were conducted to test for ATPase and GTPase, both of which have the potential to be regarded significant prognostic factors in the area of COVID-19 vaccination.

Materials and Methods:

For this investigation, samples were obtained from Ibn Al-Nafis Teaching Hospital and the Baquba Teaching Hospital/Internal Medicine and Chest Consultant between March 2022 and January 2023. The current study involved 150 individuals who were divided into three groups. The first group (G1) comprised fifty individuals and acted as the control group without contracting COVID-19. Fifty individuals with FLD who had not received the COVID-19 vaccination made up the second group (G2). fifty FLD patients who had received the COVID-19 vaccination (Pfizer) made up the third group (G3), ll patients have COVID-19. The age group of participants was 25-55 years. Every patient was subjected to a personal interview conducted using a well-crafted questionnaire. This interview included a comprehensive history. Most patients infected with COVID-19 developed FLD more than 4 months after they were diagnosed with a CT scan and by doctors in the hospital. Following an infection with COVID-19, it has been shown that several individuals have developed new instances of emphysema, cysts, and mosaic attenuation, (11, 12). It was determined that five milliliters of venous blood should be taken from each participant. The serum was utilized to determine the levels of ATPase and GTPase using the ELISA method (My bio source in the United States, America, manual procedures used in ALP, ALT, and AST determination).

Statistics: The SPSS was used for statistical analysis. The results are expressed as mean ± SD. Independent t-tests were utilized to compare the groups in the study. To compare the differences between the two groups, the Pearson Chi-square test was used. The P-P-value of 0.05 or less is considered statistically significant. ROC tests were examined for ATPase and GTPase.

Results:

It is our understanding that this is the first research that has assessed the levels of ATPase and GTPase in fibrosing lung disease, both with and without the COVID-19 vaccine.

Data in Table (1) represented a significant increase in serum ALP level in G2 compared to G1. A highly significant increase was found in G3As compared to G1, and G3As compared to G2.

However, ALT levels showed a non-significant increase in G2 in contrast to G1. A highly significant increase in $G3_3$ compared to G1 and G3 compared to G2.

Data in Table (1) revealed a highly significant increase in G2 and G3 match to G1 for AST levels. Highly-significant increase was seen in G3 compared to G2.

Table (2) shows an ANOVA analysis of GTPase and ATPase levels for G1, G2, and G3. Results of GTPase and ATPase display a highly significant increase in G2 and G3 compared to G1. A highly- significant rise in G3 compared to G2.

Analysis of the Receiver's Operating Characteristics (ROC)

1. (ROC) for GTPase:

The Receiver Operating Characteristic (ROC) curve analysis of GTPase to three distinct groups reveals an impressive area under the curve (AUC) of 0.90, which is significant at 95%, with a P-value of 0.0027, less than 0.01t threshold. The sensitivity and specificity were 88% and 94%, respectively, as shown in Table (3) and Figure (1). Results demonstrated high accuracy levels in discrimination between the three groups and efficiency of the GTPase test among the three groups.

2. (ROC) for ATPase

ROC curve analysis of ATPase illustrated a 0.91 AUC which is a significant amount in 94% levels with a 0.0023 P-value, less than the threshold of 0.001. The ATPase optimal cut–off value was 4.48. Sensitivity and specificity were 91% and 94%, respectively. Data display a high accuracy among the three groups and efficacy, as shown in Table (5) and Figure (2).

Table (1): ANOVA test of ALP, ALT, and AST for G1, G2, and G3.

Group		Mean	Std. Deviation	Std. Error	Sig.
ALP(U/L)	G_1	103.62	9.92	3.14	HS
	G_2	113.09	8.49	1.90	
	G_3	130.81	10.34	2.31	
ALT(U/L)	G_1	29.21	2.03	0.64	HS
	G_2	31.32	2.35	0.52	
	G_3	43.21	8.84	1.98	
AST(U/L)	G_1	24.15	4.19	1.32	HS
	G_2	29.48	3.18	0.71	
	G_3	39.31	6.75	1.51	

HS: Highly significant correlation between parameters (P-value ≤ 0.01)

Table (2): ANOVA test of GTPase and ATPase for G1, G2, and G3

Group		Mean	Std. Deviation	Std. Error	Maximum
GTPase(ng/ml)	G_1	17.72	0.46	0.14	HS
	G_2	24.00	3.53	0.79	
	G_3	27.27	0.65	0.15	
ATPase(ng/ml)	G_1	3.01	0.09	0.03	HS
	G_2	4.98	0.71	0.16	
	G_3	7.31	1.80	0.40	

Table (3): Difference between sensitivity and specificity of GTPase

Variable	Sensitivity	Specificity	The area under the curve	Accuracy		Cut off value
				L.B.	U.B.	
GTPase	0.88	0.94	0.90	0.83	0.97	26.20

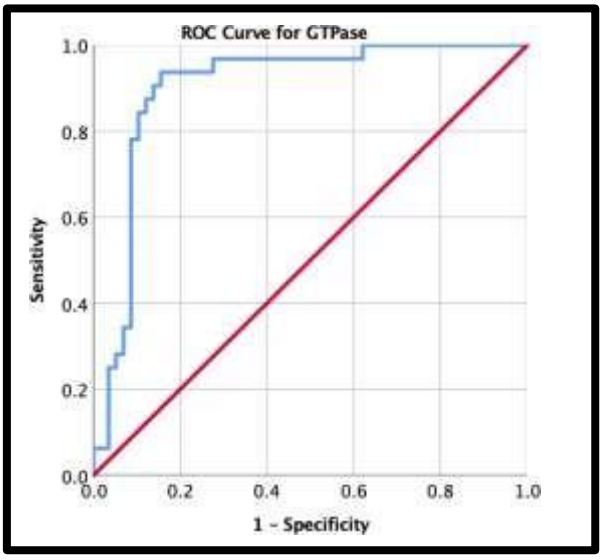

Figure (1) curve of the GTPase.

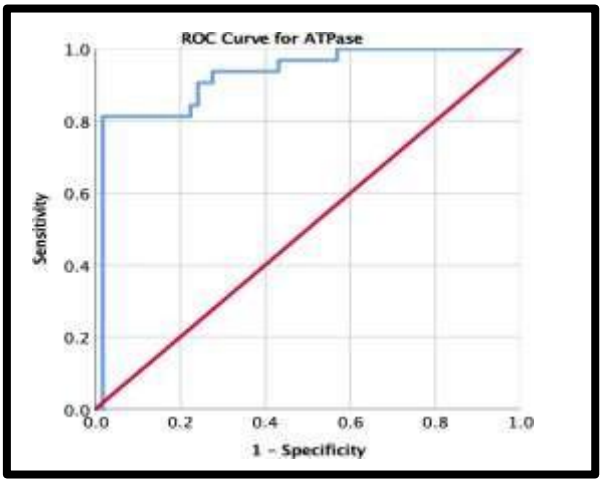

Figure (2): ROC curve of ATPase.

Discussion

There is a possibility that COVID-19 might result in several adverse effects, including lung fibrosis, pneumonia, respiratory failure, and syndrome of cytokine release (13,14). An infection caused by COVID-19 cannot be treated with any of the drugs that are presently on the market and are effective. The data will likely make it simpler to carry out clinical experiments on COVID-19 (15).

The results of analytical investigations indicated that the COVID-19 vaccination has a substantial influence on hearing thresholds for a variety of frequencies that are distributed and distinct from one another (irregular distribution). The research proposed using a larger study sample size, a novel. Design, or researching a more extended age range to accomplish the goal of validating the results (16,17). Recent research demonstrated that a strong intelligence swarm algorithm is important in addition to chest X-ray classifiers in differentiating COVID-19 patients from conventional chest X-ray pictures (18).

Another recent research concluded that mRNA sequences that were involved in the study have a length of 107 bases, and the deterioration rates scored in the first 68 bases of the sequence. The actual COVID-19 mRNA vaccine should be longer, which displays that additional study should be conducted to investigate the predicting longer sequences of algorithms reliability (19).

ALP, ALT, and AST signs of liver damage, which are used as biomarkers in sepsis, viral pneumonia, and obstructive pulmonary diseases, have been reported to increase in patients with COVID-19 [20,21]. The results of the current study agreed with the findings of another study, which demonstrated that the levels of AST, ALT, and ALP in the blood of the patients who had recovered were all high compared to the control. Researchers concluded that certain biochemical markers are relevant (22-24). The analysis of GTPase and ATPase levels for G1, G2, and G3 display a highly significant increase in G_2 and G_3 compared to G_1. A highly-significant rise in G_3 compared to G_2. To determine the levels of GTPase and ATPase for G1, G2, and G3, ANOVA test was carried out. When compared to G_1, the

results of the GTPase and ATPase tests suggested that there was a considerable increase in the levels of G2 and G3 in comparison to G1. Furthermore, in contrast to G2, there has been a very significant increase in G3, which is a trend that should be seen as beneficial. An examination of the lung cDNA library led to the discovery that the isoform subunit of V-ATPase G1 has direct contact with the 3CLpro that is generated by the SARS coronaviruses. In addition, it was found that the G1 subunit has a 3CLpro cleavage site, and it was shown that the viral protease can cleave cell culture experiments. A drop in the intracellular pH was shown to be connected with cleavage of the G_1 subunit in cells that expressed 3CLpro.

A key component of mammalian V-ATPase, Ac45, is capable of interacting with a component of the SARS CoV-2 viral replicas/transcriptase complex. It seems that V-ATPase could have a role in the viral transmission process (25,26).

The results of a study that investigated single-cell RNA sequencing (bronchoalveolar lavage fluid), bulk-RNA sequencing, and proteomics revealed that the expressions of V-ATPase were found to be enhanced in SARS-CoV-2 infection. These results revealed that S protein boosted V-ATPase in COVID-19 infection resulting in the generation of a microenvironment that was more conducive to cleavage of S protein. Activation of inflammatory cells was likely to occur as a result of calprotectin enhancement in respiratory epithelium (27,28).

As time goes on, it is becoming more apparent that several viruses can form a broad range of connections with Rho GTPase signal lings and manipulate these interactions to have its aid. Rho GTPases, in particular, have a role in the pre-entry process and endocytosis when it comes to the infection that is induced by the COVID-19 virus. (29-31). Rho GTPases are the most potent signaling cells' molecules. They are present in eukaryotic creatures and govern cell polarity via their effect on the cytoskeleton, trafficking of membranes, and adhesion cells (32). It was discovered that small GTPase has the potential to be used as an adjuvant target in the process of developing a vaccine against CoV. Within the context of the hunt for new adjuvants for CoV vaccines, this might prove to be of aid (33). It was possible to examine the response to infection in nasal swabs obtained in persons who were positive for SARS-CoV-2 and those who were negative for the virus. This was accomplished by evaluating the expression of genes in the respective host cell retarget role with Rho GTPases (34).

Conclusions:

Many COVID-19 vaccinations were used in the protection from this virus. The current study showed that lung fibrosis, a side effect appears in many people diagnosed by CT scan and some laboratory parameters such as ALP, ALT, and AST serum levels. Moreover, elevated ATPase and GTPase levels in recipients of the coronavirus vaccine showed a relationship between these markers and the vaccination in these individuals. The Receiver Operating Characteristic (ROC) study for ATPase and GTPase revealed that these parameters could act as efficient tests across patient groups.

Authors' declaration:

We hereby confirm that all Figures and Tables in this manuscript are ours. Besides, the figures and images, which are not ours, have been permitted re-publication and attached to the manuscript.

Ethical Clearance**:** The institutional Scientific Committee at the Diyala Health Department approved this study according to the Declaration of Helsinki of Human Studies (Consent number: 54 on 5 March 2023).

Conflict of interest: None

Funding: None

Author Contributions:

Study conception & design: (Raed Mahmoud Al-Azawee). Literature search: (Raed Mahmoud Al-Azawee & Zeinab M. Al-Rubaei). Data acquisition: (Raed Mahmoud Al-Azawee & Zeinab M. Al-Rubaei). Data analysis & interpretation: (Raed Mahmoud Al-Azawee & Zeinab M. Al-Rubaei). Manuscript preparation: (Raed Mahmoud Al-Azawee & Zeinab M. Al-Rubaei). Manuscript editing & review: (Raed Mahmoud Al-Azawee & Zeinab M. Al-Rubaei).

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دراسة مستويات ATPase و GTPase في مرض التليف الرئوي للملقحين وغير الملقحين لفايروس كورونا

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الخلفية : في الخلايا حقيقية النواة، تقع مسؤولية تحمض الأجزاء داخل الخلايا على عاتق عائلة من مضخات البروتون المعروفة باسم-H+ATPases الفراغية، والمعروفة أحيانا باسم V-ATPases. إن GTPases الصغيرة عبارة عن جزيئات إشارة تنظم العمليات الخلوية المهمة بالإضافة إلى الأنشطة دون الخلوية، مما يجعلها تلعب دور اساسي، خاصة في مجموعة واسعة من عمليات الإصابة بفيروس كورونا .

هدف الدراسة: كان الغرض من هذا البحث هو تقييم مستويات ATPase وGTPase لدى مرضى مرض الرئة الليفي (FLD) الذين تلقوا أو لم يتلقوا تطعيم COVID-19، ومن ثم مقارنة هذه المستويات مع تلك الموجودة في المجموعة الضابطة .

المرضى وطرق العمل : شارك في هذه الدراسة 150 شخصا تم تقسيمهم إلى ثلاث مجموعات، المجموعة الأولى (G_1)، كانت بمثابة مجموعة ضابطة وتضمنت (50) شخصا. المجموعة الثانية (G_2) تكونت من (50) مريضا بمرض التليف الرئوي غير الحاصلين على لقاح كوفيد-19 . المجموعة الثالثة (G_3) تكونت من (50) مريض بالتليف الرئوي مع لقاح (فايزر) كوفيد-19. كانت الايلايزا هي الطريقة التي تم استخدامها لتحديد كميات ATPase وGTPase.

النتائج: أظهر تحليل البيانات وجود ارتفاع معنوي في إنزيم الفوسفاتيز القلوي، الأنين أمينوترانسفيراز، الأسبارتات-أمينوترانسفيراز بين المجموعات الثلاث، كما أظهرت النتائج أن مستويات ATPase وGTPase قد زادت بشكل ملحوظ في المجموعتين 3 و2 مقارنة بمستويات المجموعة 1. كذلك، تم اكتشاف ارتفاع كبير في المجموعة الثالثة مقارنة بالمجموعة الثانية.

الاستنتاج: ارتفعت مستويات ATPase و GTPase لدى مرضى تليف الرئة بغض النظر عن حالة التطعيم ضد فايروس كورونا باستخدام لقاح (فايزر) المستخدم في العراق .

الكلمات المفتاحية : ATPase, GTPase , , , , , لقاح (فايزر) كوفيد-19, تليف الرئوي .

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Research Article

Study of Adhesion Molecules in Type 2 Diabetes Mellitus Iraqi Patients with Dyslipidemia

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Abstract:

Background: Cell adhesion molecules are protein entities that are located on the cell surface. The vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) expression is related to type 2 diabetes mellitus (T2DM) with dyslipidemia.

Objectives: To determine the levels of VCAM-1 and ICAM-1 in T2DM patients with dyslipidemia and to explore the relationship between VCAM-1 and ICAM-1 and the development of dyslipidemia in T2DM patients.

Methods: The study included 150 individuals with an age range of (35-55) years. Patients with diabetes for more than 5 years were excluded. Fifty healthy individuals constituted Group 1 (G1), fifty patients with T2DM constituted Group 2 (G2), and fifty T2DM patients with dyslipidemia constituted Group 3 (G3). Whole blood samples were drawn to measure HbA1c based on fluorescence immunoassay technology. The serum was separated to measure fasting blood glucose (FBG), triglycerides (TG), total cholesterol (TC), and high-density lipoproteins (HDL) by manual methods, while VCAM-1, and ICAM-1 were determined using the ELISA test. The study was conducted between November 2022 and April 2023 at the National Center for Diabetes Treatment and Research, Baghdad, Iraq.

Results: Significantly higher levels of FSG and HbA1c were detected in G2 and G3 compared to G1, but non-significantly so when G3 was compared to G2. Significant higher levels of TG and TC levels were detected for G3 when compared to G1 and G2, but non-significantly so when G2 was compared to G1. HDL levels were significantly lower in G3 compared to G2 and G1, but non-significantly so when G2 was compared to G1. VCAM-1, and ICAM-1 were significantly higher in G2 compared to G1, and VCAM-1 level was significantly higher in G3 compared to G2. Non-significant differences in ICAM-1 levels were found between G3 and G2.

Conclusion: VCAM-1 and ICAM-1 are potentially significant factors in the development of dyslipidemia in diabetes patients. They might serve as biomarkers to accurately predict the progression of cardiovascular disease.

Keywords: Diabetes mellitus; Dyslipidemia; VCAM-1; ICAM-1.

Introduction:

The characteristic of diabetic dyslipidemia is hyperglycemia with lipoprotein abnormalities. Over 70% of those who have T2DM are affected by dyslipidemia making it a fairly common condition. Diabetes confers a greatly increased risk of cardiovascular disease (1,2). The VCAM-1 is a protein that also contains T-cell receptors and antibodies (3). Studies demonstrate relationships among the sVCAM-1 and sICAM in patients of T2DM with cardiovascular disease (CVD) and revealed that abnormalities in endothelium cause a rise in these markers (4,5). The ICAM-1 is a glycoprotein that is found in microglial cells, astrocytes, central nervous system in addition to endothelial cells (white and grey human forebrain) (6,7). Because of dyslipidemia, cholesterol builds up and becomes oxidized, which speeds up the activation of ICAM-1 for monocyte adhesion. This causes a rise in the number of monocytes and production of cytokines (8). Hyperglycemia and dyslipidemia that last for an extended time both contribute to oxidative stress rise, which increases the production of oxidized low-density lipoprotein, stimulates immunological cells, and increases the levels of VCAM-1 and ICAM-1, which contribute to the creation of foam cells (9). Many researches demonstrated that VCAM- 1 and ICAM-1 are involved in the development of microvascular issues (10,11). The purpose of this study was to determine the levels of VCAM-1 and ICAM-1 in T2DM with and without dyslipidemia, the findings of which may be helpful in the follow-up care of diabetic patients with dyslipidemia and in preventing the development of CVD.

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Patients and Methods:

One hundred and fifty participants were included in the study with an age range of (35-55) years. They were divided into three groups: Group 1 (G1) consisting of 50 healthy individuals (control group), Group 2 (G2) consisting of 50 T2DM patients with no

dyslipidemia, and Group3 (G3) consisting of 50 T2DM patients with dyslipidemia. Venous blood samples (5 milliliters) were collected from each participant in the three groups from November 2022 to April 2023 at the National Center for Diabetes Treatment and Research, Baghdad, Iraq. The blood samples were used to measure HbA1C by the fluorescence immunoassay technology and the levels of VCAM-1 and ICAM-1 by the ELISA test (Mybiosource / USA). The concentrations of serum FBG, TG, TC, and HDL were determined by manual procedures. The Statistical Packages of the Social Sciences, version 21 (SPSS-21) was used for statistical analysis, with $P < 0.05$ accepted as significant. Quantitative variables were correlated using Pearson's correlation. The t-test was used to test the difference between two independent means. The evaluations of the Receiver Operating Characteristic (ROC) curves were investigated for the three study groups for VCAM-1 also ICAM-1.

Results:

The results of the FSG, HbA1c, TG, also HDL tests are shown in table (1). There was a significant difference between the means of FBG and HbA1c in G2 and G3 compared to G1. No such difference was found between G3 and G2. The table also shows that the mean TG level in G3 (237.9 ng/mL) was significantly higher than in G1 (93.5 ng/mL) and G2 (101.5 ng/mL). The means of G2 and G1 were not significantly different. The mean TC levels in G1 (156.1) and G2 (157.3 ng/mL) were significantly higher than in G3 (216.0 ng/mL). However, the difference between G2 and G1 was not significant. compared to G2 and G1, the mean HDL level in G3 was significantly lower. The mean levels of G2 and G1 were not significantly different.

Table 1: Mean levels of FBG, HbA1c, TG, TC, and HDL in the three study groups

Parameter	Mean ± SD			p-value		
	G1 _ n (50)	G2 _ n (50)	G3 _ n (50)	G1&G2	G1&G3	G2&G3
FBG (mg/dL)	89.0±8.82	184.5±8.41	211.5±8.29	S	S	NS
HbA1c%	4.9±0.43	8.5±1.95	8.8±1.79	S	S	NS
TG (mg/dL)	93.5±15.77	101.5±33.89	237.9±51.88	NS	S	S
TC (mg/dL)	156.1±26.3	157.3±33.14	216.0±35.54	NS	S	S
HDL (mg/dL)	51.5±7.41	46.2±10.90	31.2±4.45	NS	S	S

Table (2) shows the mean levels of VCAM-1 and ICAM-1 for the three groups. As for VCAM-1, G3 and G2 had significantly higher levels than G1. The mean for G3 is higher than G2. The mean levels of ICAM-1 for G2 and G3 were higher than for G1, but they were not significantly different for G2 and G3.

Table 2: The mean levels of VCAM-1 and ICAM-1 in the three study groups

Parameter	Mean±SD			p-value		
	G1 . n=(50)	G2 . n=(50)	G3 . n=(50)	G1&G2	G1&G3	G2&G3
VCAM-1 ng/mL	1014.3±136.5	2326.1±720.52	2888.2±722.15	S	S	S
ICAM-1 ng/mL	1.7±0.12	2.8±0.53	2.6±0.39	S	S	NS

Correlation of VCAM-1 and ICAM-1

Table (3) and figure (1) show a highly significant positive correlation between VCAM-1 and ICAM-1 in G1 (r = 0.531) and G2 (r = 0.381). A negative non-significant correlation was found in G3 (r = -0.131).

Table 3: Correlation coefficients between VCAM-1 and ICAM-1 in the three study groups

Parameters	VCAM-1		
ICAM-1	G1 r = 0.531 (HS)	G2 r = 0.381 (S)	G3 r = -0.131 (NS)

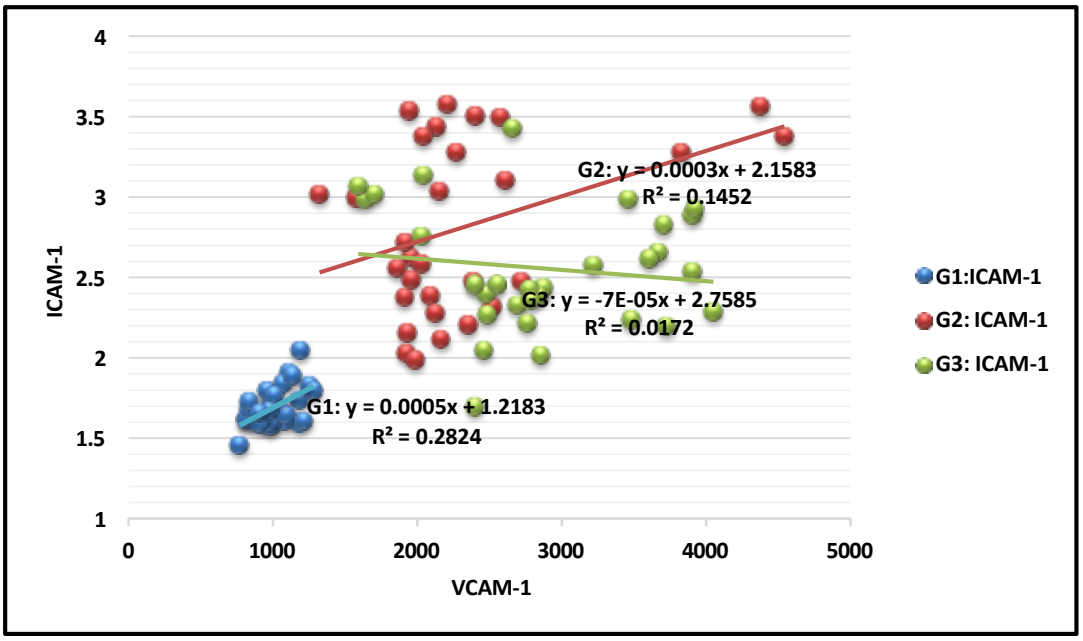

Figure (1): Correlations between VCAM-1 and ICAM-1 in the three study groups

Receiver Operating Characteristic (ROC): For VCAM-1: ROC curve analysis of VCAM-1 in the three study groups reveals an impressive area under the curve (AUC) of 0.87, which is significant at 95% with a p-value of 0.0081, that is less than the 0.01 threshold. The VCAM-1 optimal cut-off value is 1926. The sensitivity and specificity were 91% and 86%, respectively, as shown in table (4) and figure (2). The results demonstrate high accuracy levels in discriminating between the three groups, and the efficacy of the VCAM-1 test among three groups.

1- For ICAM-1: ROC curve analysis shows a value of 0.76 AUC that is significant at 95% with a 0.007 p-value, which is less than the threshold of 0.01. The ICAM-1 optimal cut–off value was 2.23. The sensitivity and specificity were 90% and 73%, respectively. This shows a high accuracy in recognition between the three groups and the test efficacy among the groups, table (4) and figure (3).

Table 4: sensitivity and specificity for VCAM-1

Variables	Statistical Values					Cut off value
	Sensitivity	Specificity	Area under the curve	Accuracy L.B.	U.B.	
VCAM-1	0.91	0.86	0.87	0.79	0.95	1926

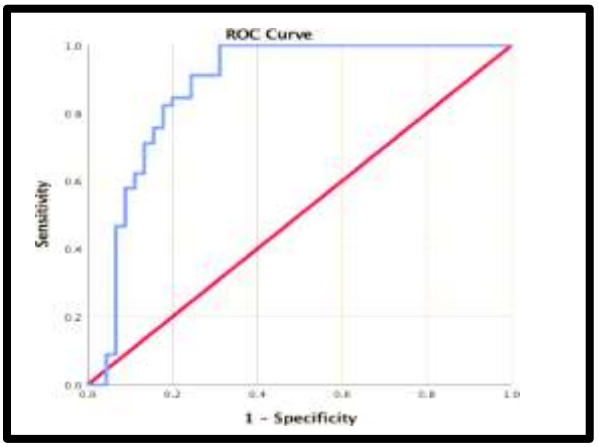

Figure (3): ROC for ICAM-1

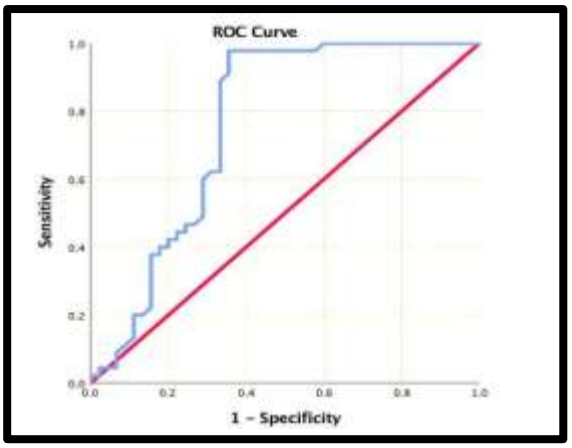

Figure (2): ROC for VCAM-1

Discussion:

The findings of the current study, reveal that diabetic patients had greater levels of FBG and HbA1c than the control which is supported by a number of other investigations (12–14). This may indicate that those diabetics were not well-controlled.

Several studies have indicated that diabetics have greater rates of dyslipidemia, a condition that is related to hyperglycemia, increased HbA1c in blood, and hypercholesterolemia (15). This condition affects the ability of the body to process fats. Diabetics have abnormal lipid profiles and high HbA1c levels (16, 17). The abnormalities of lipids include low HDL and high TG and TC (18, 19). The findings of the current study agree with previous studies which showed that dyslipidemia is more common in T2DM cases (20–22). Dyslipidemia contributes to CVD development due to the persistent buildup of lipid plaques on arterial walls (23,24).

The findings on the effect of HDL on the upregulation of VCAM-1 messenger RNA inside human umbilical vascular endothelial cells were recently made public with participants representative of the general population (25).

Adhesion molecules presence in diabetics without micro- or macro-vascular complications indicates that the endothelium is functioning, but is bound to release endothelial products, that are linked to microangiopathy development (26,27).

The levels of ICAM-1, VCAM-1, and HbA1c were shown to have a significant correlation in a recent study that measured glycemic control. Since prolonged uncontrolled hyperglycemia makes glycemic control more difficult, the levels of these molecules are greater when there is poor glycemic control (28). According to the findings of other studies, the levels of VCAM-1 in the isolated endothelial cells of diabetes patients are much greater than those of ICAM-1. This result is consistent with the concept that diabetics who have the consequences of their illness, such as macrovascular and diabetic renal disease, should have greater levels of VCAM-1 (29,30).

A recent study found that persons who had greater ICAM-1 and VCAM-1 showed higher TGs and lower HDL levels. Despite the importance of these molecules in the progression of atherosclerosis in diabetic and cardiovascular patients, studies indicated that the higher the levels of VCAM-1 and ICAM-1 the higher the risk of T2DM in individuals (31,32).

Conclusions:

VCAM-1 and ICAM-1 are potentially significant factors in the development of dyslipidemia in diabetes patients. They might serve as biomarkers to accurately predict the progression of cardiovascular disease.

Authors' declaration:

We hereby confirm that all Figures and Tables in this manuscript are ours. Besides, the figures and images, which are not ours, have been given permission for re-publication and attached to the manuscript.

Ethical Clearance: The institutional Scientific Committee at the National Diabetes Center/ Mustansiriyah University approved this study according to the Declaration of Helsinki for human studies (Consent number: 1501 on 14/11/2022).

Conflicts of Interest: None

Funding: None

Author Contributions:

Study conception & design: (*Abbas M. Alsaedy & Zeinab M. Al-Rubaei*). Literature search: (*Abbas M. Alsaedy & Zeinab M. Al-Rubaei*). Data acquisition: (*Abbas M. Alsaedy& Zeinab M. Al-Rubaei*). Data analysis & interpretation: (*Abbas M. Alsaedy & Zeinab M. Al-Rubaei*). Manuscript preparation: (*Abbas M. Alsaedy & Zeinab M. Al-Rubaei)*. Manuscript editing & review: (*Abbas M. Alsaedy& Zeinab M. Al-Rubaei*).

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دراسة جزيئات الإلتصاق في المرضى العراقيين المصابين بداء السكري من النوع الثاني وإضطراب شحميات الدم

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الخلاصة:

الخلفية: جزيئات إلتصاق الخلايا هي كيانات بروتينية موجودة على سطح الخلايا. لقد ثبت أن التعبير عن جزيء التصاق الخلايا الوعائية -1 (VCAM-1) وكذلك جزيء الإلتصاق بين الخلايا -1 (ICAM-1) مرتبط بإضطراب شحميات الدم وكذلك داء السكري من النوع الثاني. **الأهداف:** تحديد مستويات VCAM-1 و ICAM-1 لدى مرضى اضطراب شحميات الدم السكري ومعرفة ما إذا كانت هناك علاقة بين مستويات VCAM-1 و ICAM-1 مع تطور اضطراب شحميات الدم لدى المصابين بداء السكري من النوع الثاني.

المرضى والمنهجية: شملت الدراسة 150 شخصا: خمسين من الأصحاء (المجموعة الأولى - G1)، خمسين مريضا بداء السكري من النوع الثاني (المجموعة الثانية - G2)، وخمسين مريضا بداء السكري من النوع الثاني مع إضطراب شحميات الدم (المجموعة الثالثة - G3). تم سحب الدم الكامل لغرض قياس نسبة HbA1c، وفصل المصل لغرض قياس سكر البلازما الصائم، الدهون الثلاثية، الكولسترول الكلي، البروتين الدهني عالي الكثافة بالطرق اليدوية، بينما تم قياس VCAM-1، وICAM-1 عن طريق اختبار ELISA. أجريت الدراسة في الفترة ما بين نوفمبر 2022 وأبريل 2023 في المركز الوطني لعلاج وأبحاث السكري، بغداد، العراق.

النتائج: أظهرت النتائج إرتفاعا معنويا في مستويات سكر البلازما الصائم وHbA1c في G2 وG3 مقارنة بـ G1، مع زيادة غير معنوية في G3 مقارنة بـ G2. أظهرت نتائج الدراسة الحالية إرتفاعا معنويا في مستويات الدهون الثلاثية والكولسترول الكلي في G3 مقارنة مع G1 وG2، في حين لوحظت زيادة غير معنوية في G2 مقارنة مع G1. أظهرت مستويات البروتين الدهني عالي الكثافة إنخفاضا معنويا في G3 مقارنة مع G2 وG1، بينما لم تظهر فروق معنوية في G2 مقارنة مع G1. وجدت زيادة مستويات VCAM-1 وICAM-1 بشكل ملحوظ في G2 مقارنة بـ G1، وزيادة VCAM-1 بشكل ملحوظ في G3 مقارنة بـ G2 في. وجدت فروق غير معنوية في G3 مقارنة مع G2 في مستويات ICAM-1.

الإستنتاجات: قد يكون هناك دور مهم لـVCAM-1 وICAM-1 في مسببات اضطراب شحميات الدم لدى مرضى السكري ويمكن اعتبارهما كمؤشر حيوي للتنبؤ بتطور أمراض القلب والأوعية الدموية لدى هؤلاء المرضى.

الكلمات المفتاحية: داء السكري، إضطراب شحميات الدم، VCAM-1، ICAM-1.

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Research Article

The Burden of Chronic Obstructive Pulmonary Disease COPD in Stable Patients and its Association with Inflammatory Biomarkers and Body Mass Index

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Abstract

Background: Chronic obstructive pulmonary disease (COPD) is a progressive airflow limitation that is preventable but not curable. It is associated with persistent symptoms that cause a considerable burden on individual productivity at work, and daily activities, and reduced quality of life, also burdening the healthcare system and society.

Objectives: The study aims to measure the burden of COPD on patients in terms of daily activities and work productivity. It also seeks to investigate some inflammatory biomarkers' levels and their correlation with selected outcomes.

Methods: A cross-sectional study on 120 stable COPD patients who were diagnosed and treated according to the GOLD guidelines at Kirkuk General Hospital's chest and Internal Medicine consultation clinics, and in private internist clinics in Kirkuk City, Iraq between January and April 2023. The Work Productivity and Activity Impairment Questionnaire (WPAI-COPD) measured the Burden of COPD. The symptom burden was assessed by the COPD assessment test (CAT). The level of IL-6 monoclonal antibodies was measured via a Roche Cobas 6000 analyzer and TNF-α using the ELISA sandwich technique. All these devices and machines were authorized by the Ministry of Health, Iraq.

Results: The mean age was (54.1 ± 8.12) years, 62.5% being males and 37.5% being females; 41.7% were public sector employees, and 39.2% were self-employed. Due to COPD, the overall work loss was (45.9±18.91) days, the mean percentage of absenteeism was (10.9% ± 12.31%), activity impairment was (47.5% ±17.79%) among all patients, presenteeism was (40.8 ± 15.05%,) and COPD-related retirees were 14.2% of the study group. The mean CAT score (19.3±5.63) was high and the most troubling symptom among patients was chest tightness. WPAI-COPD scores were higher in older age and longer disease duration. The levels of biomarkers were above the reference ranges, the mean IL-6 level was (69.4 ± 35.29) pg/ml, and the mean TNF-α was (72.3 ± 22.45) pg/ ml.

Conclusions: COPD patients exhibit a disease burden in terms of productivity loss at work and activity impairment that increases with aging and disease duration. COPD patients with low BMI are more prone to a decline in lung functions and to worse symptoms.

Keywords: Disease burden; COPD, productivity; tumor necrosis factor-α; interleukin- 6.

Introduction:

Chronic Obstructive Pulmonary Disease COPD is a progressive respiratory condition that imposes a significant burden on patients. It is characterized by chronic productive / non-productive coughing, wheezing, and dyspnea which is the hallmark sign of COPD and a significant contributor to the disease's impairment and anxiety (1). It is expected that by 2030, COPD will be the third most common cause of mortality globally, accounting for 3 million deaths yearly, and over 10% of people worldwide will suffer from COPD. (2,3). Increased rates of smoking, indoor and outdoor air pollution, and other exposures, together with aging, increase the burden and prevalence of COPD (4).

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Cytokines and chemotactic factors that compromise the immune system are secreted excessively due to infections, smoking, air pollution, and other reasons. Previous studies showed that the inflammatory damage caused by inflammatory reactions is the main contributor to the progression of COPD (5).

In Iraq, two recent studies have paid more attention to asthma and its management, assessing asthmatic patients' response to different medication regimens (6,7). Another study compared treatment regimens and their influence on the quality of life of pediatric asthmatics (8), and another one assessed the effects of pharmacist counseling for asthmatic children to ensure correct inhaler use (9). Another study addressed problems associated with inhaler shortages and adherence to treatment guidelines and the assessment of Iraqi doctors' compliance with

treatment guidelines (10). Not much is known about the burden and the immunological complications caused by COPD in the Iraqi population. A prior study examined this issue and found that the quality of life is significantly deteriorated as a result of COPD (11). Further research examined type 2 diabetes mellitus (T2DM) prevalence in patients with severe COPD and showed that they are more likely to have T2DM (12). Another study addressed the prevalence of COPD among smokers over 40 years of age (13). Employees with COPD have an approximately five-fold increase in work productivity loss and a three-fold increase in activity impairment compared to those without COPD (14). It is important to examine the influence of COPD on patients' everyday activities, and work environment, which is not currently done in Iraq.

The goal of this study is to describe COPD burden among stable cases in terms of everyday activity and impact on work productivity, with a focus on exploring the relationship between symptoms burden and work productivity with essential biomarkers involved in COPD inflammatory response.

Patients and Methods

This is a cross-sectional study on 120 COPD-stable patients who attended the Department of Respiratory Diseases and Internal Medicine consultation clinic at Kirkuk General Hospital, Kirkuk City between January and April 2023. Inclusion criteria were patients > 18 years of age, already diagnosed by the pulmonologist since at least one year according to the GOLD criteria (a post-bronchodilator FEV1/FVC ratio <0.7), and clinically stable. Patients who refused to perform a pulmonary function test (PFT) or had a disease exacerbation that needed systemic corticosteroid, antibiotic treatment, or an emergency department visit in the previous three months and had any medical condition that impacted quality of life were excluded. Verbal consent was obtained from all patients.

Data Collection Tool: A questionnaire form was used to collect clinical and demographic variables (age, gender, work status, level of education, employment status, disease duration, smoking index, and comorbidities).

Pulmonary function test (PFT): The procedure is done using spirometry under a skilled technician's supervision. After 15 minutes of bronchodilator inhalation (short-acting agonist, 4×100 µg salbutamol) via a spacer device, a post-bronchodilator test was carried out. A test with forced expiratory volume in the first second / forced vital capacity (FEV1/FVC) ratio < 70%) confirmed the diagnosis of COPD (1).

Body mass index (BMI): Is measured by dividing weight by height square (kg/m²) "and is categorized as: Underweight (<18.5), normal (18.5–24.9), overweight (25.0–29.9), obese (≥30)".

Biomarkers level: Blood interleukin-6 (IL-6) is determined using Roche Cobas 6000 analyzer, elecsys IL-6 kit (ref. code 05109442190) and tumor necrosis factor alpha (TNF-α) is determined using ELISA sandwich technique, and TNF-α Camilo biological kit.

COPD assessment test: disease symptoms were assessed using the Arabic version of the (CAT) questionnaire, a short validated, and easy-to-use self-administered questionnaire to evaluate the impact of COPD symptoms on the patient's life. It includes eight items (cough, sputum production, dyspnea, chest tightness, self-confidence leaving home, activity, sleep, and energy level). Each item has a score of 5, with total ratings ranging from 0 to 40. The CAT questionnaire score is presented as a percentage (0 - 100%). Higher scores reflect a greater impact of illness on the health status and a greater symptom burden (15).

Work Productivity and Activity Impairment Questionnaire (WPAI): The impact of COPD on work productivity was assessed by the Arabic version of the (WPAI-COPD) Questionnaire which is a reliable tool to estimate the burden of COPD in terms of work and activity impairments in the previous seven days prior to the study. The WPAI generates four scores presented as percentages: Activity impairment, presenteeism (impact of illness on work performance), overall work productivity loss (combined impact of absenteeism and presenteeism), and absenteeism (hours missing from work due to COPD), with larger values reflecting greater impairment and poorer productivity (16).

Statistical analyses:

SPSS v 25 was used for data analysis. Descriptive statistics were conducted for demographic and clinical characteristics of the disease. Continuous variables were expressed as (means ± standard deviation SD), whereas categorical variables were expressed as percentages and frequencies. To determine the correlations between the continuous variables, the Pearson correlation was utilized. A P-value of less than 0.05 was considered statistically significant.

Results:

Demographic characteristics of the participating patients

The mean age of the patients was (54.1 + 8.12) years. Males constituted 62.5% of the cases. The majority (86.7%) were married, and 47.5% had a college degree or above. Three-quarters were urban residents. Most of the respondents were employed with (41.7%) working for the government, (39.2%) with private employment, and 14.2% were COPD-related retirees. Former smokers constituted (56.7%) and (20%) were current smokers, table 1.

Table 1: Demographic characteristics of the participating patients

Characteristics	Subcategories	Frequency	Percent
Gender	Male	75	62.5
	Female	45	37.5
Marital status	Married	104	86.7
	Unmarried	16	13.3
Education	Illiterate	16	13.3
	Primary school	15	12.5
	Secondary school	32	26.7
	College or higher	57	47.5
Residence	Urban	91	75.8
	Rural	29	24.2
Employment status	Governmental job	50	41.7
	Private job	47	39.2
	Housewife	5	4.2
	Retired due to COPD	17	14.2
	Unemployed	1	.8
Smoking status	Current smoker	24	20.0
	Ex-smoker	68	56.7
	Never smoked	28	23.3

Clinical parameters of the participating patients: The mean age of the patients was (54.1 ±8.12) years. The mean COPD duration was (7.5 ±3.71) years, and the duration of treatment was (4.8 ±3.0) years. The mean BMI was (24.1 ± 3.97 kg/m²), with a range of (16.3–30.5 kg/m²). The lung function tests (FEV1 and FEV1/FVC ratio) of the participating patients were below the normal range. Biomarker levels were elevated: The mean IL-6 level was (69.4 ±35.29) and the mean TNF-α level was (72.3±22.45). The mean of work time loss (absenteeism) due to COPD was 10.9% ± 12.31%, and patients estimated presenteeism (productivity loss at work) was 40.8% ± 15.05%, for an estimated 45.9% ± 18.91% overall work impairment.

Activity impairment is seen in (47.5% ±17.79%) of stable COPD patients, table 2.

Table 2: Clinical parameters of the participating patients

Variable	N	Minimum	Maximum	Mean	SD
Age (year)	120	31	68	54.1	8.12
BMI (kg/m²)	120	16.3	30.5	24.1	3.97
Pack-years smoking	96	24	50	39.4	4.74
Disease duration (years)	120	2	15	7.5	3.71
Treatment duration (months)	120	2	24	4.8	3.00
IL-6 (pg/ml)	120	5.60	142.40	69.4	35.29
TNF-alpha (pg/ml)	120	32.80	128.40	72.3	22.45
FEV1	120	38.80	82.20	60.4	11.06
FEV1 /FVC	120	40.20	69.00	62.9	5.42
CAT score	120	8.00	32.00	19.3	5.63
Work time (hours)	97	40.00	70.00	47.0	6.97
Actual work time (hours)	97	27	60	41.9	8.29
Absenteeism (% per week)	97	0.00	41.00	10.9	12.31
Presenteeism (% per week)	97	10.00	60.00	40.8	15.05
Activity impairment	120	10.00	80.00	47.5	17.79
Overall work loss score	97	10.00	76.00	45.9	18.91

Assessment Test (CAT): COPD

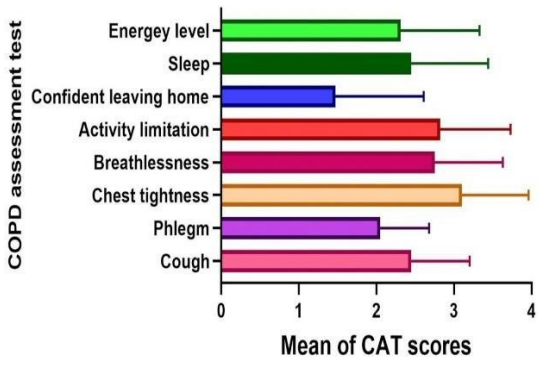

Figure 1: Means of the eight items of the COPD Assessment Test

The mean (CAT) score was (19.3 ±5.63) (Table 2). On average, the majority of patients felt tight chests and were limited in their ability to walk upstairs and do activities at home. On the other hand, the majority felt confident leaving home despite their lung condition (Figure 1).

The correlations between patient characteristics and WPAI-COPD outcomes: Patient age and disease duration had significant positive correlations, while BMI had significant negative correlations with absenteeism, presenteeism, activity impairment, and overall work loss. In other words, old age and longer COPD disease duration are associated with absenteeism, presenteeism, activity impairment, and overall loss job. In contrast, lower BMI is associated with greater loss of function, table 3.

Table 3: The correlations between patient characteristics and outcome measures

Patient characteristics		Actual work time	Absenteeism	Presenteeism	Activity impairment	Overall work loss score
Age (year)	Pearson Correlation	-0.148	0.320	0.448	0.421	0.455
	P-value	0.147	0.001*	0.000*	0.000*	0.000*
BMI (kg/m²)	Pearson Correlation	0.203	-0.262	-0.281	-0.399	-0.287
	P-value	0.046*	0.010*	0.005*	0.000*	0.004*
pack-years Smoking	Pearson Correlation	0.096	0.057	0.146	0.223	0.119
	P-value	0.405	0.619	0.202	0.029*	0.301
Disease duration	Pearson Correlation	-0.338	0.473	0.667	0.750	0.638
	P-value	0.001*	0.000*	0.000*	0.000*	0.000*

*Significant at P<0.05 level

The correlations between patient characteristics and the disease biomarkers, lung function tests, and CAT: The BMI also had significant negative correlations with IL-6, TNF-alpha, FEV1, FEV1 /FVC, and CAT scores. Patient age and disease duration had similar significant correlations with the five parameters: two biomarkers, two lung function tests, and a CAT score, table 4.

Table 4: The correlations between patient characteristics and the disease biomarkers, lung function tests, and CAT

Patient/disease characteristics		IL-6	TNF-alpha	FEV1	FEV1 /FVC	CAT score
Age (year)	Pearson Correlation	0.368	0.214	-0.366	-0.258	0.406
	P-value	0.000*	0.019*	0.000*	0.004*	0.000*
BMI (kg/m²)	Pearson Correlation	-0.458	-0.729	0.438	0.465	-0.497
	P-value	0.000*	0.000*	0.000*	0.000*	0.000*
Smoking index	Pearson Correlation	0.225	0.439	-0.204	-0.194	0.163
	P-value	0.028*	0.000*	0.046*	0.058	0.113
Disease duration	Pearson Correlation	0.635	0.387	-0.676	-0.586	0.726
	P-value	0.000*	0.000*	0.000*	0.000*	0.000*
Treatment Duration	Pearson Correlation	-0.159	-0.087	0.109	0.119	-0.221
	P-value	0.083	0.345	0.237	0.196	0.015*

*Significant at P<0.05 level

Discussion:

The findings of this study indicate that even in stable cases, COPD places a significant burden on patients to perform everyday life activities and tasks, resulting in productivity loss at work. Symptom burden (CAT score 19), mainly chest tightness, had a significant impact on health status, restricted the patient's activity and performance of household tasks, and resulted in poor productivity. This not only affects the patients but also disturbs the work environment. Consistent with our result, a study done on mild and moderate airflow obstruction demonstrated that decreased job productivity was more common in COPD patients with a high symptom burden (17). In consistence with the findings of Foo et al (18), our findings showed a significant loss in work productivity of COPD participants who had greater degrees of dyspnea and other symptoms.

The current study showed that work productivity loss, activity impairment, and missed hours at work increased as the age and duration of disease increased, I agreement with a study done in Korea (19). Age-related changes in lung structure and function in COPD patients are accompanied by a reduction in the effectiveness of the pulmonary protective mechanisms against oxidative stress, which enhances the risk of lung infections, all leading to a decline in lung function (20).

Additionally, owing to aging reduced muscle mass causes a decrease in muscular strength, which in turn causes a decline in exercise tolerance (21).

In COPD patients with low BMI, a decline in lung functions (FEV1, FEV1 /FVC) and CAT score was detected, with patients with lower BMI showing greater productivity loss, impairment in activity, and absenteeism. This agrees with other studies that suggested that one of the risk factors for accelerated deterioration in lung function is a low BMI (22). Studies demonstrated that a low BMI is a significant prognostic factor in patients with COPD. BMI reduction in COPD should be carefully monitored, as it is linked to exacerbations and increased mortality (23). COPD is an inflammatory process in which the levels of TNF-α and IL-6 are elevated in stable COPD patients and associated with an increase in age and disease duration. This finding is consistent with previous studies which showed that many inflammatory mediators are released even in stable COPD condition such as IL-6 released from endothelial cells, fibroblasts, and epithelial cells and macrophages releasing TNF-α which can damage

lung structure and worsen lung functions (24,25). The BMI of the participants was shown to be negatively correlated with their TNF-α level, which is consistent with the findings of Webster et al. which showed that TNF-α releases nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) which causes atrophy in the skeletal muscles (26).

Conclusion

COPD patients exhibit a disease burden in terms of productivity loss at work and activity impairment that increases with aging and disease duration. COPD patients with low BMI are more prone to a decline in lung functions and to worse symptoms.

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Authors' declaration:

We hereby confirm that all the Figures and Tables in the manuscript are ours. Besides, the Figures and images, which are not ours, have been given permission for re-publication attached to the manuscript. Ethical Clearance: The project was approved by the local ethical committee in Kirkuk General Hospital, Iraqi Ministry of Health (according to code 952.14- 12-2022). In addition, approval of the ethical board College of Pharmacy, University of Baghdad according to the code number (REAFUBCP7122022).

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Author Contributions:

Study conception & design: (*Ali L. Jasim*). Literature search: (*Samaa D. Ibrahi*). Data acquisition: (*Samaa D. Ibrahi*). Data analysis & interpretation: (*Samaa D. Ibrahi*). Manuscript preparation: (*Samaa D. Ibrahi)*. Manuscript editing & review: (*Ali L. Jasim*).

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عبء مرض الإنسداد الرئوي المزمن لدى المرضى المستقرة حالتهم وارتباطه بالمؤشرات الحيوية الإلتهابية

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الخلاصة:

خلفية البحث: م مرض الانسداد الرئوي المزمن (COPD) هو تقييد تدريجي لتدفق الهواء يمكن الوقاية منه ولكن لا يمكن علاجه. ويرتبط بالأعراض المستمرة التي تسبب عبئًا كبيرًا على إنتاجية الفرد في العمل، والأنشطة اليومية، وانخفاض جودة الحياة، مما يثقل كاهل نظام الرعاية الصحية والمجتمع..

الأهداف: تحديد عبء مرض الإنسداد الرئوي المزمن على المرضى من حيث الأنشطة اليومية وإنتاجية العمل وقياس مستوى بعض المؤشرات الحيوية الإلتهابية ودراسة العلاقات المحتملة بين النتائج المختارة.

المنهجية: تم إجراء دراسة مقطعية على 120 من مرضى مرض الإنسداد الرئوي المزمن المستقرين الذين تم تشخيصهم بالفعل من قبل المتخصصين في عيادات الصدر والباطنية الإستشارية في مستشفى كركوك العام، وكذلك العيادات الباطنية الخاصة في مدينة كركوك، العراق. بدأت الدراسة في شهر كانون الثاني ولغاية شهر نيسان من سنة 2023. وتم قياس عبء مرض الإنسداد الرئوي المزمن من حيث النشاط وضعف الإنتاجية في العمل من خلال إستبيان ضعف إنتاجية العمل والنشاط (WPAI-COPD) وعبء الأعراض الذي تم تقييمه بواسطة إختبار تقييم مرض الإنسداد الرئوي المزمن (CAT). تم قياس مستوى IL-6 باستخدام محلل Roche Cobas 6000 وTNF-α باستخدام تقنية ELISA.Sandwich

النتائج: كان متوسط العمر (54.1 ± 8.12) سنة وأغلبية المرضى من الذكور (62.5%) و37.5% إناث. نسبة المرضى العاملين لدى الحكومة (41.7%)، والمرضى العاملين في القطاع الخاص (39.2%)، اجمالي فقدان العمل (بسبب التغيب وقلة الانتاجية) هي (45.9 ± 18.91). متوسط نسب التغيب عن العمل (10.9٪ ± 12.31٪)، وقلة النشاط خلال العمل هو (47.5٪ ± 17.79٪). متوسط نسبة قلة الانتاجية خلال العمل هي (40.8 ± 15.05٪). إجمالي نسبة المتقاعدين بسبب مرض الإنسداد الرئوي المزمن هي 14.2%. كانت درجة تقييم عبء الأعراض مرتفعة (5.63 ± 19.3)، وكان العارض الأكثر إثارة للقلق بين المرضى هو ضيق الصدر. وكانت درجات الإنتاجية خلال العمل وضعف النشاط أعلى في كبار السن ومع زيادة مدة المرض. إرتفع مستوى المؤشرات الحيوية حتى في الحالات المستقرة، وكان متوسط مستوى IL-6 (69.4 ±35.29) بيكوغرام/مل ومتوسط TNF-α (72.3 ±22.45) بيكوغرام/مل.

الإستنتاجات: يُظهر مرضى مرض الانسداد الرئوي المزمن عبئًا مرضيًا من حيث فقدان الإنتاجية في العمل وضعف النشاط الذي يزداد مع تقدم السن ومدة المرض. مرضى الانسداد الرئوي المزمن الذين يعانون من انخفاض مؤشر كتلة الجسم هم أكثر عرضة لانخفاض وظائف الرئة ولأعراض أسوأ.

الكلمات المفتاحية: عبء المرض، مرض الإنسداد الرئوي المزمن، الإنتاجية، عامل نخر الورم-α، الإنترلوكين-6.

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Research Article

Evaluation of some Biochemical and Hematological Parameters in Patients with Chronic Kidney Disease

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Abstract:

Background: Chronic kidney disease is a condition that results from an indefinite change in the structure and function of the kidneys. A slow, steady progression characterizes it and is irreversible.

Objectives: This study aims to evaluate the findings of certain biochemical and hematological tests in samples from Iraqi CKD patients.

Methods: This study included 90 subjects, where 70 patients with chronic kidney disease and 20 healthy individuals. Blood samples were collected from the patients during their visits to Ghazi Al-Hariri Surgical Specialties' Hospital- Medical City, Baghdad, Iraq. Age, sex, and body mass index were assessed for each participant followed by renal function tests [serum blood urea, creatinine, uric acid and estimated glomerular filtration rate], and complete blood count. Also, serum levels of uromodulin and cystatin C were measured statistically studies were carried out using analysis of variance (ANOVA).

Results: the study demonstrated a highly significant (P<0.001) increase in blood urea, serum creatinine and uric acid levels, while a significant (P<0.05) decrease in estimated glomerular filtration rate levels in patients compared to the control group. On the other hand, it showed a highly significant (P<0.001) decrease in hemoglobin and hematocrit values and a significant (P<0.05) decrease in the red blood cell count. Patients had revealed a significant (P<0.05) increase in cystatin C level and a decrease in uromodulin level when compared to the control group.

conclusion: the present study shows that chronic kidney disease patients have upregulated renal function parameters blood urea, serum creatinine and with downregulated estimated glomerular filtration rate, while hematological disorder was more prevalent in patients. On the other hand, cystatin C level revealed an increase while uromodulin level showed a decrease in Iraqi patients.

Keywords: Chronic Kidney disease; Cystatin C; Hematological; Hemoglobin; Uromodulin.

Introduction:

Chronic kidney disease (CKD) is a disorder that affects multiple systems and organs, the increased rates of cardiovascular morbidity and mortality, as well as bone disease development. The main risk factors for developing renal dysfunction into uremia include arterial hypertension (AH), diabetes mellitus (DM), dyslipidemia, and glomerular or congenital abnormalities (1). The kidney structure or function persists for more than three months abnormal are diagnosed according to the Kidney Disease Improving Global Outcomes (KDIGO) initiative as CKD (2)

Since 1990, the incidence of CKD has risen by about 30%, mainly due to renal replacement therapy (RRT) and the long-term use of dialysis for patients with end-stage kidney disease (ESRD). CKD will develop a serious public health problem due to its prevalence, risk of death, recurrent hospitalizations, and economic burden (3).

Anemia is a communal significance of CKD and its incidence increases as the eGFR falls.

Several mechanisms have been proposed to explain CKD-related anemia, including relative erythropoietin insufficiency, shortened red cell life span, aberrant iron metabolism, chronic inflammation, metabolic abnormalities (3). Blood urea, creatinine, and eGFR are the typical methods for measuring kidney function (4). Cystatin C (Cys C) is a fairly reliable substance that can be examined quickly, correctly, and precisely by an automated analyzer. Furthermore, in CKD and lower renal filtration disorders, cys C levels predict mortality and morbidity more strongly than S. Cr. Levels (5)

Uromodulin (Umod) is separated as a highly glycosylated mucoprotein that inhibits viral hemagglutination, is expressed primarily in the kidney. Almost all uromodulin in the kidney is released from the luminal surface of tubular epithelial cells between the thick ascending limb (TAL) of Henle's loop and the early distal convoluted tubule. Uromodulin is cleaved by proteases and eliminated in the urine(6). It is the most abundant protein in urine, and kidney tubular epithelial cells manufacture and secrete it (7). The decline in Umod levels associated with the stages of CKD recommends reduced cell viability in the TAL segment (8).

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Material and Methods:

Study subjects

Ninety subjects were analyzed in this study, who were divided into two groups. The first group comprised 70 CKD patients, while the second group consisted of 20 healthy individuals who served as the control group. The study was conducted between October 2022 and February 2023, and the study subjects were patients at Ghazi Al-Hariri Surgical Specialties Hospital - Medical City in Baghdad, Iraq. The study design included descriptive data such as (age, sex and BMI), as well as clinical data such as (disease duration and stages of the disease (G2, G3, G4).

Collection of blood samples

Blood samples of five millilitres were collected from CKD patients and the controls through a venous blood draw and the samples were divided into two tubes: the first tube contained ethylene diamin tetraacetic acid (EDTA) for assessing complete blood count (CBC); the second tube contained gel, and was then centrifuged at 4000 rpm for 4 min to collect the serum used in the renal function tests.

Renal function tests

The levels of B. urea and S.Cr. were measured by Siemens's diagnostic equipment to obtain the results for patients and control. The level of UA was measured by spectrophotometry with BioSystems kit. The CKD-EPI 2021 equations, on the other hand, are used to calculate the level of eGFR. It is possible to program the eGFR was creatinine equation for age's ≥18 years in a single sentence for eGFRcr:

eGFRcr = 142 × min (Scr/ κ, 1(^{a} ×max (Scr /κ, 1) $^{1.20}$ ×0.9938^{Age}× 1.012 [if female]

Where κ = 0.7 (females) or 0.9 (males).

a = -0.241 (female) or -0.302 (male).

Scr = serum creatinine in mg/dL; divide by 88.4 for creatinine in mmol/L

Age (years) (9).

Hematological tests

The complete blood count (CBC) was measured by a NIHON KOHDEN auto hematological analyzer to measure several hematological parameters namely hemoglobin (Hb) level, hematocrit (HCT), red blood cells (RBC) count, total white blood cells (WBC) count differential WBC (neutrophile, eosinophile, basophile, lymphocyte and monocyte), and platelets (PLT) count.

Cystatin C and Uromodulin levels

Enzyme-linked immunosorbent assay (ELISA) was employed to estimate levels of cys C and Umod using the Cloud-Clone Crop kit from the USA.

Statistical analysis

The data was then analyzed using the Statistical Package for Social Sciences (SPSS; Version 28) (IBM) program. The statistical studies were carried out using analysis of variance (ANOVA). The data were provided as mean standard error (M± S.E.) and a *P*-value of (P<0.05) was considered significant.

Results:

Descriptive data of the study groups

Table (1) present descriptive information about the study subjects. The results presented indicate that there were non-significant (P > 0.05) differences between patients with CKD and control in regards to age, (47.56±1.55 vs 40.65±2.74 years, respectively), and BMI values (31.79±4.21, vs 30.79±1.64 kg/m^2 respectively). The sex comparison was made between the two groups as follows; patients [male (59%) and female (41%)], and control [male (55%) and female (45%)]. The findings revealed that the percentage of the males were significantly higher (p< 0.05) than that of the percentage of female.

Table (1): Descriptive data of the study groups

Groups	Descriptive data (Mean ± SE)			
	Age (year)	BMI (kg/m^2)	Gender No (%)	
			Male	Female
Patients	47.56±1.55	31.79±4.21	41 (59%)	29 (41%)
Control	40.65±2.74	30.79±1.64	11(55%)	9 (45%)
P-value	0.902(NS)	0.036(NS)	0.985(NS)	0.995(NS)
Ns: Non-Significant				

Rena faction tests of study groups

Data presented in figure (1), shows the results of the renal function tests (B.U., S.Cr. UA, and eGFR) of patients with CKD and the control group. A highly significant (P<0.001) increase was found in the levels of B.U. and S.Cr. (74.26±3.82, 2.31±0.11 mg/dL, respectively) compared with values in the control group (30.0±2.08, 0.76±0.04 mg/dL, respectively). There was a significant (P<0.05) increase in UA levels in the patients (6.63±0.19 mg/dL) as compared to the control group (5.73±0.35 mg/dL). The eGFR value showed a highly significant (P<0.001) decrease in the patients (37.46±2.26 ml/min/1.73m^2) compared with the control group (112.25±4.19 ml/min/1.73m^2).

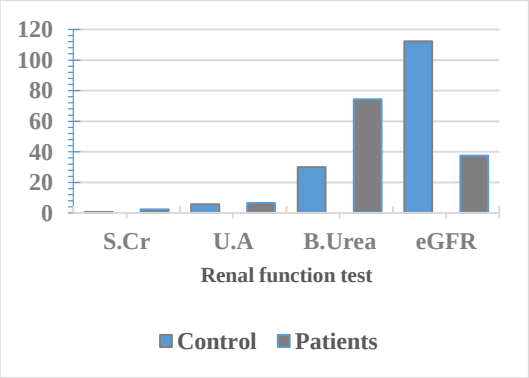

Figure (1): The result of renal faction tests of study groups

Hematological parameters of study groups

Table (2) presents the hematological data, which demonstrated a highly significant (P<0.001) decrease in Hb and HCT values in the patients (11.22±0.23 gm/dL, 34.03±0.62 %, respectively) compared with the control (13.06± 0 .48 gm/dL, 39.48±1.22 %, respectively). While there was a

significant (P < 0.05) decrease in the RBC count in the patients (4.35±0.09 10^6/µL) compared with the control (4.77± 0.14 10^6/µL).

Non-significant (P>0.05) differences in the MCV, MCH, and MCHC values with patients CKD (79.29±1.13 fL, 26.22±0.48 pg, and 32.55±0.34 g/dL, respectively); as compared with the control (82.10±1.21 fL, 27.42±0.52 pg, and 33.04±0.28 g/dL, respectively). Also, non-significant (P>0.05) differences were found in the numbers of total WBC (7.95±0.33 10^3/ µL) and differential WBC (neutrophils, eosinophils, basophils, lymphocytes, and monocytes), (58.83±1.27, 2.23±0.198, 1.70±0.21, 31.00±1.13, 6.28±0.30 %, respectively). In addition, a non-significant (P>0.05) differences in platelet count was reported in a CKD patient (245.47±8.78 103/ µL) compared with the control (260.35±17.05 10^3/ µL)

Table (2): The results of hematological parameters of study groups

Hematological tests	Groups (Mean ± SE)		
	Patients	Control	P-value
Hb (gm/dL)	11.22±0.23	13.06± 0 .48	<0.001*
HCT (%)	34.03±0.62	39.48±1.22	<0.001*
RBC (10^6/µL)	4.35±0.09	4.77± 0.14	0.031*
MCV (fL)	79.29±1.13	82.10±1.21	0.100 NS
MCH (pg)	26.22±0.48	27.42±0.52	0.205 NS
MCHC(g/dL)	32.55±0.34	33.04±0.28	0.462 NS
WBC (10^3/ µL)	7.95±0.33	8.07±0.45	0.846 NS
Ne. (%)	58.83±1.27	55.94±1.77	0.257 NS
Eo. (%)	2.23±0.198	2.04±0.49	0.684 NS
Ba. (%)	1.70±0.21	1.32±0.22	0.365 NS
Lym. (%)	31.00±1.13	34.33±1.62	0.150 NS
Mon. (%)	6.28±0.30	6.41±0.49	0.833 NS
PLT (10^3/ µL)	245.47±8.78	260.35±17.05	0.430 NS

NS: Non-significant, *: Significant (P≤0.05), **: High significant (P< 0.001)

Levels of Uromodulin and Cystatin C in the study groups

The data in figure (2), shows the levels of Urom and cys C in the studied groups. Statistically, there was a significant (P < 0.001) decrease in Uomd level in patients (5.45±0.14 ng/mL) when compared with the control group (21.84±1.13 ng/mL). While the level of cys C revealed a significant (P < 0.001) increase in the patients (18.5±0.39 ng/mL) when compared with the control group (9.37±0.48 ng/mL).

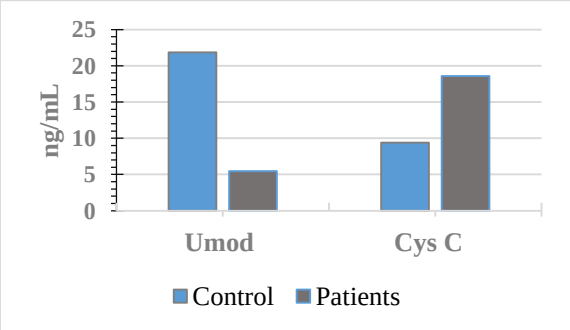

Figure (2) Levels of uromodulin and cystatin C in the study groups

Correlation between Uromodulin and studied parameter

The data presented in table (3) shows that the correlation analysis between the uromodulin level and the other parameters tested. Current study reported a significant negative correlation between uromodulin and the following parameters: B. urea (r= -.0321, P=.015), S.Cr. (r= -.341, P=.014), UA (r= -.294, P= .014) and cys C (r= -.452, P= .000). While a significant positive correlation was found between the levels of Umod and eGFR (r= .425, P= .003). On the other hand, a non-significant (P>0.05) correlation was found between Umod and the other parameter.

Table (3): Correlation analyses between the level of uromodulin and studied parameters

Parameters	*R*	P-value
Blood urea (mg/dL)	-.0321*	.015
Serum creatinine (mg/dL)	-.341*	.014
Uric acid (mg/dL)	-.294*	.014
eGFR ml/min/1.73m^2	.425*	.003
Hb (gm/dL)	-.103	.397
HCT (%)	-.069	.569
RBC (10^6/µL)	.037	.764
MCV (fL)	-.130	.283
MCH (pg)	-.147	.226
MCHC (g/dL)	-.135	.263
WBC (10^3/ µL)	-.017	.886
Ne. (%)	-.086	.477
Es. (%)	-.008	.946
Ba. (%)	.042	.728
Lym. (%)	.090	.460
Mon. (%)	.012	.919
PLT (10^3/ µL)	.047	.697
Cystatin C (ng/mL)	-.452**	.000

Correlation between Cystatin C and studied parameters

The data presented in table (4) shows the results of the correlation between of the levels of cys C and the other studied parameters. The current finding revealed a significant positive correlation between the levels cys C and S.Cr. (r= .440, P= .000), while a significant negative correlation was found between the level of cys C and those of eGFR (r= -.399, P=.001) and Umod (r= -.452, P= .000). On the other hand, a non-significant (P>0.05) correlation was found between the level cyst C and the rest of parameters.

Table (4): Correlation analyses between the level cystatin C and the other studied parameters

Parameters	*R*	P-value
Blood urea (mg/dL)	.234	.051
Serum creatinine(mg/dL)	.440**	.000
Uric acid (mg/dL)	-.208	.083
eGFR ml/min/1.73m^2	-.399**	.001
Hb (gm/dL)	-.031	.800
HCT (%)	-.011	.931
RBC (10^6/µL)	.121	.317
MCV (fL)	-.179	.139
MCH (pg)	-.176	.144
MCHC (g/dL)	-.107	.380
WBC (10^3/ µL)	-.077	.526
Ne. (%)	-.129	.289
Es. (%)	-.020	.873
Ba. (%)	-.061	.616
Lym. (%)	.117	.334
Mon. (%)	.126	.299
PLT (10^3/ µL)	-.039	.746
Uromodulin (ng/mL)	-.452**	.000

Discussion:

The current study showed no age disparity among the subjects involved,, which agreed with a previous study (10) which revealed that age has no statistically significant effect on the presence of CKD. However, it disagreed with other studies (11, 12) which discovered that the average age of CKD patients was older than the control population. The current study also discovered a non-significant difference in BMI values between studied group. which disagreed with other authors (13) who reported that increased unhealthy BMI levels were pointedly related to higher risk of CKD. On the other hand, the study found a non-substantial difference in the sex between the patients and control groups. These findings were in agreement with previous studies (14, 15) which found non- significant sex differences association with the frequency and distribution of the main causes of CKD. Nevertheless, the finding in the present study that male were a majority in both study groups agreed with the results of an earlier study (16). This could be due to premenopausal women having a lower incidence of hypertension, less diabetic microvascular disease and a slower damage in renal function (17).

Regarding the findings of the increased levels of B. urea and S.Cr., a similar study was previously conducted (18) showed that B. urea and S. Cr levels were considerably advanced in CKD patients. The reasons may be due to the fact that patients lose their ability to effectively filter waste products from the blood by the kidneys. Additionally, increased protein intake contributes to higher levels of S.Cr. and B. urea due to muscle wasting and protein metabolism abnormalities (19, 20). Furthermore, this study showed an increased level of UA in CKD patients, which is consistence with previous reports (21). These finding may be due to the fact that the kidneys are impaired by reduced uric acid excretion with subsequent accumulation of UA in the

bloodstream, which was already reported (22, 23). On the other hand, The decrease in GFR indicated in the current study was consistent with the findings of a previous study (24) which found that the S.Cr. level-increased whereas the level of eGFR decreased due to abnormal kidney function that led to lower efficiency in filtering creatinine and its accumulation in the bloodstream.

The other findings of current study were also in agreement with other studies (25, 26) which found significant decreases in the values of RBCs, Hb, and HCT among CKD patients leading to symptoms of anemia. These values of hematological parameters are reduced due to the decreased synthesis rate of the hormone erythropoietin caused by kidney failure. Increased breakdown of RBCs in chronic renal disease due to reduced erythropoietin production leads to a drop in red blood cell count, which decreases Hb concentration and HCT in those suffered from CKD with mild to moderate renal injury (27). The present finding also showed non-significant differences in MCV, MCH, and MCHC values, which agreed with previously published findings (28). This was, however, in disagreement with other data (29) who found that the values of MCV, MCH, and MCHC decrease significantly as the disease progresses in more advanced stages of chronicity. The present results found no significant differences in the levels of PLT, which is in agreement with earlier data(30) which found that platelets function remained constant or even improved as CKD progressed. Also, non-significant changes in counts of total and differential WBCs presented in the current study, were in agreement with other authors (31) who revealed that WBC, neutrophils, lymphocytes, monocytes, eosinophils, and basophils numbers were found to have no correlation with CKD progression. The current finding of decreased Umod value in CKD patients was in agreement with those published by other researcher (32) who found that reduced levels of Umod are reflected indirectly as impairments in renal function. This finding may be due to the tubular cells of the kidney being damaged or undergoing structural changes that lead to a decrease in Umod production, which may be due to fibrosis within kidney tissue (8, 33). Moreover, the present results showed an increase in cys C levels in CKD patients, which was in agreement with earlier finding (34) which found serum cys C is a reliable biomarker for CKD. It is especially useful in patients where traditional methods of measuring creatinine and GFR are ineffective. CKD patients usually have increased levels of cys C due to inflammation, renal tubular failure, and decreased muscle mass. Additionally, smoking, diabetes, hypertension, and cardiovascular disease are risk factors that contribute to higher levels of serum cys C (35).

The current study showed negative correlations between the level of Umod and B. urea, S.Cr. and Cys C, while showed a positive correlation between

Umod and eGFR. These findings were in agreement with previously published reports (36) which stated the positive correlation between Urom and eGFR and

the negative correlation of Umod with B. urea, S.Cr., and cys C may predict that Umod level may be a marker of renal function with similarly high diagnostic accuracy. Umod measurements may become a method for estimating the number of functional nephrons that is independent of nonrenal variables and thus greater to GFR calculation-based S.Cr. Alternatively, it may be used to supplement GFR in the assessment of total renal function (33, 37). On the other hand, there was negative correlation between Umod and UA value, which was in agreement with finding of other authors (38) who stated an inverse relationship between Umod and uric acid. The current study revealed a positive correlation between Cys C and S.Cr, which was consistent with previous research (39). The findings of the present study can be explained on the ground that the levels of Cys C and S.Cr. increased as kidney function declined. The findings indicated that Cys C could be a dependable marker of GFR. This is especially useful when S.Cr. may not accurately reflect kidney function. A combination of Cys C and S.Cr can provide a more comprehensive evaluation of kidney function (40, 41). The result regarding the negative correlation between cyst C and eGFR was in agreement with earlier findings (42) which stated the precision, sensitivity, and specificity of cys C to detect GFR relative to creatinine are valuable in clinical research, according to the study. The primary rationale for the increased use of cys C tests has been their ability to predict the effects of declining GFR. The present finding of no correlation between cys C and B.urea was in disagreement with another previous study (43) which stated a significant positive correlation between cys C level with B. urea and uric acid.

Conclusions: The present study revealed that CKD patients with impaired kidney function show elevated values of renal function parameters (B. urea and S. Cr) and a decrease in eGFR, in addition to the development of anemia. Furthermore. The study revealed that cys C level was increased while the Umod level was decreased in our patients with CKD. Also, a significant correlation was shown between uromodulin and cystatin C, on one side, and other studied parameters, on the other side. Finally, the level of Umod appears to be in better correlation with the results of renal function test than the level of cys C in CKD patients.

Limitation:
The study was based and only on center (Kidney Disease and Transplant Center) at Ghazi Ai-Hariri Hospital For surgical hence, the findings don't represent the whole population.

Authors' declaration
We confirm that all the tables in the manuscript are ours.

Authors sign on ethical consideration's approval-Ethical Clearance: accepted by the Researcher Ethical Committee and Scientific Committee designated by Biology Department, College of Science, University of Baghdad under the reference number (No. CSEC/0922/0105).
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Study conception & design: (Hadeel T.A. AL-Ani and Makarim Q. D. Al-Lami.). Literature search: (Hadeel T.A. AL-Ani). Data acquisition: (Hadeel T.A. AL-Ani and Makarim Q. D. Al-Lami.). Data analysis & interpretation: (Hadeel T.A. AL-Ani and Makarim Q. D. Al-Lami). Manuscript preparation: (Hadeel T.A. AL-Ani and Makarim Q. D. Al-Lami.). Manuscript editing & review: (Hadeel T.A. AL-Ani and Makarim Q. D. Al-Lami.).

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تقييم بعض المعلمات الكيموحيوية والدموية في المرضى الذين يعانون من مرض الكلى المزمن

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الخلاصة:
خلفية البحث : مرض الكلى المزمن هو متلازمة تنتج عن تغيير غير محدد في هيكلية الكلى و / أو وظيفتها. يتميز بتطوره البطيء والمطرد وعدم روجوعه. يصنف المريض البالغ مع ظهور مرض الكلى المزمن ، لفترة تساوي أو تزيد عن ثلاثة أشهر ، معدل ترشيح كبيبي (GFR) أقل من 60 مل / دقيقة / 1.73 م 2. مرض السكري وارتفاع ضغط الدم وأمراض الكلى المتعدد الكيسات والتهاب كبيبات الكلى المزمن وأمراض الكلى الحادة لفترات طويلة هي الأسباب الرئيسية لمرض الكلى المزمن.
الاهداف : الهدف من هذه الدراسة هو تقييم نتائج بعض المعلمات الكيموحيوية والدموية في عينات من المرضى العراقيين الذين يعانون من مرض الكلى المزمن.
المواد وطرق العمل: تم استخدام تصميم دراسة الحالات حيث تم جمع 90 عينة, حيث 70 مريضا منهم يعانون مرض الكلى المزمن و 20 شخصا سليم. تم جمع عينات الدم من الرضى خلال زياراتهم من مستشفى غازي الحريري للتخصصات الجراحية- المدينة الطبية, بغداد , العراق. تم تقييم العمر و الجنس ومؤشركتلة الجسم (BMI) لكل مشارك متبوعا باختبارات وظائف الكلى [اليوريا في الدم (B.urea), الكرياتينين (S.Cr), حمض اليوريك (UA) وeGFR], و(CBC) تحليل الدم الكامل . ايضا تم قياس مستويات المصل للسيستاتين سي و اليوروموديولين.
النتائج: بينت هناك زيادة معنوية في مستويات B.urea, S.Cr. وUA ، بينما تم الكشف عن انخفاض معنوي في مستوى eGFR في المرضى مقارنة بالمجموعة السليمة. من ناحية أخرى, تم تسجيل انخفاض في قيم الهيموغلوبين (Hb)والهيماتوكريت (HCT)وفي عدد خلايا الدم الحمراء (RBC). لم يتم الكشف عن فرق بين المرضى والمجموعة السليمة فيما يتعلق باختبارات الدم الاخرى. كانت هناك زيادة معنوية في مستوى السيستاتين سي وانخفاض معنوي في مستوى يوروموديولين في المرضى. علاوة على ذلك, كشفت النتيجة الحالية عن وجود علاقة سلبية بين مستوى اليوروموديولين ومستوى B.urea, S.Cr , UAوالسيستاتين سي بينما تم وجود علاقة ايجابية كبيرة بين اليوروموديولين و eGFR. كما لوحظ وجود ارتباط ايجابي بين مستويات السيستاتين سي و الكرياتينين, بالاضافة الى ارتباط سلبي مع eGFR.
الاستنتاجات: تظهر الدراسة الحالية أن مرضى CKD لديهم ارتفاع في معايير وظائف الكلى B. ureaو S. Cr وانخفاض في eGFR، إلى جانب ظهور فقر الدم. من ناحية أخرى ، كشف مستوى السيستاتين سي عن زيادة بينما أظهر مستوى يوروموديولين انخفاضا في المرضى العراقيين الذين يعانون من مرض الكلى المزمن. أيضا ، تم الكشف عن علاقة كبيرة بين اليورموديلين وسيستاتين سي من جانب ومع معايير دراسية اخرى من جانب اخر.
الكلمات المفتاحية: يوروموديولين ، سيستاتين سي ، أمراض الكلى المزمنة ، أمراض الدم.

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Research Article

The Correlation of P53 and MSI Immune Markers in Gastric Adenocarcinoma

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Abstract:

Background: The known risk factors for gastric adenocarcinoma are chromosomal instability, TP53 mutations, aneuploidy, translocations, proto-oncogenes, and tumor suppressor gene changes. Microsatellite instability (MSI) affects DNA replication accuracy and is detected by the heterodimeric protein complex hMSH2/hMSH6, which recruits hMLH1 and hPMS2 for re-synthesis. MSI can cause sporadic gastric cancer and Lynch syndrome.

Objectives: To examine the relationship between P53 and MSI immune markers expression with the clinicopathological parameters of gastric adenocarcinoma by using immunohistochemistry.

Methods: The study examined 40 formalin-fixed, paraffin-embedded gastric adenocarcinoma tissue blocks. The samples were retrieved from archived materials in the histopathology department of the Gastroenterology and Hepatology Teaching Hospital, Teaching Laboratory Institute, and some private laboratories in Baghdad, Iraq. The samples were taken from patients between 2020 and 2023, while their retrieval spanned from October 2022 to October 2023 for the sake of examining primary cases, surgical tissues, and available clinicopathological data. The immunohistochemical (IHC) expression was assessed using a scoring system. Data were analyzed using SPSS, Chi-square, and Fisher's exact tests, with a 95% confidence level and a 0.05 P-value or less considered significant.

Results: IHC staining for P53 was positive in 65% of the samples, while MSI findings were positive in 97.5% of the samples. The MLH1/PMS2 heterodimeric couple showed 32.5% positive results, while the MSH2/MSH6 heterodimeric couple showed 87.5% positive results. P53 stain was significantly correlated with lymph node involvement and grade, but not with the other parameters. No significant association was found between MSI markers and the studied parameters. There was no significant association between MSI heterodimeric couple (MLH1/PMS2) and the clinicopathological parameters, but there was a significant association between MSI heterodimeric couple (MSH2/MSH6) markers and metastasis only.

Conclusion: P53 is a key biomarker for evaluating lymph node involvement and aggressiveness in grading, indicating prognosis, and identifying high-risk cancer patients for metastasis.

Keywords: Gastric adenocarcinoma; molecular classification; immunohistochemistry; P53; microsatellite instability.

Introduction:

The Global Cancer Observatory (GLOBOCAN) in 2020 indicated that stomach cancer was the fifth largest cause of cancer deaths, with 1.1 million cases of which 75% were in Asia. Five-year survival is 20%, with Eastern Asians having the greatest incidence (22.4 per 100,000) (1,2). Stomach cancer, which is Iraq's second-leading cause of cancer mortality, killed 783,000 people worldwide in 2018 and caused over 1,000,000 new cases with clinical, genetic, morphological, epidemiological, and developmental abnormalities (3). It is the fifth most prevalent cancer and the third leading cause of cancer death worldwide, caused by environmental and genetic factors, including Helicobacter pylori. [4]. GLOBOCAN 2021 reported Iraqi stomach cancer incidence, death, and prevalence as follows: New cases (all ages) 1149 (3.4%) with a rank of 9 and a cumulative risk of 0.56. There were 966 deaths (4.9%) with a rank of 6 and a cumulative risk of 0.48. Presence at 5 years was 1579, or 3.39 per 100,000 (5). Most non-cardia stomach cancers are caused by H. pylori, the first bacterial carcinogen. Diffuse gastric cancer, non-cardia intestinal gastric adenocarcinoma, and gastric B-cell lymphocyte mucosa-associated lymphoid tissue lymphoma can result from childhood acquisition (6). As H. pylori eradication may restore atrophic gastritis but not intestinal metaplasia, targeted intervention before stomach precancerous changes may help high-risk individuals prevent gastric cancer (6). Among the factors that increase the risk of gastric cancer are dietary nitrite-secondary amines, high-temperature cooking of protein-rich foods, high salt intake, smoking, and excessive alcohol consumption. On the other hand, Vitamin C, onions, garlic, and shallots reduce stomach carcinogenesis (6).

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Hereditary diffuse gastric cancer, BRCA2, HNPCC, Lynch II, Li Fraumeni syndrome, and FAP syndrome increase stomach cancer risk (6). The 2019 WHO categorization of malignant epithelial tumors includes tubular, papillary, poorly cohesive signet ring phenotype, poorly cohesive other cell type, mucinous, and mixed histologic types (7). Intestinal and diffuse gastric cancer subtypes have diverse shapes, epidemiology, pathogenic mechanisms, and genetic profiles. Intestinal tumors are tubular or glandular, with better prognosis in males and older individuals. Poorly cohesive carcinomas invade glandular structures (8). The Cancer Genome Atlas (TCGA) reveals that 20% of stomach cancers are genetically stable (GS), aneuploid, and early-identified, with 73% diffuse subtype enrichment, cadherin-1(CDH1) somatic mutations (8-10). Fifty percent of stomach cancers are chromosomally unstable (CIN), more common in esophageal gastric junction tumors. P53, a cell cycle regulator, prevents DNA replication errors during synthesis, minimizing cancer progression. Mutation or heterozygosity loss usually inactivates it on 17p. (11). TP53 mutations and histological P53 overexpression are important molecular factors in understanding stomach cancers, as trastuzumab can treat 10–20% of gastric adenocarcinomas if HER2 is overexpressed [8]. MSI results from DNA mismatch repair deficiencies. The Mismatch Repair (MMR) system—hMLH1, hMSH2, hMSH6, and hPMS2 proteins—corrects base mismatches, insertions, and deletions for DNA replication accuracy (8). Lynch syndrome, caused by autosomal dominant MMR gene defects, increases cancer risk at younger ages, particularly in colorectal, endometrial, ovarian, and gastric cancers. Microsatellite-unstable gastric tumors, accounting for 22% of cases, have a high mutation rate (8). Epstein-Barr, a herpes virus, infects B-cells in the oropharyngeal epithelium, leading to various cancers like breast, lung, stomach, colon, and lung, due to complex interactions between cell environment and viral gene expression. (12). EBV gene expression and host genome control impact oncogenesis, affecting stomach cancer's host gene expression and cell cycle pathways. Recent research links viral latent profiles to latency I or II. EBV-positive gastric tumors, primarily affecting men, have the best prognosis, with genetically stable subtypes having the worst prognosis (8,13). The Asian Cancer Research Group (ACRG) found four molecular categories for gastric cancer. The second classification algorithm firstly includes the mesenchymal group with microsatellite stability (MSS) and epithelial-mesenchymal transition (EMT), which accounts for 15.3% of cases and is usually found in advanced stages. In 80% of cases, signet ring cell carcinomas occur. The second MSS/TP53-negative subtype accounts for 35.7% of cases, while the third subtype MSS/TP53+ positive which has more EBV infections. The fourth subtype, microsatellite unstable, starts in the distal stomach and has the best prognosis (8).

Patients, Materials and Methods:

Our study was conducted on 40 stomach cancer patients using formalin-fixed paraffin-embedded tissue blocks. The samples were collected from the Gastroenterology and Hepatology Teaching Hospital, Teaching Laboratory Institute, and private laboratories between 2020 and 2023. After possibly curative gastrectomy and lymphadenectomy, the patients were histologically divided into 19 intestinal, 14 diffuse, and 7 mixed adenocarcinoma groups. The study focused on cases with primary gastric adenocarcinoma, available clinicopathological data, and surgical specimens with available tissue for paraffin blocks. Immunological markers were investigated, including P53 and MSI (MLH1, MSH2, MSH6, and PMS2). The study was performed in a private laboratory and excluded other gastric tumors, secondary gastric adenocarcinoma, endoscopic biopsies, and gastric cancers with pre-operative neoadjuvant therapy.

Table 1: Materials

Material	Type
Xylene	Analar (England)
Ethanol (absolute)	Merck (Germany)
Distilled water	
Rinse buffer	TBS (DakoCytomation)
Target retrieval solution (heat-induced epitope retrieval (HIER) DAKO PT LINK (code PT100/PT101)	Tris EDTA pH 9.0 (Dakocytomation) EnVision FLEX Target retrieval solution HIGH pH 50x code (K8000 /K8004)
Primary antibody	DAKO FLEX monoclonal mouse anti-human p53 protein (clone DO-7). Isotype: IgG2b, kappa. Ready-to-use (Link) Code IR616
	DAKO FLEX monoclonal mouse Anti-Epstein-Barr Virus, LMP, (Clone CS.1-4). Isotype: IgG1, kappa. Ready-to-use (Link) Code IR753
	DAKO FLEX monoclonal mouse Anti-Human E-Cadherin, (clone NCH-38) Isotype: IgG1, kappa. Ready-to-use (Link), Code IR059
	DAKO MLH1 Clone ES05 Ready-to-use (Prediluted) Product no/lot no.:IR079/IS079/ 11450820
	DAKO MSH2 Clone FE11 Ready-to-use (Prediluted) Product no/lot no.: IR085 / 10148024
	DAKO MSH6 Clone EP49 Ready-to-use (Prediluted) Product no/lot no.: IR086 / 11166400
	DAKO PMS2 Clone EP51 Ready-to-use (Prediluted) Product no/lot no.: IR087 / 11170264
Hematoxylin	Counter stain EnVision FLEX Hematoxylin (link) (code K80008)
Mounting media	Dakocytomation
Secondary detection system	HRP/DAB detection (Dakocytomation)
Visualization system	EnVision FLEX High pH (Link) (code K8000) for p53, EBV. EnVision FLEX+ mouse High pH (Link) (code K8002) for E-CADHERIN, MLH1, MSH2, MSH6 and PMS2

Methods: The process involved deparaffinizing blocks in an oven at 60°C for 1 hour, followed by xylene swaps. The tissue was rehydrated with ethanol, and a hematoxylin nuclear stain was applied. Differentiation was done in a 1% acid-alcohol solution, and eosin counterstain was used. Mounting was done using DPX, and H&E slides were examined to choose the best sections for IHC. The immunohistochemistry process involved sectioning tissue blocks, incubating them in a water bath, deparaffinization, applying Xylene, rehydration in alcohol solutions, and rinsing with tap water. The antigen retrieval phase involved using a tris ethylenediamine tetra-acetic acid (TRIS EDTA) solution heated to 80°C and maintained for 20 minutes before being lowered back to 65°C for each cycle. A PAP pen was used as a reagent blocker, and a wash buffer solution was used. Two drops of peroxidase blocker were applied to stop the endogenous antigen activity. Primary antibodies were applied to the samples, targeting five markers: P53, MLH1, MSH2, MSH6, and PMS2, and each was incubated for 30 minutes. Anti-mouse and anti-rabbit antibodies labeled with horseradish peroxidase were applied and washed. (3,3-diaminobenzidine) DAB was prepared by adding poly detector DAB chromogen per milliliter of poly detector DAB buffer. The samples were washed with wash buffer, and hematoxylin counterstain was applied to the background for one minute. For the MSI markers, Gastric cancer is considered negative if no tumor cell staining is present for all the markers, and tumors with all markers' expression are considered microsatellite stable (14), while other articles suggested that the loss of expression of a single protein or a heterodimeric couple supports MMRD, which is indirect evidence of MSI. Proteins hMLH1 and hMSH2 are stable without their dimeric partners, hPMS2 and hMSH6, but these components are rarely stable (15).

For the quality control, basal epithelial cells in the colon and appendix show a moderate to strong staining reaction, while germinal centers in cells of the tonsil show a moderate to strong staining reaction; both are considered positive controls for MLH1, MSH2, MSH6, and PMS2. Colonic adenocarcinoma with loss of MLH1, MSH2, MSH6, and PMS2 expression can serve as a negative control, and stromal cells show a distinct nuclear staining reaction serving as an internal positive tissue control, as mentioned in the antibody leaflet. The expression of P53 cells is typically detected through nuclear staining. Two patterns are considered abnormal: Strong nuclear staining in at least 70% of tumor cells and complete loss of p53 expression, or less than 5%. Stromal cells and benign epithelium served as controls for normal and reactive mesothelium, with mesotheliomas showing negative cells (14). Neoplastic cells of colonic adenocarcinoma with a moderate to strong staining reaction were considered a positive control, and normal colonic mucosa was considered a negative control, as mentioned in the antibody leaflet. The interpretation of the slides and the correlation of the immune markers' expression and the clinicopathological parameters: Age, sex, location of the tumor, type of surgery, morphological tumor pattern, TNM staging, tumor grade, lympho-vascular invasion, and perineural invasion were done by the authors.

Statistical analysis: The study used Statistical Package for Social Sciences (SPSS) version 26 to describe variables, with serial numbers being the only reference for participant details. Data were managed daily and expressed using mean, standard deviation, and frequency/ percentage. The Chi-square and Fisher's exact tests were used to assess the association between categorical variables, with a 95% confidence level and a P-value of 0.05 or less being considered significant.

Results

The study examined 40 cases of gastric adenocarcinoma, with 57.5% being males and 42.5% being females. The age distribution of the patients showed that one case (2.5%) was between 20-29 years of age, 6 cases (15%) were between 30-39 years, 10 cases (25%) were between 40-49 years, 10 cases (25%) were between 50-59 years, and 13 cases (32.5%) were 60 years or over.

The samples were from the proximal stomach gastroesophageal junction and cardia (10%), (2.5%) in the fundus, (50%) in the body and antrum, and (37.5%) in the distal stomach. The cases were treated with total gastrectomy (62.5%), proximal gastrectomy (5%), and distal gastrectomy (32.5%). There were 19 cases (47.5%) of intestinal type adenocarcinoma, 14 cases (35%) of diffuse type adenocarcinoma, and 7 cases (17.5%) of mixed type adenocarcinoma, (table 2). Four stages were identified: 1A and 1B, 2A and 2B, 3A and 3B, and 4, (table 4). The cases were graded into G1 well-differentiated adenocarcinoma, G2 moderately differentiated adenocarcinoma, G2/G3 moderately to poorly differentiated adenocarcinoma, and G3 poorly differentiated adenocarcinoma. Only 17). 42.5% of the cases showed lympho-vascular invasion, and 18. While 45% showed perineural invasion.

Table 2: Distribution of the samples by site, specimen and diagnosis

Variable	Category	NO.	%
Site	Proximal stomach gastroesophageal junction and cardia	4	10.0
	Fundus	1	2.5
	Body and antrum	20	50.0
	Distal stomach	15	37.5
Specimen	Total gastrectomy	25	62.5
	Proximal gastrectomy	2	5.0
	Distal gastrectomy	13	32.5
Diagnosis	Intestinal type adenocarcinoma	19	47.5
	Diffuse type adenocarcinoma	14	35.0
	Mixed type adenocarcinoma	7	17.5

Table 4: Distribution of the samples by stage and grade

Variable	Category	NO.	%
Stage	1A	1	2.5
	1B	5	12.5
	2A	5	12.5
	2B	11	27.5
	3A	6	15.0
	3B	10	25.0
	4	2	5.0
T	1	2	5.0
	2	7	17.5
	3	26	65.0
	4A	4	10.0
	4B	1	2.5
N	0	11	27.5
	1	9	22.5
	2	8	20.0
	3	4	10.0
	3A	4	10.0
	3B	2	5.0
	X	2	5.0
M	0	1	2.5
	1	2	5.0
	X	37	92.5
Grade	G1 well differentiated	1	2.5
	G2 moderately differentiated	24	60.0
	G2/G3 moderately to poorly differentiated	2	5.0
	G3 poorly differentiated	13	32.5

The study found that P53 was positive in 26 cases (65%) and MSI was positive in 39 samples (97.5%). Immune markers as heterodimeric couples were found to be positive as follows: MHL1/PMS2 heterodimeric couple (32.5%) of the samples, MSH2/MSH6 heterodimeric couple (87.5%) of the samples, and 35% of the samples were found to be positive for P53 and negative for MSI expression, tables 4 and 5

Table 4: Distribution of the samples by the main stains

Variable	Category	Number	%
P53	Positive	26	65.0
	Negative	14	35.0
MSI	Positive	39	97.5
	Negative	1	2.5

Table 5: Distribution of the samples by t e stain couples

Variable	Category	Number	%
MLH1/PMS2	Positive	13	32.5
	Negative	27	67.5
MSH2/MSH6	Positive	35	87.5
	Negative	5	12.5

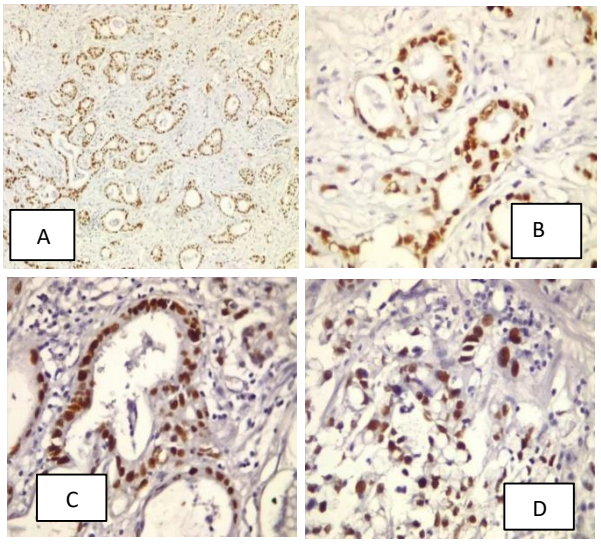

Figure (6): P53 positive immune markers overexpression

- A: P53 positive nuclear staining >70 % overexpression (40x)
- B, C: P53 positive nuclear staining >70 % overexpression (100x)
- D: P53 positive nuclear staining >70 % overexpression (400x)

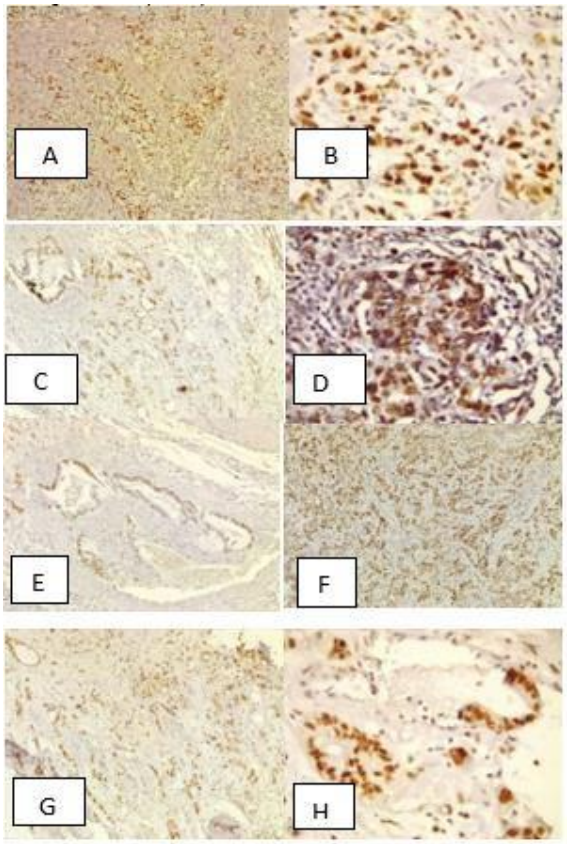

Figure (7): MSI immune markers positive expression
A: MLH1 positive 100x, B: MLH1 positive 400x
C: MSH2 positive 100x, D: MSH2 positive 400x
E: MSH6 positive 100x, F: MSH6 positive 400x
G: PMS2 positive 100x, H: PMS2 positive 400x

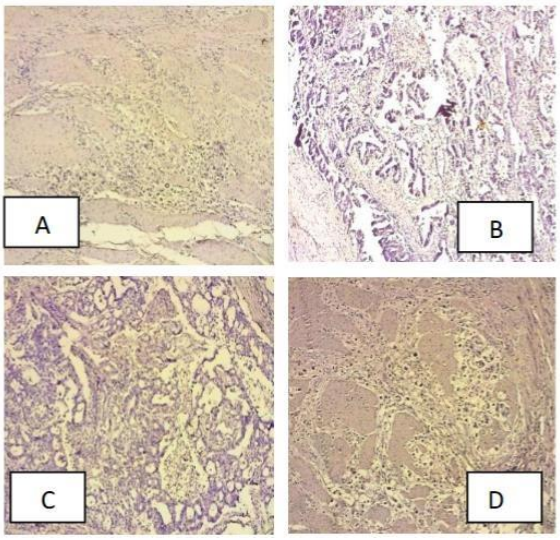

Figure (8): MSI immune markers negative expression
A: MLH1 negative 100x
B: MSH2 negative 100x
C: MSH6 negative 100x
D: PMS2 negative 100x.

A significant association was found between the P53 score and lymph node involvement (P = 0.047) and grade (P = 0.012), table (5). There was no significant association between the P53 score and age group (P = 0.135), sex (P = 0.191), site of the tumor (P = 0.245), specimen (P = 0.754), diagnosis (P = 0.677), stage (P = 0.124), tumor size (P = 0.371), metastasis (P = 0.693), lymphovascular (P = 0.973), or perineural invasion (P = 0.257).

Table 5: Distribution of P53 stain by the sample characteristics

Variable	Category	P53 Positive No. = 26	%	Negative No. =14	%	P value
N	0	8	30.8	3	21.4	
	1	3	11.5	6	42.9	
	2	7	26.9	1	7.1	
	3	4	15.4	0	0.0	0.047*[1]
	3A	3	11.5	1	7.1	
	3B	1	3.8	1	7.1	
	X	0	0.0	2	14.3	
Grade	G1 well differentiated	0	0.0	1	7.1	
	G2 moderately differentiated	12	46.2	12	85.7	0.012*[1]
	G2/G3 moderately to poorly differentiated	2	7.7	0	0.0	
	G3 poorly differentiated	12	46.2	1	7.1	

*Significant result [1]Fisher's exact test

There was no significant association between MSI markers when they were considered as one marker and age group, sex, site of the tumor, specimen, diagnosis, stage, tumor size, metastasis, lymph nodal involvement, tumor grade, lympho-vascular, and perineural invasion (P > 0.05). As for the heterodimeric MSI couple (MLH1/PMS2) with the clinicopathological parameters, there was no (MLH1/PMS2) and age group, sex, site of the tumor, specimen, diagnosis, stage, tumor size, metastasis, lymph node involvement, tumor grade, lympho-vascular, and perineural invasion (P > 0.05). For the significant association between MSI markers when they were considered as a heterodimeric couple heterodimeric MSI couple (MSH2/MSH6) relationship with the clinicopathological parameters, there was a significant association between MSI markers when they were considered as a heterodimeric couple (MSH2/MSH6), and metastasis (P = 0.036), table (6). No significant association was found between MSI markers when they were considered as a heterodimeric couple (MSH2/MSH6, and age group, sex, site of the tumor, specimen, diagnosis, stage, tumor size, lymph node involvement, tumor grade, lymphovascular, and perineural invasion (P > 0.05).

Table 6: Distribution of MSH2/MSH6 stain by sample characteristics

Variable (M)	MSH2/MSH6				P value
	Positive		Negative		
	N=35	%	N=5	%	
0	0	0.0	1	20.0	
1	1	2.9	1	20.0	0.036*
X	34	97.1	3	60.0	

*Significant result Fisher's exact test used

Discussion

The results of the current study agree with the study of Al-Badri et al, who found a significant association between p53 expression and tumor grade and lymph node involvement in gastric carcinoma and gastric dysplasia. However, no significant association was found between p53 protein expression and tumor depth or histological type. [11]. Grosser et al found that abnormal p53 expression negatively impacts patients' prognosis in resection specimens. The study found that P53 did not predict response or survival in the biopsy cohort before CTx. The expression of P53 varied across molecular subtypes in surgical resection and biopsied specimens, with a clear correlation between P53 and MSI-L. Individuals with MSI-H and abnormal P53 had the worst survival outcomes in biopsy patients. Our results are in disagreement with these results as they found a relationship between p53 and MSI expression that had the worst survival outcome which we didn't investigate. [16]. Hwang et al conducted a study using deep-targeted sequencing on surgical or biopsy materials from 120 individuals with gastric cancer. They found that high P53 expression was linked to TP53 missense mutations, negative expression was related to other mutations, and weak expression was seen in cases with wild-type TP53.

The preliminary diagnostic TNM staging showed a strong association with both TP53 mutation type and P53 expression status. A survival study on 109 stage II and III gastric cancer cases revealed that patients with TP53 missense mutations had significantly worse overall survival compared to wild-type and other mutation groups. A higher level of P53 expression was associated with a worse overall survival rate. For the comparison with our study, we partially agree because we used IHC to examine the protein product and found a significant relation to lymph nodal involvement and grading which gave a prognostic insight, while in the comparative study, they used gene sequencing and found a relation of p53 expression to TP53 missense mutation which was an important poor prognostic factor and worse overall survival rate. [17]. A study by Kim et al found that among 3608 gastric cancer patients, 37% had P53 overexpression. In intestinal-type gastric cancer, overexpression was associated with less
invasion depth and early-stage disease. In diffuse-type gastric cancer, overexpression was linked to advanced TNM stage and advanced disease. Patients with P53 overexpression had reduced overall survival and gastric cancer-specific survival, with the significance being more prominent in diffuse-type gastric cancer [18]. Zhang et al in a study on gastric cancer found a significant positive correlation between Her-2 and P53 expression. The study found that Her-2 expression intensity varied significantly in patients with varying degrees of gastric cancer cell differentiation, with signet-ring cell carcinoma being strongly associated with Her-2 expression. The proportion of positive P53 expression was correlated with tumor differentiation grade and positive Ki67 expression, suggesting that HER-2 and P53 collaborate in gastric cancer. The study revealed a significant correlation between positive P53 expression, age, tumor differentiation grade, and Ki67 expression, with significant differences observed across groups with higher differentiation degrees, and a positive correlation between high P53 expression and poor differentiation [19]. Regarding the MSI immune stain reaction. The study of Karpińska-Kaczmarczyk et al on 107 patients with gastric cancer found an MSI deficit, with 5.6% of the patients showing MMR proteins. The loss of MMR protein expression was linked to intestinal gastric cancer in the Lauren classification and tubular and papillary architecture in the WHO classification. Negative MMR expression was not associated with age, sex, tumor site, depth of invasion, lymph node status, ulceration, or lymphocytic infiltration. Our results are not in agreement with these results as the mismatch repair protein expression does not show any correlation with the histological types [20]. Hanon et al in a study in Baghdad, Iraq, focusing on the prevalence of MSI in colorectal carcinoma (CRC) found that MSI prevalence was higher in women (38.1%) and older individuals (34.6%). Morphological features of CRC specimens showed a higher percentage (47.1%) in poorly differentiated cases. Mucinous CRC had 100% MSI compared to 27.7% for non-mucinous cases. MSI was more common at the right site (52.9%) than in MSI L and MSS, Hanon et al used PCR to study the MSI profile in colorectal carcinoma, while we used IHC for MMR proteins to assess gastric adenocarcinoma. [21]. Hiroki in a study on Japanese patients with early gastric malignancies found 54 adenocarcinomas, including high-grade dysplasia, treated with endoscopic resection over five years. The WHO characteristics re-evaluation revealed that EBV-positive carcinomas were poorly differentiated (83.8%), while MSI-H tumors were common in well- to moderately differentiated adenocarcinomas (85.7%). This highlights the importance of understanding the WHO criteria in subdividing Japanese early gastric malignancies, which may help compare precursor lesions and early carcinoma.

Significant differences in macroscopic characteristics and histological subtypes were observed between these groups, which was not relevant in our study. [22]. Reitsam et al found that 1.4% and 5.1% of patients with dMMR had loss of MLH1, PMS2, and MSH6 immuno-expression. The study examined MLH1 promotor hypermethylation and BRAF exon 15 status and sequenced DNA repair genes using next-generation sequencing. Pathogenic germline variants and sporadic mutations were identified in the MMR and HRR genes, affecting ATM, BARD1, BRCA1, CDK12, CHEK1, CHEK2, FANCA, MLH1, MSH6, PALB2, and TP53. This study considers the biological function of MMR proteins and next-generation sequencing as potential drug targets and the low frequency of most of these mutations in the digestive system which was not in the capability of our scope. [23]. Evaristo et al found that 12.3% of gastroesophageal junction tumors had the MMR-deficient (dMMR) immunophenotype, with most cases lacking the BRAF V600E mutation. The dMMR phenotype was not significantly associated with tumor grade but was associated with lower pathologic staging than the pMMR. Patients with pMMR tumors had a higher median number of positive lymph nodes than those with DMMR tumors, leading to higher pathologic lymph node staging groups which was irrelevant in our investigation regarding lymph nodal staging [24]. Zhang et al found that mismatch repair-deficient gastric cancer patients have higher programmed death ligand-1 expression and a higher incidence of MSI. They found 126 (6%) MLH1/PMS2-negative individuals and 14 (0.9%) MSH2/MSH6-negative ones. The study found a high association between d-MMR status and intestinal group, but not with tumor differentiation which was irrelevant in our investigation regarding histological subtyping and differentiation (25). Elrefaey et al in a study in Egypt, examined the IHC expression of MLH1, MSH2, and P53 proteins to correlate them with tumor differentiation, lymph node status, and TNM staging in 70 gastric adenocarcinomas. They found a significant correlation between the MSI status and tumor differentiation, invasion depth, lymph node status, and TNM staging, while in our investigation, we only had a significant association between MSH2/MSH6 expression and metastasis [26]. A quick reference to the studies that tested other markers related to gastric adenocarcinoma, Ashour et al found a significant correlation between MUC5AC expression and lymph node involvement in gastric cancer patients, with a decrease in expression compared to the control group. However, there was no significant correlation between MUC5AC expression and age, sex, histopathological subtypes, grade, and stage of gastric cancer. The results suggest that MUC5AC can be used as an ancillary marker for diagnosing lymph node involvement and malignant transformation of gastric cancer, but not for predicting grade and stage outcomes [4]. Mwafaq et al found that there were significant differences in PARP1 expression levels between patients and control groups, with significant correlations between histopathological subtype, grade, invasion depth, lymph node involvement, and stages in patients. However, no significant associations were found with age or sex [3]. These two later studies throw a light on the other IHC markers expression that was significantly correlated with some of the clinicopathological parameters so as P53 and mismatch repair proteins that we were interested in our research. The disagreement in all the mentioned studies above was regarded due to the small sample size, different methodology, different antibody clones, and subjective interpretation of the immune markers' expression.

Conclusions

P53 is a key biomarker for evaluating lymph node involvement and aggressiveness in grading, indicating prognosis, and identifying high-risk cancer patients for metastasis.

Authors' declaration:

All of the tables in the text are original works by the authors. Authors signed off based on their acceptance of ethical considerations. The research ethical committee in the pathology and forensic medicine department (college of Medicine/university of Baghdad) gave their stamp of approval to the study, according to code No.145 (4THOCT-2022).

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Author Contributions:

Study conception & design: (*Ali M.J Al-Shakarchi & Sazan A.W. Mirza)*. Literature search: (*Ali M.J Al-Shakarchi*). Data acquisition: (*Ali M.J Al-Shakarchi*). Data analysis & interpretation: (*Ali M.J Al-Shakarchi & Sazan A.W. Mirza*). Manuscript preparation: (*Ali M.J Al-Shakarchi).* Manuscript editing & review: (*Ali M.J Al-Shakarchi & Sazan A.W. Mirza*).

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علاقة المعلمات المناعية ل (P53) وعدم استقرار الساتل الميكروي (MSI) مع سرطان المعدة الغدي

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الخلاصة

الخلفية: يحدث سرطان المعدة الغدي بسبب عدم إستقرار الكروموسومات، وطفرات TP53، وإختلال الصيغة الصبغية، والإنتقالات، والجينات الورمية الأولية، والتغيرات الجينية المثبطة للورم. يؤدي عدم إستقرار الساتل الميكروي (MSI) إلى فشل إصلاح عدم تطابق الحمض النووي، مما يؤثر على دقة تكرار الحمض النووي. يتم الكشف عن أخطاء النسخ المتماثل المبكرة بواسطة مجمع البروتين المتغاير (hMSH2/hMSH6)، الذي يقوم بتفعيل (hMLH1 و hPMS2) لإعادة تكوين الحمض النووي. يحدث عدم إستقرار الساتل الميكروي في حالات سرطان المعدة المتفرق ومتلازمة لينشز.

الهدف من الدراسة: دراسة العلاقة بين تعبيرالمعلم P53 وتعبير المعلمات المناعية لعدم إستقرار الساتل الميكروي (MSI) مع العوامل السريرية المرضية لسرطان المعدة الغدي بإستخدام الكيمياء النسيجية المناعية.

المواد والطرق: تم فحص 40 كتلة من نسيج سرطان المعدة الغدي المثبت بالفورمالين والمطمور بالشمع في بغداد، العراق. تناولت الدراسة حالات سرطان المعدة الأولية، مع البيانات السريرية المرضية المتاحة، والأنسجة الجراحية. تم تقييم التعبير المناعي الكيميائي بواسطة نظام تسجيل النقاط. تم إستخدام برنامج SPSS لتحليل البيانات، كما تم إستخدام إختبارات Chi-square وإختبارات Fisher الدقيقة لتقييم الإرتباطات. تم إعتبار مستوى الثقة 95٪ والقيمة الإحتمالية P 0.05 أو أقل مهمًا.

النتائج : كان التصبيغ المناعي النسيجي الكيميائي لـ P53 إيجابيًا في 65٪ من الحالات، بينما كانت نتائج MSI إيجابية في 97.5٪ من الحالات. حصل الزوجان المتغايران MLH1/PMS2 على نتائج إيجابية بنسبة 32.5% ونتائج سلبية 67.5%، في حين حصل الزوجان المتغايران MSH2/MSH6 على نتائج إيجابية بنسبة 87.5% ونتائج سلبية 12.5%. ارتبطت صبغة P53 بشكل كبير بانتشار العقدة الليمفاوية ودرجة الورم، ولكن لم يكن هنالك إرتباط مع العوامل الأخرى. لم يتم العثور على إرتباط كبير بين معلمات MSI والعوامل المدروسة ولم يكن هناك إرتباط كبير بين معلمات MSI غير المتجانسة (MLH1 / PMS2) والمعلمات المرضية السريرية، ولكن كان هناك إرتباط كبير بين علامات MSI الزوجين غير المتجانسة (MSH2 / MSH6) والنقائل فقط.

الإستنتاج: يعد P53 معلما حيويا مهما لتقييم انتشار العقدة الليمفاوية وعدوانيتها في التصنيف النسيجي، مما يشير إلى التشخيص، وتحديد مرضى السرطان الأكثر عرضة لخطر الإصابة بالورم النقيلي. على الرغم من أن MSH2/MSH6 أظهر إرتباطا مهمًا مع النقائل الورمية، إلا إن معلمات MSI كان لها أقل قيمة إنذارية في دراستنا. هناك حاجة إلى مزيد من البحوث لإثبات فعاليتها في علاج سرطان المعدة.

الكلمات المفتاحية: سرطان المعدة الغدي، الكيمياء المناعية النسيجية، عدم إستقرار الساتل الميكروي، التصنيف الجزيئي، بروتين مثبط الورم 53.

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Research Article

Correlation between Demographic Characteristics and Oxidized Low-Density Lipoprotein (oxLDL-IgM and oxLDL -IgG) Antibodies Levels in Patients with Systemic Lupus Erythemat

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Abstract:

Background: Systemic Lupus Erythematosus (SLE) may affect one or more organ systems; as time goes on, other manifestations may start to appear. Musculoskeletal, cutaneous, renal, and endocrine systems are all involved in SLE. The nervous system, hematological, vascular, pulmonary, gastrointestinal, and ocular imbalance of the immune response and the production of autoantibodies such as anti-oxidized Low-Density Lipoprotein LDL antibodies have a clear impact on the body's organs and the development of complications of the disease.

Objectives: To assess the levels of anti-oxLDL (IgM-Abs) and anti-oxLDL (IgG-Abs) as biomarkers for disease activity in SLE patients and their relationship to demographic characteristics.

Methods: The study comprised 100 SLE patients admitted to the Rheumatology Unit at Baghdad Teaching Hospital, age range (33.4 to 9.95) years, including 7 males and 93 females. An enzyme-linked immunosorbent Assay ELISA was used to measure the levels of serum oxLDL (IgM- Abs) and oxLDL (IgG-Abs).

Results: The results of the present study showed that there was a significant difference between the levels of anti-oxLDL IgM antibodies(Abs) in SLE patients in obese and non-obese groups since the levels of anti-oxLDL-IgM Abs in obese patients were (3.14 µg/L) and non-obese patients were (5.13 µg/L) (P=0.005), while in SLE patients with Diabetes Mellitus (D.M.), the levels of anti-ox LDL-IgM Abs were (3.80 µg/L) and in SLE patients with no DM were (5.13 µg/L). Also, the results showed that there were no significant differences between levels of anti-oxLDL IgG Abs in obese patients with SLE (6.28 µg/L) and non-obese patients with SLE (10.25µg/L) P > 0.05.

Conclusion: There was a significant difference between levels of anti-oxLDL IgM Abs in obese and non-obese patients with SLE, and no significant differences between the levels of anti-oxLDL IgG Abs in the same groups of patients.

Keywords: Immunoglobulins G; Immunoglobulins M; Oxidized Low-Density Lipoprotein antibody; Obesity; Systemic Lupus Erythematosus.

Introduction:

Systemic lupus erythematosus (SLE) is an autoimmune condition that can affect virtually any organ in the body and is relapsing-remitting. The result is tissue damage and systemic inflammation, which is marked by the creation of autoantibodies, the growth of immune complexes, and the deposition of autoantibodies. [1].

The development of lupus is strongly predisposed in females of reproductive age. In women between the ages of 15 and 44, the female-to-male ratio for the prevalence of lupus can reach 13:1, whereas in children and the elderly, it is only 2:1[2] Chronic Obstructive Pulmonary Disease (COPD), malignancies, and cardiovascular disorders are smoking. Smoking appears to be a significant risk factor for SLE in addition to its regular side effects, and it has a negative impact on both the progression of the condition and the effectiveness of available treatments [3]. Despite the inherent heterogeneity and research design limitations, there are signs that smoking causes illnesses such as rheumatoid arthritis, Grave's disease, and multiple sclerosis. [3, 4]. Additionally, smoking increases comorbidities in lupus patients, such as Atherosclerosis, at a risk comparable to Diabetes mellitus [5] Obesity has been linked to the pathophysiology of SLE because it can create a systemic milieu that is low-gradely inflamed by increasing the production of cytokines such as tumor necrosis factor-alpha (TNF-) and interleukin 6 (IL-6) [6]. SLE is also linked to a higher risk of developing Diabetes Mellitus (DM). Type 1 Diabetes and other autoimmune diseases were more likely to occur in SLE patients [7]. The relationship was first demonstrated in the context of hypercholesterolemia, where the lowest risk of Coronary artery disease for a given degree of hypercholesterolemia was associated with the

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highest IgM levels. IgG and IgM have been demonstrated to be independent predictors of Coronary artery disease (CAD) development, as well as potential moderators of the CAD risk linked to rising levels of oxidative Biomarkers, in an epidemiological cohort of initially healthy individuals [8]. The increased risk of Atherosclerosis and CVD in SLE cannot totally be accounted for by the known risk factors [9] Their significance must not be overlooked. The onset of CVD is significantly correlated with age. An increased risk of clinical CVD has been linked to male sex, hypertension, and dyslipidemia [10,11,12]. Oxidized LDL (ox-LDL) is thought to be a major Atherosclerosis antigen. In both atherosclerotic lesions and human plasma, anti- OxLDL antibodies have been identified. It has not yet been conclusively determined whether the immune response is primarily pro- or antiatherogenic. The majority of research has demonstrated a link between Atherosclerotic disease and higher IgG titers against OxLDL Although fewer studies have looked into IgM titers, the majority of studies appear to show an inverse link between IgM titers and Atherosclerotic disease. immunization with oxLDL induces antibody formation (both IgG and IgM) and protects against Atherosclerosis development [13]

The current study aimed to assess the levels of oxLDL (IgM-Abs) and oxLDL (IgG-Abs) as Biomarkers for disease activity in SLE patients and their relationship to demographic characteristics.

Patients and Methods:

The current study involved (100) patients (7 males,93females) with SLE and the age range was 33.4±9.95 years for the patients admitted Rheumatology Unit in the Baghdad Teaching Hospital from 09/11/2021 2021 to 18/01/2022.

The rheumatologist used the 2012 Systemic Lupus Erythematosus International Collaborating Clinics (SLICC) [14] criteria and the 1997 updated Systemic Lupus Erythematosus (SLE) criteria of the American College of Rheumatology (ACR)[15], which are based on clinical examination and laboratory evaluation, to make the diagnosis.

The current study received approval from the College of Medicine scientific ethics committee at the University of Baghdad. Blood samples were collected, to get baseline information for each participant. After that, sera were kept at -20°C. Each serum sample underwent evaluation for detection of anti-oxLDL (IgM) Abs and anti-oxLDL (IgG) Abs utilizing an enzyme-linked immunosorbent assay (ELISA), as directed by the manufacturer (Sun Long Biotech Company, China). The absorbance was measured at 450 nm. All immunological tests were carried out in the Medical Research Unit at the College of Medicine, Al-Nahrain University.

Statistical analyses: The SPSS statistics software for Social Sciences was used to perform the statistical analysis (version 20.0 for Windows, SPSS, Chicago, IL, USA). Because of the non-normal distribution of the oxLDL (IgM) Abs and oxLDL- (IgG) Abs, median and IQR (Inter Quartile Range) were used to describe them (Kolmogorov-Smirnov test). Mann-Whitney test was used to study the difference between the two groups. Qualitative data is represented as count and percentage. The chi-squared test was used to test the relation of qualitative data. Pearson correlation test was used to test the relation between quantitative data. P- value of <0.05 was considered statistically significant.

Results

Table (1) shows some characteristics of SLE patients, such as active disease hypertension, obesity, hair loss, smoking, thyroid disease, anemia, diabetes, and CVD. The results showed that 50 (69.4%) of the SLE patients had disease activity, while 22 (30.6%) patients with inactive phases of SLE**.** Also,33.3% of patients with SLE had hypertension, compared to 66.7% of patients with SLE who did not have hypertension, while 82% of SLE patients had no obesity, and 17.7% of SLE patients were obese. Anemia was seen in 56.2% of SLE patients, compared to 43.8% of SLE did not have anemia. The percentage of SLE patients who had hair loss was 58.3%, compared to 41.7% of those who did not have While 25.8% of patients had thyroid disease compared with 74.2% of SLE patients who did not have.

Table (1): Demographic Characteristics of patients SLE

		Count	%
Disease activity	Active	50	69.4
	Inactive	22	30.6
Hypertension	Yes	32	33.3
	No	64	66.7
Obesity	Yes	17	17.7
	No	79	82.3
Anemia	Yes	54	56.2
	No	42	43.8
Hair loss	Yes	56	58.3
	No	40	41.7
DM	Yes	18	18.6
	No	79	81.4
Thyroid	Yes	25	25.8
	No	72	74.2
CVD	Yes	3	3.1
	No	94	96.9
Smoking	Yes	4	4.1
	No	93	95.9

Figure (1) showed that there were significant differences between obese patients with SLE(5.13%) and non-obese patients with SLE (3.14%)for oxLDL IgM-Abs, P- value (P<0.005) and significant differences between SLE patients with DM for anti oxLDL IgM Abs (5.13%) and patients with SLE non-diabetic (3.8%) (P<0.005). Also, the results showed in Figure (2) that there were no significant differences in oxLDL IgG Abs levels in Diabetic patients with SLE(10.82%) and non-DM patients with SLE(5.45%) (P< 0.005).

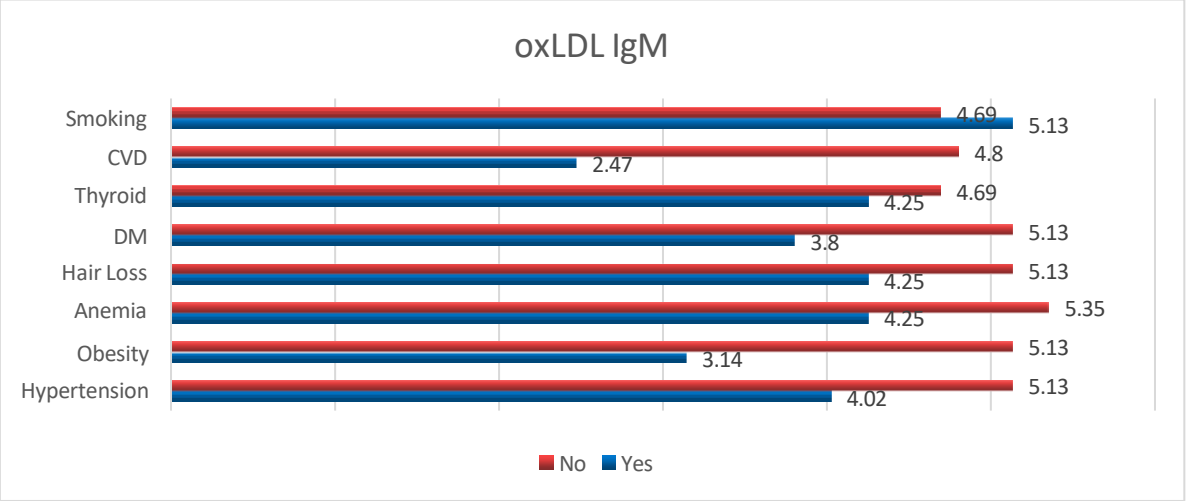

Fig.1: The percentages of oxLDL-IgM Abs in SLE patients according to demographic characteristics of SLE patients

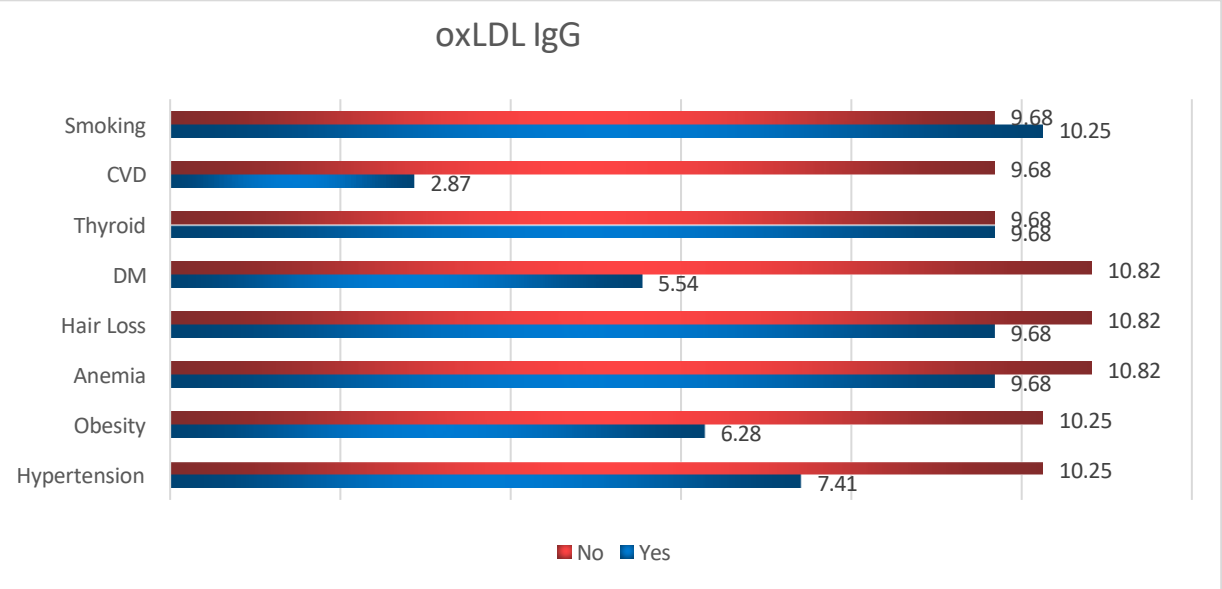

Fig.2: The percentages of oxLDL-IgG Abs in SLE patients according to demographic characteristics of SLE patients.

Discussion:

Table (1) demonstrates the various characteristic features of SLE patients such as the disease activity, and this may be due to the failure of SLE patients to adhere to their treatment, whereas patients who undergo customized treatment have shown good results in reducing SLE activity and these results in agreement with that reported by Petri *et al*,1992, that primary and secondary prevention strategies directed at hypertension, hypercholesterolemia, and obesity, as well as other known CAD risk factors, should be routinely employed in the management of patients with SLE(16). According to the results of the represented study, 32 (33.3%) SLE patients were suffering from hypertension, this result was in agreement with that reported by (Nived, *et al*,2020) who revealed that 15% of SLE patients had high blood pressure [17], while Mungu-Realpozo reported that cardiovascular disease had a higher prevalence in people with systemic lupus erythematosus (SLE) (CVD), which was partly due to traditional vascular risk factors like hypertension. was found that 66.7% of SLE patients had high blood pressure. According to WHO, hypertension affects 14% to 60% of SLE patients, making it more common than it is in the general population [18]. The current study found that the number of SLE patients was 17 (17.7%)who suffering from obesity and had high body mass index, as shown in Table (1) and these results were in agreement with that reported by (Patterson, *et al*, 2019) that although the exact function that obesity plays role in disease activity is unknown, it has been linked to the accumulation of SLE damage, particularly lupus nephritis, as well as other risk factors such as disease duration, aging, and higher steroid use. The risk of atherosclerosis rises with increasing waist size [19]. According to earlier research on SLE-affected women, obesity is independently linked to the disease's negative consequences, such as depression, disease activity, exhaustion, and pain. Obesity reduction is a crucial objective for patients' health. [20]. Table (1) shows that there were 58.3% of SLE patients had hair loss, these results were in agreement with that reported by Segura *et al*,2020 that there are several complications that result in SLE patients as a result of the long period of disease, including the period of taking steroid medications, and as a result, it leads to exposure to several complications, including diabetes, thyroid disorders, anemia, and hair loss due to the exposure of various parts of the body to damage [21,22].

There were18.6% of SLE patients had D.M.and there were 81.4% did not have D.M., these results in agreement with that reported by Masztalewicz *et al*, 2014 that cardiovascular disease is more likely to be the reason for death in those with SLE who had it longer than five years (CVD) Epidemiological findings in inflammatory disorders like SLE, additional processes (atypical/disease-specific factors) accelerate atherosclerosis when combined. Age, hypertension, diabetes mellitus, dyslipidemia, a history of a vascular event, such as ischemic heart disease or cerebrovascular accident, menopause, and smoking are all traditional causes of cardiovascular disease risk factors, with more conventional risk elements. [23].The results of the present study showed that the levels of ox-LDL IgM Abs in diabetic patients with SLE were 5.13µg/L, and 10.82 µg/L of ox LDL-IgG Abs, and these results were in agreement with that reported by Omer *et al*,2017and Van den berg *et al*,2019 that many studies showed that the high levels of (ox-LDL)antibodies in people who suffer from various diseases such as diabetes, hypertension, thyroid disease, Atherosclerosis Rheumatoid arthritis, in addition to other autoimmune diseases [24,25,26,27] Furthermore, there were 5.13% of OxLDL IgM Abs and 10.28% of OxLDL IgG Abs, in non-diabetic patients with SLE Conversely, the percentage of oxLDL- IgM Abs and OxLDL -IgG Abs was (3.8%, and 5.54%) in diabetic patients with SLE respectively. Additionally, the levels of OxLDL- IgM Abs and OxLDL- IgG Abs in non-obes patients with SLE were (5.13% and 10.26%) respectively. Furthermore, the percentage of obese patients with SLE in both OxLDL IgM and OxLDL IgG Abs were 3.14% and 6.28% respectively. In addition, the results of the present study showed that SLE patients with

D.M. had higher levels of ox LDL- IgG Abs which were10.82µg/L than oxLDL- IgM Abs which were 5.54 µg/L and these results agreed with that reported by Maria *et al*.,2011 that human-modified LDL, Abs are predominantly of the IgG Abs isotype easily across the endothelial barrier (28).

Conclusion:

The results of the present study showed that SLE patients with D.M. had higher levels of ox LDL- IgG Abs than oxLDL- IgM Abs. Also, there was a significant difference between levels of OxLDL- IgM Abs in obese patients with SLE and non-obese patients with SLE, and no significant differences between the levels of OxLDL- IgG Abs.

Authors' declaration:

We confirm that all the Figures and Tables in the manuscript belong to the current study. Besides, the Figures and images, which do not belong to it have been given permission for re-publication attached to the manuscript. Authors sign on ethical consideration's approval-

Ethical Clearance: This study was approved by the Scientific Ethics Committee of Communicable Clinical infectious Diseases Research Unit. It is also approved by the Iraqi Ministry of Health and the

Ministry of Education and Scientific Research according to the code number (88 IN 25/10/2021)

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Author Contributions:

Study conception & design: (Suha A. Al-Fakhar, Muhammed H. Al-Asami). Literature search: (Nusaibah Kh. Saddam). Data acquisition: Nusaibah Kh. Saddam, Suha A. Al-Fakhar). Data analysis & interpretation: (Nusaibah Kh. Saddam, Suha A. Al-Fakhar). Manuscript preparation: Nusaibah Kh. Saddam, Suha A. Al-Fakhar). Manuscript editing & review: (Nusaibah Kh. Saddam, Suha A. Al-Fakhar, Muhammed H. Al-Asami).

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العلاقة بين الخصائص الديموغرافية لمرضى داء الذئبة الحمامي المجموعي مع مستوى الاضداد المناعية المضادة للاكسدةOxLDL-IgM وOxLDL-IgG.

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الخلاصة:
داء الذئبة الحمامي المجموعي (SLE) قد يؤثرعلى واحد أو أكثر من أجهزة الجسم، ومع مرور الوقت، قد تبدأ المظاهر الأخرى بالظهور. الجهاز العضلي الهيكلي، والجلد، والكلى، وأنظمة الغدد الصماء كلها متورطة في مرض داء الذئبة الحمامي المجموعي. إن اختلال توازن الجهاز العصبي، الدم، الأوعية الدموية، الرئوي، الجهاز الهضمي، والعين في الاستجابة المناعية وإنتاج الأجسام المضادة الذاتية كأجسام مضادة للأكسدة LDL (مضادات oxLDL) لها تأثير واضح على أعضاء الجسم وتطور مضاعفات المرض.
الهدف من الدراسة: تقييم مستويات اضداد oxLDL (IgM) واضداد(oxLDL (IgG-Abs كمؤشرات حيوية لنشاط المرض لدى مرضى داء الذئبة الاحمامي المجموعي وعلاقتها بالخصائص الديموغرافية.
المرضى وطرق العمل: اشتملت الدراسة على 100 مريض بمرض داء الذئبة الحمامي المجموعي 7 ذكور (7٪) و93 (93٪) إناث تتراوح أعمارهم بين 33.4 ± 9.95 سنة والذين دخلوا وحدة االمفاصل بمستشفى بغداد التعليمي. تم تقييم مستويات اضداد oxLDL IgM وoxLDL IgG باستخدام المقايسة الامتصاصية المناعية بالإنزيم المرتبط (ELISA).
التحليل الاحصائي: تم استخدام البرنامج الإحصائي للعلوم الاجتماعية لإجراء التحليل الإحصائي (SPSS؛ الإصدار 20.0 لنظام التشغيل Windows، SPSS، Chicago، IL، USA), يتم استخدام المتوسط والانحراف المعياري والمدى لتصوير البيانات الكمية. تم استخدام اختبار الطالب لفحص الاختلافات بين مجموعات المريض والسيطرة. بسبب التوزيع غير الطبيعي لـ (IgM) oxLDL و(IgG)oxLDL، تم استخدام الوسيط وIQR المدى الرباعي) لوصفهما (اختبار .Kolmogorov-Smirnovوتم استخدام اختبار مان ويتني لدراسة الفرق بين المجموعتين. يتم تمثيل البيانات النوعية كعدد ونسبة مئوية. تم استخدام اختبار مربع كاي لاختبار العلاقة بين البيانات النوعية. تم استخدام اختبار ارتباط بيرسون لاختبار العلاقة بين البيانات الكمية. واعتبرت قيمة P <0.05 ذات دلالة إحصائية
النتائج: أظهرت الدراسة الحالية أن هناك فرقا معنويا بين مستويات الأجسام المضادة OxLDL IgMفي مرضى داء الذئبة الحمامي المجموعي الذين يعانون من السمنة المفرطة ومجموعات غير البدينين، حيث أن مستويات اضداد oxLDL-IgM في المرضى الذين يعانون من السمنة كانت (3.14 ميكروغرام / لتر) والمرضى غير البدينين كانت (5.13 ميكروغرام / لتر). كانت قيمة P 0.005 بينما مرضى داءالذئبة الحمامي المجموعي كانت مستويات اضدادox LDL-IgM (3.80 ميكروغرام / لتر) وفي مرضى داء الذئبة الحمامي المجموعي الذين ليس لديهم مرض السكري كانت (5.13 ميكروغرام / لتر)، بينما أظهرت النتائج عدم وجود فروق ذات دلالة إحصائية. بين مستويات اضداد oxLDL IgG في مرضى داء الذئبة الحمامي المجموعي الذين يعانون من السمنة المفرطة وغير البدينين. ومرضى داء الذئبة الحمامي المجموعي مع مرض داء السكري. كانت قيمة P>0.05.
الاستنتاجات: عند مقارنة مرضى داء الذئبة الحمامي المجموعي، تم العثور على فروق ذات دلالة إحصائية بين مستويات اضداد oxLDL IgM في المرضى البدناء والمصابين بداء الذئبة الحمامي المجموعي وغير البدناء وعدم وجود فروق ذات دلالة إحصائية بين مستويات اضداد oxLDL IgG.
الكلمات الرئيسية: داء الذئبة الحمامي المجموعي , الاضداد المضادة للأكسدة (oxLDL (IgMو(oxLDL (IgG , مرض السمنة مع داء الذئبة الحمامي المجموعي ,التهاب المفاصل الرثوي,امراض المناعة الذاتية.

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Research Article

Evaluation of Two Readings for the QuicGM *Aspergillus* Lateral Flow Assay in a Group of Immunocompromised Patients in Iraq

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Abstract

Background: *Aspergillus* spp. causes a wide range of diseases called Invasive Aspergillosis (IA) which is a fatal illness that affects a variety of immunocompromised people worldwide.
Objective: Using the lateral flow test for the detection of Galactomannan antigen in immunocompromised patients suspected to have IA.
Methods: This study was conducted on 72 patients, whose samples (serum, Bronchoalveolar lavage, Blood) were collected from the Hematology-Oncology Unit at Baghdad Teaching Hospital and Pediatric Welfare Hospital, and ICU in Ghazi AL-Hariri Surgical Specialties Hospital, and Bone Marrow Transplantation Center in period between November 2022 to February 2023. Patients' blood and sputum were sent for fungal culture to confirm the diagnosis in most cases.
Results: QuicGM *Aspergillus* Lateral Flow Assay was conducted on 72 patients, of whom 34 (47.2%) were positive and 38 (52.7%) were negative (*P*-value of 0.001 and 0.5) respectively. This screening aimed at detecting IA. One week later, the second confirmative result was obtained from 24 patients to determine the response to antifungal drugs or recovery from neutropenia on which 15 readings were negative and nine were positive. Out of 48 single readings, 25 were positive and 23 were negative. All for 72 members of the control group gave negative results. This study is the first in Iraq to use this assay.
Conclusion: QuicGM Aspergillus Lateral Flow Assay was found to be reliable, sensitive, and specific, and proved to be a very good guide for the early diagnosis of IA in immunocompromised patients and in monitoring treatment outcomes and follow-up.
Keywords: Aspergillus diagnostics; Galactomannan; Invasive Aspergillosis (IA); Lateral Flow Assay.

Introduction:

Invasive Aspergillosis (IA) is a fatal fungal illness that affects a variety of immunocompromised persons worldwide, including people with immunocompetent and non-neutropenic lung disease and viral or bacterial pulmonary infections, as well as those with hematological malignancies receiving chemotherapy and/ or immunosuppressive/ immunomodulatory drugs, neutropenia, stem cell transplantation, and organ transplantation (1). IA is caused by the very common and opportunistic fungus called *Aspergillus* spp (2). *Aspergillus* spp. is the second most common cause of fungus-related respiratory infections in critically ill patients, following fungi from the order Mucorales. They are not a part of the normal flora. There are numerous species of *Aspergillus* that are common in the environment around us, including *Aspergillus flavus* which represents the more common type of fungus that can grow on fruits, vegetables, and even in the air and soil(3). Regular inhalation of their spores has no adverse side effects, but some species, most notably *A. fumigatus*, are capable of spreading a variety of diseases, including allergic bronchopulmonary Aspergilloma, when the fungus spreads from the lungs, and causes widespread illness in the immunocompromised patient (4).

*Corresponding Author: hiba.altaee12@gmail.comMorphological

illness in the immunocompromised patient (4). and microscopic methods were used to identify this mold. The identification process was based on cultural characteristics such as colony morphology, the presence of septate hyphae, and the shape of conidial heads (5). The Taiwan Food and Drug Administration (TFDA) approved the Galactomannan (GM) assay in 2003 as a widely used enzyme immunoassay for the detection of IA. Galactomannan, a polysaccharide component of the *Aspergillus* cell wall produced in varying amounts by the fungus hyphae in the serum and nearby fluids of infected organs such as bronchoalveolar lavage (BAL) during invasive growth (6). it had been discovered by Reiss and Lehman as a potential indicator IA (7). For the diagnosis of the more invasive form of aspergillosis. The detection of GM might be regarded as a suitable assay.

The benefit of utilizing this assay, particularly its capacity to identify IA in its earliest stages (8). However, the GM assay has several methodological limitations since the test findings can be influenced by many circumstances and because it takes a lot of time and labor (9). The early diagnosis of IA is particularly important, the BAL fluid/serum Galactomannan assay is a useful auxiliary diagnostic

modality. Since the sensitivity of existing microbiological procedures is low, they take a long time to complete. Lateral flow assays (LFA) for the diagnosis of IA have recently been CE (Conformité Européenne) marked and commercialized. These assays can be used to test samples quickly (10), (11). Galactomannan cannot be used as a diagnostic indicator by itself. Clinical correlation, radiologic results, and underlying risk factors are all important considerations when deciding whether to start empiric treatment (12). It was done by using QuicGM *Aspergillus* LFA is based on fluorescent immunochromatography (13), (14). The main goal of our research is to aid physicians in rapid assessment of patients with invasive aspergillosis and monitoring patients during course of treatment with antifungal drugs. Patients who benefit from it is hematological malignancies with neutropenia, allogeneic hematopoietic stem cell transplantation, glucocorticoids use for over three weeks, immunosuppressant use, graft-versus-host disease. This assay may evolve to include all body fluids such as urine and cerebrospinal fluid.

Patients and Methods:

Seventy-two patients were included in this study. Samples of serum and Bronchoalveolar lavage were collected from patients in the Hematology-Oncology Unit at Baghdad Teaching Hospital and Pediatric Welfare Hospital (52 patients), Bone Marrow Transplantation Center (six patients), intensive care unit (ICU) in Ghazi Al-Hariri Surgical Specialties Hospital (four patients), and respiratory care unit (RCU) (10 patients). All of these were referred by Hematology-Oncology department specialists. Blood and sputum samples were cultured to confirm diagnosis. Seventy-two healthy controls were also enrolled in this study, selected as a disease-free and immunocompetent and healthy people from the community, their age ranged from 14-64 years old, while the patients' age ranged from 1-80 years. The data for the study was collected between November 2022 to February 2023. Azole drugs included in the present study are fluconazole and voriconazole. Mixed include combination of voriconazole + traditional ambisome, caspofungin + liposomal amphotericin, fluconazole+ liposomal amphotericin. Other antifungal drugs used for suspected IA with non-diagnostic causes. Point of care testing was performed using the QuicGM LFT according to the manufacturer's guidelines. Briefly, 300 μL of serum/BAL pipetted into 1.5 ml screw-cap polypropylene tubes. 100 μL of sample treatment solution added to the tubes containing the serum/BAL, then vortexed for 10 seconds to thoroughly mix the contents. The tubes were centrifuged at 10,000 × g for 1–5 seconds to shake the sample out of the tubes. The tube was heated in a water bath for 3–4 minutes at 100 °C. The tubes were centrifuged for 10 minutes at 10,000 g, then the supernatant was collected for testing. This assay was conducted on different groups patients within the Medical City Hospitals suspected of having invasive fungal infections, serving as a rapid means that take around (45 minutes) of identifying infection before initiating treatment thereby avoiding empirical therapy. Subsequently, in cases where the LFT yielded a positive outcome, coupled with a positive culture and corresponding positive radiological findings, the researchers administered tailored treatment based on medical assessment. Most of the patients were suffering from potential fungal infections affecting the lungs as observed through MRI or CT, complemented by sputum samples and blood culture.

Statistical Analysis:

All data of the current study were analyzed by using Statistical Package for Social Science version 26 and Excel 2016. Scale parameters were calculated by student t-test, and P values were less than 0.05 were considered significant. Sensitivity and specificity were calculated by receiver operating characteristic curve (ROC curve).

Results:

Of the 72 cases, 31 (43.1%) were females and 41 (56.9%) were males. The patients age range was 1-80 years. The control group consisted of 72 healthy persons who were negative for IA. Out of 72 blood cultures among the cases, 27 cases were positive for the following organisms (Candida spp. 10, Aspergillus spp. 9, Cryptococcus 8, Gram negative bacteria 5, Sporotherix schenkii 1, Histoplasma capsulatum 1). For sputum culture, only 15 out of 31 were positive for the following organisms Candida spp. 13, Cryptococcus 4, Gram-negative bacteria 2, Aspergillus spp. as shown in figure (1).

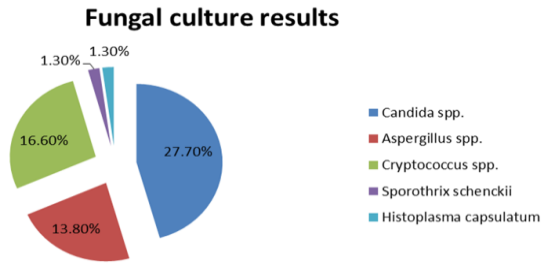

Figure (1): Distribution of types of microorganisms from blood and sputum cultures.

In this study, 72 LFT were conducted on the samples of 72 patients: 48 patients were with a single reading, of whom 25 (52.1%) were positive and 23 (47.9%) were negative (P-value 0.079 and 0.002, respectively, calculated by t-test) this is not a construction, t-test used in this study to see the significant differences between the means of two Galactomannan result, the percentage was demonstrated to show number of positive cases to be negative once. Most patients in this study were neutropenic (61%) (500-1000 cell\ml). A second reading was taken after one week for 24 patients to determine the response to antifungal drug or recovery from neutropenia, 9 of whom

(37.5%) were positive and 15 (62.5%) were negative (P-value of 0.01 and 0.012, respectively, calculated by t-test) table 1. There was no significant difference between the first and the second readings of QuicGM Aspergillus LFT. Thirty-four (47.2%) tests were positive and 38 (52.7%) were negative (P-value of 0.000 and 0.506) respectively, calculated by t-test)

Table 1: The mean values for the QuicGM LFT first and second readings

LFT reading	No.	Mean	±SD	*P* value(t-test)
Single				
+ve	25	1.17	1.055	0.079
-ve	23	0.35	0.099	0.002
Total	48	0.76	0.850	0.488
Second				
+ve	9	0.74	0.208	0.01
-ve	15	0.34	0.100	0.012
Total	24	0.49	0.245	0.288

The current study found that there is a highly significant difference between patients who had a positive fungal growth in either blood or sputum culture where 16 patients showed a positive LFT and 20 patients showed a negative LFT, (*P*-value < 0.001 calculated by t-test) table 2.

Table (2): Relationship between positive fungal growth and the readings of QuicGM *Aspergillus* LFT

Fungal growth	LFT Level	N	Mean	Std. Deviation	*P* – Value
Positive growth	+	16	0.91	0.318	< 0.001
	-	20	0.32	0.101	
	Total	36			

According to antifungal treatment, for patients treated with traditional Ambisome, four of them were positive for LFT, while seven patients were negative, (*P* -value < 0.003, calculated by t-test). Twenty patients were treated with liposomal amphotericin B, of whom only nine were positive for LFT and 11 were negative with highly significant differences (*P*-value 0.001, calculated by t-test). Nine patients were treated with caspofungin, seven of whom were positive and two were negative for the LFT with a highly significant difference (*P*-value 0.001, calculated by t-test). Other azole drugs were used (fluconazole and voriconazole) for three LFT positive cases and seven negative patients, (*P*-value 0.015, calculated by t-test). Drug combinations include (voriconazole + traditional ambisome), (caspofungin + liposomal amphotericin), (fluconazole + liposomal amphotericin), for five positive and five negative patients (*P*-value 0.008, calculated by t-test). Other antifungals drugs were used for suspected IA and non-diagnostic causes for five positive and five negative patients (*P*-value 0.014, calculated by t-test), table (3).

Table 3: Relationship between the type of antifungal drugs used and results of QuicGM *Aspergillus* LFT

Antifungal	LFT Level	N	Mean	Std. Deviation	*P* – Value
Traditional AB	+	4	2.145	2.503	0.003
	-	7	0.37	0.084	
	Total	11			
Liposomal	+	9	1.03	0.406	0.001
	-	11	0.39	0.091	
	Total	20			
Caspofungin	+	7	0.89	0.270	0.001
	-	2	0.24	0.049	
	Total	9			
Azole	+	3	0.73	0.272	0.015
	-	7	0.32	0.158	
	Total	10			
Mixed	+	5	0.79	0.262	0.008
	-	5	0.334	0.109	
	Total	10			
Other	+	5	0.91	0.142	0.014
	-	5	0.29	0.142	
	Total	10			

Forty-four out of seventy-two patients had neutropenia 44 (61.1%), 19(43.1%) of them were positive for QuicGM *Aspergillus* LFT and 25 (56.8%) of them were negative for QuicGM *Aspergillus* LFT with significant difference *P*-Value (<0.05) as in table (4).

Table 4: Relationship of neutropenia with QuicGM *Aspergillus* LFT readings:

	LFT Level	N	Mean	Std. Deviation	*P* - Value
Low WBC (neutropenia)	+	19	0.87	0.344	< 0.05
	-	25	0.384	0.260	
	Total	44			

Standard curve:

QuicGM *Aspergillus* LFA (using 0.5 cutoffs) had a sensitivity of 89% and a specificity of 100%. Figure (2)

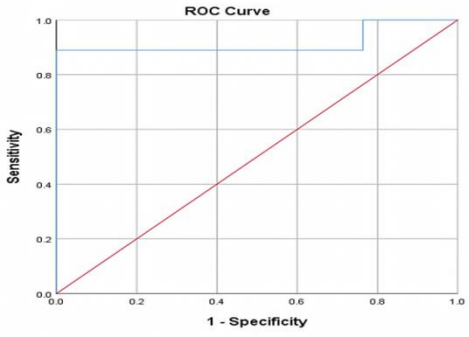

Figure (2): The standard curve

Discussion:

QuicGM *Aspergillus* Lateral Flow Assay is one of the novel methods for IA diagnosis. (15).

The LFA is a self-contained fluorescent immunochromatographic assay for the qualitative and quantitative identification of *Aspergillus* GM in serum and BAL sample. (16) QuicGM *Aspergillus* LFT was widely used in the hospitals of Belgium, a wide range of LFAs are implemented in Jodhpur, Rajasthan, and Turkey. (17) (18) (19) The LFT test was conducted twice on 24 patients, initially on days

1 and 7 days later, with the majority of the patients having decreased Galactomannan levels in the second test, as a result of their positive response to the treatment they received. Follow up of patients is very useful in two readings measure within the first week of antifungal therapy as rising titer refers to active fungal infections while decreased values refereed to good response to adequate antifungal therapy. In the current study, the second test revealed lower Galactomannan antigen levels in seven patients, and that was in agreement with Taghavi et al. who stated that patients with a high risk of IA should have a baseline serum test and have their levels of GM antigen monitored twice a week. (20)

The distribution of the study groups based on blood disease data showed that IA is more prevalent in Acute lymphoblastic leukemia (ALL) due to lack of lymphocyte generation and trafficking, as well as changes in the way lymph organs operate, which are characteristics of lymphocytic leukemia. Contrary to earlier research that claimed patients with acute myeloid leukemia most usually develop IA, as the adaptive immune system is linked to modified and defective lymphocytic function. (21) (22) The distribution of fungal infection in blood and sputum samples varied significantly, with the majority of these samples showing yeasts, molds, and Gram-negative bacteria this was in agreement with other studies where the presence of bacteria in the blood is frequently linked to serious diseases. (23) (24) Liposomal amphotericin was the drug of choice for treating invasive pulmonary Aspergillosis (IPA) because it is the medication that is most readily available in Iraq and this was in agreement with other studies. (25) (26) According to the blood count data, the majority of the patients had neutropenia, which is the body's main defense against infection. When exposed to *Aspergillus* spores, neutropenic individuals are more likely to develop a fungal infection, particularly when intense chemotherapy causes the polymorph nuclear cell level to be below 1000 cells/ml. (27)

Conclusion:

QuicGM *Aspergillus* Lateral flow assay can be used as a diagnostic method in conjunction with other diagnostic procedures and as an aid in the diagnosis of IA. The two readings were very useful in the follow up of patients' response to antifungal therapy.

Authors' declaration:

We confirm that all the Figures and Tables in the manuscript belong to the current study. Besides, the figures and images, which do not belong to the current study, have been given permission to be republicated and attached to the manuscript. Authors sign on ethical consideration's approval-Ethical Clearance: The project was approved by the local ethical committee in (Hematology-Oncology Unit at Baghdad Teaching Hospital and Pediatric Welfare Hospital, and ICU in Ghazi AL-Hariri Surgical Specialties Hospital, and Bone Marrow Transplantation Center) according to the code number (0211) on (16/ 07/ 2022).

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Author contributions:

Study conception & design: Wifaq M. AL-Wattar Literature search: Hiba S. Kareem Data acquisition: Hiba S. Kareem Data analysis & interpretation: Hiba S. Kareem. Manuscript preparation: Hiba S. Kareem. Manuscript editing & review: Hiba S. Kareem & Wifaq M. AL-Wattar

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تقييم قراءتين لأختبار التدفق الجانبي السريع لكالاكتومانان الرشاشيات في مجموعة المرضى الذين يعانون من النقص المناعي في العراق

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الخلاصة

الخلفية: يسبب جنس الرشاشيات مجموعة واسعة من مرض داء الرشاشيات والذي هو مرض قاتل يصيب مجموعة متنوعة من الاشخاص الذين يعانون من نقص المناعة في جميع انحاء العالم. يعد الكالاكتومانان علامة حيوية مهمة تم استخدامها تاريخيا في تشخيص ومراقبة الالتهابات الفطرية وخاصة داء الرشاشيات الغازي الذي يمكن اكتشافه في مصل الدم وسائل غسيل القصبات

.الاهداف: استخدام مقايسة التدفق الجانبي للكشف عن مستضد الكالاكتومانان في مرضى العوز المناعي الذين يعانون من عدوى فطرية هوائية مشتبه بها.

المرضى والطرائق: اجريت هذه الدراسة على 72 مريضا وتم جمع عيناتهم (مصل الدم، غسيل القصبات) من وحدة امراض الدم والاورام في مستشفى بغداد التعليمي، مستشفى الطفل المركزي، مركز زرع نخاع العظم، ووحدة العناية المركزة في مستشفى غازي الحريري للتخصصات الجراحية بين تشرين الثاني 2022 وشباط 2023. تم إرسال دم المرضى والبلغم لإجراء زراعة فطرية لتأكيد التشخيص في معظم الحالات. باستخدام اختبار التدفق الجانبي السريع للرشاشيات ووسط زرع الدم المناسب ومن ثم زرع النخامة.

النتائج: تم اجراء مقايسة التدفق الجانبي للرشاشيات على 72 عينة في قراءة اولى حيث تبين ان 34 (47.2%) كانت موجبة و 38 (52.7%) عينة سالبة وكانت (*P*-value of 0.001 and 0.5) يهدف هذا الفحص إلى الكشف عن داء الرشاشيات الغازي وبعد أسبوع واحد تم الحصول على النتيجة التأكيدية الثانية من 24 مريضًا لتحديد الاستجابة للأدوية المضادة للفطريات أو التعافي من قلة العدلات حيث كانت (15 كانت سلبية 9 كانت ايجابية) وومن بين 48 قراءة منفردة (25 كانت موجبة و 23 كانت سالبة). كل القراءات ل 72 حاله ضابطة اعطت نتائج سلبية. تعتبر هذه الدراسة الاولى التي استخدمت هذاالاختبار في العراق.

الإستنتاجات: كان مقايسة التدفق الجانبي للرشاشيات موثوقا، ذو حساسية و خصوصية، واثبت انه دليلا جيدا جدا لتشخيص داء الرشاشيات الغازي في مجموعة المرضى الذين يعانون من نقص المناعة ومراقبة متابعة العلاج.

الكلمات المفتاحية: اختبار التدفق الجانبي; الكالاكتومانان; داء الرشاشيات الغازي; تشخيص الرشاشيات

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Research Article

Extraction and Identification of the Main Components of Cloves (*Syzygium aromaticum* L.) Oil Extract and its Antimicrobial Activity against Methicillin-resistant *Staphylococcus aureus* strain

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Abstract

Background: Methicillin-resistant *Staphylococcus aureus* is widely recognized as a significant etiological agent responsible for infections around the world. One of the biggest problems in world health care is antibiotic resistance to the MRSA strain. The use of herbal medicines is one of the promising techniques for countering bacterial resistance to antibiotics.

Objectives: The study is designed to investigate the chemical composition of clove oil extract and its *in-vitro* antibacterial activities against MRSA.

Methods: The clove oil extract was obtained by using hydro-distillation by Clevenger apparatus. After that, phytochemical analysis was done to determine the secondary metabolites by Chromatography-Mass Spectrometry. *In-vitro* antimicrobial activity of clove oil extract against Methicillin-resistant *Staphylococcus aureus* was carried out by agar well diffusion method, the broth microdilution method, and *in-vitro* time-kill curve kinetic. Least significant difference –LSD test (Analysis of Variation-ANOVA) was used to significant compare between means of results in this study.

Results: The results of this study revealed that the extraction percentage of the clove yielded 50%. The Chromatography-Mass Spectrometry results of the clove oil extract analysis showed that caryophyllen at 28.9%, Humulene at 21.6% and eugenol at 13.06% were the primary bioactive ingredients of the prepared extract. Furthermore, the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of clove oil extract against *Methicillin-resistant Staphylococcus aureus* were found to be 2.5µg/ml and 5.0 µg/ml respectively. Time killing curve of 2xMICs and 4xMICs of clove extract achieved the highest significant bactericidal effect (P≤0.05) in comparison to other concentrations.

Conclusions: The clove oil extract exhibited good *in-vitro* antibacterial properties and this can be attributed to the presence of phenolic compounds such as caryophyllene, humulene and eugenol.

Keywords: Antimicrobial, Caryophyllen, Clove extract, *Staphylococcus aureus* (MRSA), Time killing curve.

Introduction

Multidrug-resistant bacteria causing infectious diseases were responsible for significant mortality, particularly in developing countries (1). High prevalence of methicillin-resistant *Staphylococcus aureus* strains (MRSA) was recorded in both health-care and community environments (2,3,4). To develop new drugs to treat multidrug-resistant pathogens, the search for novel antibacterial compounds, including herbal products, has increased quickly (5,6). Traditional treatments frequently use pharmaceuticals made from aromatic herbs to treat bacterial infections (7). Numerous investigations claim that utilizing essential oils can help to decrease antibiotic-resistant bacteria (8).

These oils have a wide range of biological and pharmacological effects and are very volatile, lipophilic, and hydrophobic (9).

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Syzygium aromaticum (family- *Myrtaceae*), more popularly known as clove, is indigenous to the Indonesian islands. Almost there is thirty compounds have been identified in Clove Essential Oil Composition **(**CEO), eugenol is the main component, composing at least half of its components. Caryophyllene, Eugenyl acetate, and humulene compose the remainder, which ranges from 10%–40%. The remainder is less than 10% which is considered minor elements(10). It is now grown all over the world as a flavoring ingredient, a medicine, and an ingredient in perfumes (11,12). Due to its antibacterial abilities, it is frequently used as a food preservative (13). Analgesic, antioxidant, anti-inflammatory, anesthetic, and insecticidal action has also been documented in addition to its antibacterial property (14) Additionally, the antibacterial activity of clove oil have been studied against a large number of multi-resistant *Staphylococcus* and oral pathogens(15). The

development of innovative antimicrobial treatment agents is necessary due to the high prevalence of bacterial strains that are resistant to many drugs. Thus, the goal of the current study was to determine the chemical composition as well as the *in-vitro* antibacterial effect of clove oil extracts against pathogenic MRSA bacterial strains.

Materials and Methods

Collection of plant: The origin of clove *(S.aromaticum*) utilized in this study is from a local market in Baghdad, Iraq, and classified by the National Center For Herbal Medicine and Al-Razi Center for Medical Herbs. Clove buds were cleaned with 5% sodium hypochlorite solution (NaOCl), rinsed three times with distilled water, and then kept to dry. Using a mechanical mortar, the dried plant was ground into powder.

Extraction of clove oil (*Syzigyum aromaticum)*: Clove oil was extracted from dry buds using hydro-distillation by Clevenger apparatus. After drying with sunlight, it was placed in a 1000 ml glass flask then 500 ml of distilled water was added. The flask was connected to the apparatus and operated for three hours. The flask's temperature was first raised to about 80°C and then gradually increased to 100°C. The oil was collected after isolating the water, and to increase the water disposal a little of anhydrous sodium sulfate was used (16).

Extraction percentage yield of essential Clove oil The collected cloves sample was used to extract the essential oil by hydrodistillation method. The extraction yield has been accomplished by the equation below (17):

Oil yield % =Volume of oil extracted *100/weight of the sample

GC-MS analysis of *S. aromaticum* Oil extract*:* To identify the active components of clove extraction, Gas chromatography-mass spectrometry (GC-MS) was used to study the phytochemicals of the clove *(S. aromaticum)* extracts. By injecting 1 L of the sample (0.1% in absolute methanol) and operating in scan mode on the GC/MS Thermo Trace GC Ultra / TSQ Quantum GC-MS, the GC/MS analysis was carried out. Using an Agilent HP- 5ms Ultra Ineit capillary column (30 m 0.25 m film thickness), the phytochemical investigation was conducted. The rates of the four ramps were as follows: ramp 1 was 60 °C hold to 3 min, ramp 2 was 60 °C to 180 °C hold for 7 min, ramp 3 was 180 °C to 280 °C holding to 8 min, and Ramp 4 was 280 °C holding to 3 min. The following describes the operation's conditions: The carrier gas was helium, with a 99.99% purity, and the injector and detector were 250 °C hot. Comparing the results of the GC-MS analysis with the reference retention time and spectral mass data from the NIST database allowed the chemical components of the clove bud extract to be identified.

Bacteriological Examination

Test Organism: The Methicillin resistance *staphylococcus aureus* (MRSA) was obtained from the Physiology, Biochemistry, and Pharmacology Department/ College of Veterinary Medicine /University of Baghdad.

Activation and Maintenance of MRSA: Ten mL of brain heart infusion agar slants were used to activate bacterial cultures in screw-capped tubes and then placed in the incubator at 37 °C for 24 hrs. For maintenance of isolates, the bacterium was cultured on brain heart infusion agar and kept at 4°C, then the bacterium was activated every 14 days. These bacteria were established for microscopic morphological, cultural, and biochemical studies (18).

Preparation of standard bacterial suspension: By comparing to the Standard McFarland solution (0.5), the quantity of MRSA bacteria in each milliliter of the stock suspension was standardized (18). Briefly, bacterial suspension equivalent to 0.5 McFarland (1.5×10^8 CFU /ml) was arranged from overnight bacterial culture. The absorbance of this index was 0.136 as noted by the spectrophotometer at a wavelength of 450 nanometers. About 0.1 ml of the prepared bacterial suspension was diluted in 14.9 ml of Mueller-Hinton broth and incubated at 37 ºC for 1 hr. to obtain 10^6 CFU/ml bacterial suspensions to prepare bacterial suspension for the time-kill curve kinetic assay in order to bring MRSA bacteria to the logarithmic phase of bacterial growth (19).

Measurement of Antimicrobial Activity of clove oil extract:

Agar well diffusion method: The agar well diffusion method was carried out to evaluate the antibacterial activity of *S.aromaticum* extract against MRSA according to **(**20). Standardized bacterial suspension (1.5×10^8cfu/ml) of *S. aureus* was carefully mixed with sterile Mueller Hinton agar. Twenty-five ml of this agar was dispersed into sterile Petri dishes and left for 10 minutes at room temperature to dry, and six mm. diameter wells were bored in the agar. The *S.aromaticum* extract was reconstituted in distilled water to a concentration from5µg/ml to 0.312µg/ml and then 100µl was added to wells. The plates were incubated at 37 C° for 24 hours after allowing the extract to diffuse into the agar at room temperature. Three plates were made for each concentration with negative control using buffer phosphate and the strength of the clove oil extract was dictated by measuring the inhibition zone diameter around every well against the tested bacteria and used as positive bioactivity compared with negative control. Standard error and mean were calculated.

Microtiter plate Dilution: Clove oil extract (10 mg/ml) was made in Mueller-Hinton broth, from this broth, two folds dilution was downgraded from 40 µg for clove oil in U- shape (200 µl well capacity) 96 well micro-titer plate. Each well was inoculated with 100 µl of 1.5×10^8 CFU/ml *S. aureus* and

incubated at 37ºC for 24hrs. For colorimetric identification of bacterial growth, 15µl of 0.125% triphenyl tetrazolium chloride dye (TTC) which was used as an indicator of cellular viability was added to each well of the test and re-incubated for two hours to determine MIC by observing whether or not the red color that results from the reductions of TTC (colorless) to formazan (red) develops (21). While the minimum bactericidal concentration (MBC) was determined by subculturing 50 µl from the well that showed no apparent growth (clear) onto fresh nutrient agar plates. After the incubation period, if there was no growth this concentration was taken as MBC which is considered the lowest concentration of extracts that kill the bacteria (22).

Time Kill Curve Kinetic Assay*:* The time-kill curve assay of clove extract against MRSA bacteria was done according to the procedure described by (19). Briefly, bacterial suspension was prepared as mentioned before to obtain 10^6 CFU/ml bacterial suspensions, and the Clove extract had been dissolved in Mueller-Hinton broth to prepare 10 mg/ml stock solution. After that, clove extract concentrations from 4x MIC to 0.25x MIC were prepared. Bacterial colonies were calculated at 0, 1, 2, 4, 6, and 24 hr. through the incubation time by making serial dilutions and spreading of 20 µl of each dilution on Mueller-Hinton agar plate (triplicate); colonies range 30-300 CFU/plate was accepted (19).

The trapezoidal method was used to estimate the area under the time-kill curve of the concentration of clove extract as below (23):

$$AUC_{kill} = \sum \left(\frac{\log(Cn) + \log(Cn+1)}{2} . \Delta t \right)$$

Where the AUC_{kill} is the area under the killing curve of clove extract, log (Cn) is the logarithm of a specific concentration at a specific time, log (Cn+1) is the logarithm of the next concentration while (Δ t) resembles the difference between their times.

Statistical Analysis: The Statistical Analysis System- SAS program was used to detect the effect of different factors on study parameters. Least significant difference –LSD test (Analysis of Variation-ANOVA) was used to significantly compare between means in this study.

The authentication of the plant: The clove that utilized in this study was classified by the National Center For Herbal Medicine and Al-Razi Center for Medical Herbs as *Syzygium aromaticum* L. family of *Myrtaceae* to be used as oil extract in this study.

Extraction of essential Clove oil: The collected cloves sample was used to extract the essential oil by hydrodistillation method. The oil obtained was yellow in color as show in (Figure 1) with the highest extraction yield of 50%.

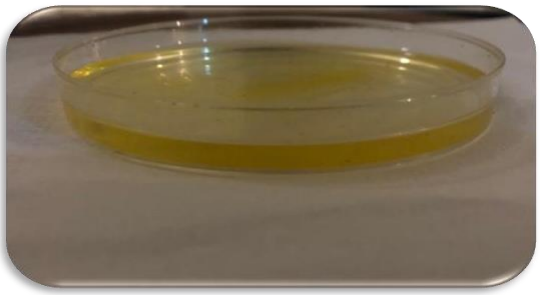

Figure1: Clove oil extract

Results:

Gas chromatography-mass spectrometry (GC-MS): Seventeen peaks related to separate components were obtained from gas chromatography–mass spectrometry (GC/MS) in (*S. aromaticum)* extract by hydrodistillation method (Figure 2).

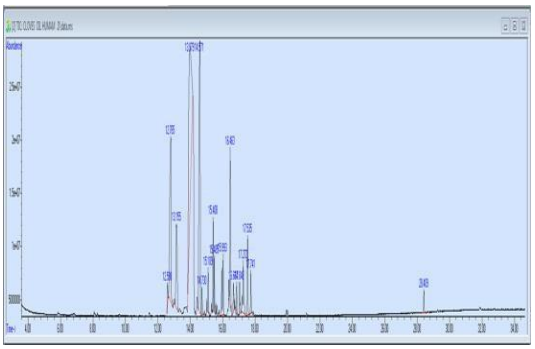

Figure 2: Gas chromatogram of clove oil extract

The main constituents were caryophyllen with 28.9%, eugenol 13.06%, and Humulene 21.6% from the GC-MS database. Additionally, other compounds were listed in (Table 1).

Table 1: Phytochemical analysis of *S. aromaticum* essential oil.

Compounds	Retention time	Percentage of total %
Caryophyllene	13.9	28.9
Humulene	14.5	21.6
Eugenol	12.7	13.06

Antibacterial activity of clove oil extract

Agar well diffusion method: Different concentrations of clove oil extract were used in agar well diffusion methods, resulting in different sizes of inhibition zones against methicillin resistance *Staphylococcus aureus*. The sizes of inhibition zones were different according to the concentration of the Clove oil extract. Results indicated that MRSA bacteria were sensitive significantly ($P<0.05$) to clove oil extract in a concentration-dependent manner 5, 2.5 ,1.25,0.625, and 0.312 µg/ml. Increasing the diameter measurement of the zone of inhibition in MRSA growth was proportionally related to clove oil extract concentrations, (Table 2 and Figure 3).

Table 2: Antibacterial activity of Clove oil extract in different concentrations against MRSA (measured as the diameter of the inhibitory zone in millimeters).

Groups	Con.(µg/ml)				
Conc.	5µg/ml	2.5µg/ml	1.25µg/ml	0.625µg/ml	0.312µg/ml
Clove oil extract	18.0±0.75 A a	15.0±0.58 B a	13.0 ±0.61 BC a	11.0 ±0.52 C a	9.0 ±0.38 C a
Buffer phosphate	0.0±0.0 A b	0.0±0.0 A b	0.0±0.0 A b	0.0±0.0 A b	0.0±0.0 A b
LSD value	3.337*				

- **Values represent mean ± S.E**
- **Different capital letters mean significant *(P<0.05)* results between different concentrations.**
- **Different small letters mean significant *(P< 0.05)* results between buffer and clove oil extract.**
- ***LSD: least significant difference**

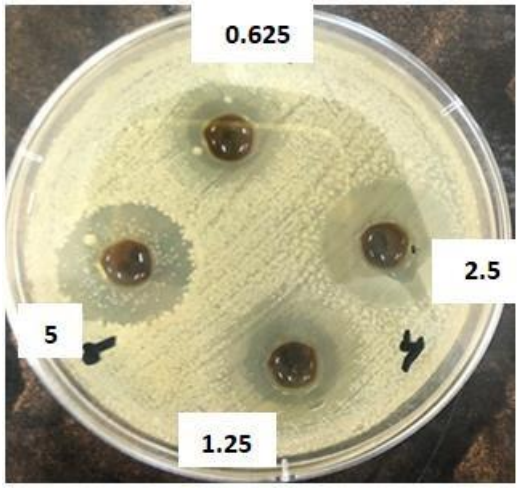

Figure 3: Susceptibility of MRSA strain to different concentrations of clove extract

Microtiter plate Dilution: The Minimum Inhibitory Concentration plays a key role in the determination of an antibacterial potency (24). The microdilution assay was used to determine MIC. The results showed that the concentration of 2.5 µg/ml of clove oil was effective in preventing MRSA from growing, this concentration was shown to have a positive value that inhibited the growth and 5.0 µg/ml of clove oil extract killed MRSA so they were considered as the (MBC). MBC value was tested in micro-dilution assay by sub-culturing 50 µl from the well that showed no apparent growth (clear), if there was no growth this concentration was taken as MBC.

Time Kill Curve Kinetic Assay: The time-kill curve kinetics of clove oil extract is based on the highest MIC recorded from the micro-dilution assay which was 2.5µg/ml for the MRSA strain. The concentrations used *in vitro* study were 0.25x MIC, 0.5x MIC, 1x MIC, 2x MICs, and 4x MICs.

The clove oil extract at a concentration of (4×MIC, 2×MIC) showed significant killing activities both at 6 and 24 hrs. respectively by reducing ≥ 3 log10 of the total number of CFU/ml in comparison to other concentrations used as shown in (Figure 4).

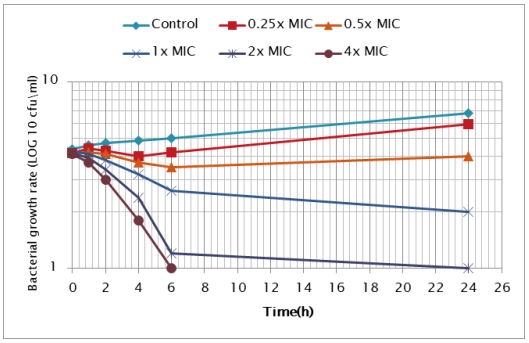

Figure 4: Time kill curve kinetics of clove extract against MRSA

The area under the time of killing curve of clove extract was calculated and compared to the control inoculum growth rate, and the difference in the area under the curve values among different treatments was set as an endpoint whereas the lowest area under the curve refers to the highest bactericidal effect as reported in the (Table 3). The results showed that both 2xMICs and 4xMICs achieved the highest significant bactericidal effect (*P≤0.05)* in comparison to other treatments. The 1xMIC concentration achieved a purely bacteriostatic effect (*P≤ 0.05*) in comparison to all concentrations and control groups; both 0.5xMIC and 0.25xMIC failed to achieve a significant bacteriostatic or bactericidal effect *(P≥0.05*) in comparison to 1xMIC, 2xMIC and 4xMIC.

Table 3: Area under the time-kill curve of clove extract against MRSA (h*log 10 CFU/ml).

Antibacterial	Control	0.25x MIC	0.5xMIC	1xMIC	2xMIC	4Xmic
Clove extract MIC=2.5µg\ml	125.3 ±0.31 A	111.5 ±0.3 A	86.81 ±0.30 B	57.22 ±0.19 C	29.87 ±0.5 D	17.25 ±0.9 D
SD				13.3*		

- **Values represent mean ± S.E**
- **Different capital letters denoted a significant difference (*p≤0.05*) among the groups.**
- ***** LSD: least significant difference**

Discussion

Clove oil extraction using hydro-distillation (water distillation) by Clevenger apparatus gave a bright yellow color oil and brown color extract with a typical clove oil smell. The extraction yield of clove extract was 50% as the amount of clove buds that were used in the current procedure was 200 g. However this outcome is nearly in line with the outcomes of the study of Ishaq and his colleagues(2019) who discovered that when clove bud powder was extracted using a soxhlet equipment, the yield of hexane extract was 48.84%
(25). Importantly, one of the reason of the resulting higher percentage of extraction may belong to the

reduction in the particle size as reported by Ratri and his colleagues(2020) as they suggested the percentage yield of clove extract is influenced by the cloves' particle size, with smaller clove particles producing higher extraction yields (26). However, the extraction procedure can also be influenced by several other factors, such as the quality and freshness of the clove buds, the temperature and pressure used during the distillation process, and the duration of the distillation process **(**27, 28).

To identify the active components of clove extraction, GC/MS was used and the main compounds of the *S. aromaticum* extract were caryophyllen, humulene, and eugenol. According to reports, these substances make up the majority of the clove buds' active ingredients which have a therapeutic use such as using as analgesic, antioxidant, anti-inflammatory, anesthetic, and antibacterial agents (29). In this study ground clove was used and found β-caryophyllene to be the most abundant since the volatile profile from whole buds showed a different pattern when compared to the volatile composition of ground clove. This is in agreement with Gaspar and his colleagues (2018) who approved that the most prevalent compound is β--caryophyllene. (49.31% concentration) as they compared compositions from whole and ground clove (30). However, other studies, have found a difference in the majority of compounds, such as the study of Lee (2009) who has shown that eugenol, β- caryophyllene, 2-propanone, and methylhydrazone are the main composites of *(S. aromaticum)*essential oil (31).

The anti-MRSA potential of clove oil extract has been reported, and the requirement to investigate bioactive components has been highlighted (32). The clove extract exerted the highest antimicrobial efficacy against the pathogenic bacterial strains (MRSA), which may be assigned to the high percentage of a phenolic compound of clove oil extract as presented in GC– MS results and this complies with the result of Alanazi and his colleagues (2022) who demonstrated the lowest concentration of clove oil that inhibit the growth of MRSA found to be 2.5 µL/Ml.(33).

The phytochemical constituents that are present in clove oil extract are responsible for the antibacterial activity. Eugenol is one of the bioactive components that may have considerably contributed to the antibacterial effects of clove oil (34). Humulene is another component that present in clove oil similar to caryophyllene with antibacterial properties (35). However, the minor components could potentially contribute by combining them with other main components to limit MRSA development. The oil's hydrophobic properties may also aid in interactions with the outer cytoplasmic membrane of MRSA, which impairs the integrity and functionality of the cell membrane (36).

Time killing-curve kinetic is a combined and extensive tool to assess both bacteriostatic and bactericidal effects of the antibiotics; it depends on the change in the logarithmic number of bacterial colonies through the defined chronological pattern (19). More accurate descriptions of antimicrobial activity are provided by a measure of bacterial killing (kill kinetics) than by the MIC, and it has also shown better sensitivity developments to physicians than disc diffusion methods (37, 38)

The current time-kill kinetic outcomes required a little more time to completely eradicate the bacteria when compared with the study conducted by Mandal and his colleagues (2011) who found that clove extract showed significant killing activities against MRSA, both at 3 and 6 hrs at a concentration of 256 µg/ml (1× MIC) (38). These differences are possibly caused by a variety of bacterial species used, the concentration of the antibacterial used, and the method used.

Conclusion: The clove oil extract at different concentrations had antimicrobial activity against MRSA bacteria. The GC-MS analysis of the clove oil showed the presence of 17 volatile components previously reported to possess antibacterial effects. Hence the antibacterial properties demonstrated by the clove oil extract can be attributed to the compounds identified caryophyllen, humulene, and eugenol. Therefore, clove oils may be used in the medicinal formulation of antimicrobial drugs.

Authors' declaration:

We confirm that all the Figures and Tables in the manuscript are mine/ ours. Besides, the Figures and images, which are not mine /ours, have been given permission for re-publication and attached to the manuscript. ethical consideration's approval-Ethical Clearance: The project was approved by the local ethical committee in the Collage of Veterinary Medicine/ University of Baghdad) according to the code number (1604 on 26-7-2023).

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Authors' contributions:

Study conception & design: (Orooba MS). Literature search: (Mays Uday & Orooba MS). Data acquisition: (Mays Uday). Data analysis & interpretation: (Mays Uday & Orooba MS). Manuscript preparation: (Mays Uday & Orooba MS). Manuscript editing & review: (Mays Uday)

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استخراج وتحديد المكونات الرئيسية لمستخلص زيت نبات القرنفل (*Syzygium Aromaticum L.*) وفعاليته المضادة للميكروبات ضد جرثومة المكورات العنقودية الذهبية المقاومة للميثيسيلن.

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الخلاصة:

خلفية: تُعرف بكتيريا المكورات العنقودية الذهبية المقاومة للميثيسيلن على نطاق واسع كعامل مسبب مهم مسؤول عن العدوى في جميع أنحاء العالم. وان واحدة من أكبر المشاكل في مجال الرعاية الصحية العالمية هي مقاومة المضادات الحياتيه لهذه البكتيريا.حيث يعد استخدام الأدوية العشبية إحدى التقنيات الواعدة لمواجهة مقاومة البكتيريا للمضادات الحياتية.

الأهداف: دراسة التركيب الكيميائي واختبارالنشاط المضاد للبكتيريا في المختبر لمستخلص زيت القرنفل ضد جرثومة المكورات العنقودية الذهبية المقاومة للميثيسيلن .

الطريقة: تم الحصول على مستخلص زيت القرنفل باستخدام التقطير المائي بواسطة جهاز Clevenger. بعد ذلك ، تم إجراء التحليل الكيميائي النباتي عن طريق التحليل الكروماتوجرافي-مطياف الكتلة. تم إجراء فحص لمضادات الميكروبات لزيت نبات القرنفل في المختبر باستخدام طريقة انتشار القرص المزدوج ، وطريقة التخفيف الدقيق ، وطريقة منحنى وقت القتل.تم استخدام اختبار اقل فرق معنوي – LSD(تحليل التباين-ANOVA) لاجراء مقارنه معنويه بين النتائج في هذه الدراسه.

النتائج: اظهرت هذه الدراسه ان نسبة استخلاص مستخلص القرنفل 50٪. وقد أظهر التحليل الكيميائي النباتي وجود مكونات مختلفة وبشكل رئيسي هي بنسبه28% Caryophyllen و 21.6%Humulene و13.06% Eugenol. حيث تبين ان المكورات العنقودية الذهبية كانت حساسة لمستخلص القرنفل بتركيزات مختلفة علاوة على ذلك ، وجد أن أقل تركيز مثبط (MIC) وأقل تركيز مبيد للجراثيم (MBC) كان 2.5 ميكروغرام / مل ، 5.0 ميكروغرام / مل على التوالي.وقد حقق منحنى وقت القتل من xMICs2 و xMICs4 لمستخلص القرنفل أعلى تأثير قاتل للجراثيم ($P \geq 0.05$) مقارنة بالتراكيز الأخرى.

الاستنتاجات: ظهر تحليل GC-MS لزيت القرنفل وجود 17 مكونًا متطايرًا في مستخلص القرنفل ويمكن أن تُعزى الخصائص المضادة للبكتيريا الجيدة التي أظهرها مستخلص زيت القرنفل ويعزى ذلك إلى المركبات الموجودة في الفينوليه مثل الكاريوفيلين , الهيمولين , والاوجينول.

الكلمات المفتاحية: المكورات العنقودية الذهبية المقاومة للميثيسيلن(MRSA), الكاريوفيلين, مضادات الميكروبات ، مستخلص القرنفل ، منحنى وقت القتل

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Research Article

Oral Findings and Salivary Alpha-Amylase in Major Depressive Disorder Patients

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Abstract

Background: The increasing global prevalence of major depressive disorder (MDD) has become an important challenge, leading to a heightened demand for oral medicine in developed nations. This demand arises from the recognition of the association between psychiatric disorders and other conditions, including various orofacial pain disorders.

Objective: This study are to evaluate oral conditions such as recurrent aphthous ulcers, burning mouth syndrome, and altered taste and to assess salivary alpha-amylase in individuals diagnosed with major depressive disorder.

Methods: This research uses a cross-sectional study design that includes a sample of 49 patients who have been diagnosed with major depressive disorder and who have undergone treatment for at least two weeks. The control group consists of 34 healthy subjects with no signs or symptoms of systemic disease. The study group received the diagnosis in Najaf City according to the criteria in the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5). With respect to recurrent aphthous ulcers, the results of this study show the percentage of patients with oral ulcers is significantly higher than in the control group.

Results: The results also show that the prevalence of burning mouth syndrome is significantly higher in patients with major depressive disorder than in healthy controls. A highly statistically significant difference was found between the study group and the control group regarding altered taste. There is also a significant difference in salivary alpha-amylase levels between the study and control groups ($p = 0.009$).

Conclusion: Major depressive disorder patients have much higher incidences of reported recurrent aphthous ulcers, burning mouth syndrome, and altered taste than healthy subjects, indicating the importance of psychological factors in these conditions. Additionally, salivary alpha-amylase levels were higher in patients with major depressive disorder than in the control group.

Keywords: Altered taste; Burning Mouth syndrome; Major depressive disorder; Recurrent Oral ulcerations; Salivary Alpha-amylase.

Introduction:

Major depressive disorder is a highly prevalent and incapacitating condition that has significant global implications for individuals and public health (). Several studies indicate that the prevalence of depression in Iraq is notably high and that rates of depression are particularly high in medical students (2-7). A recent study conducted in Iraq identified depression as a significant criterion that had previously been overlooked; this study suggested that the consideration of depression as a diagnostic factor may contribute to the early detection of individuals with Behcet's disease (8). The occurrence of depression has been observed to have adverse effects on oral health, specifically in relation to the development of dental caries (9,10). Recurrent aphthous stomatitis (RAS) is widely recognized as the prevailing ulcerative condition affecting the oral mucosa. The observed condition manifests as either solitary or multiple instances of recurring shallow ulcers. These ulcers typically exhibit a circular morphology and are accompanied by distinct erythematous borders. Additionally, they present yellow or grey pseudomembranous surfaces (1). Recurrent aphthous stomatitis (RAS) is characterized by a prodromal burning sensation that persists for 2–48 hours before the manifestation of an ulcer. This condition can occur in individuals who are in good health. Typically, it is found on the buccal or labial mucosa, as well as on the tongue. However, it is uncommon to find it on the gingiva or the heavily keratinized palatal mucosa. On average, RAS affects approximately 20% of the worldwide population (2). Various factors have been suggested as potential etiological agents for recurrent aphthous stomatitis (RAS). The factors contributing to this condition encompass genetic factors, local factors such as trauma, nutritional factors such as deficiencies in vitamin B complex or folate, hematologic and immunologic factors, food allergies, the influence of drugs, and psychological problems such as stress, anxiety, and depression (2,13,14) Burning mouth syndrome (BMS) frequently presents as sensations of burning, prickling, tingling, itching, or numbness that specifically affect the tongue, lips, palate, gums, and other mucous membranes within the oral cavity (5). The level of pain experienced by individuals tends to

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escalate progressively during the day, reaching its maximum intensity during the late evening hours (6). Frequently, patients express dissatisfaction with dysgeusia, xerostomia, and altered sensation in the oral mucosa, as well as psychological problems such as anxiety and depression. The etiology of burning mouth syndrome (BMS) is postulated to be linked to psychological disorders as well as peripheral and central neuropathy (7,21). Taste dysfunction can arise from various factors, including upper respiratory tract infections (URIs), head trauma, medication usage, and idiopathic origin (22,23,24). The primary enzyme responsible for digestion in the oral cavity is known as alpha-amylase. Alpha-amylase fulfills a dual function, encompassing roles in both digestive and immunological functions (e.g., it protects the oral cavity against microbial pathogens). Alpha-amylase serves multiple purposes and offers various advantages; it is involved in the digestive process, which initiates in the mouth, and has the capacity to bind to oral bacteria and teeth (25,27). Moreover, prior research has suggested that salivary alpha-amylase serves as a reliable indicator of the sympathetic nervous system's response to various stimuli, such as adrenaline (27,31). Salivary alpha-amylase (sAA) is released in response to neurotransmitter stimulation, and its secretion is regulated by both sympathetic and parasympathetic innervation of the salivary glands. Consequently, salivary alpha-amylase has been acknowledged as a significant biomarker for assessing autonomic activity (32). The secretion of salivary alpha-amylase (sAA) by the parotid gland is influenced by adrenergic activity, which is inhibited by beta-blockers (33). The objectives of this study are to assess oral findings and salivary alpha-amylase in patients with major depressive disorder (MDD) and to compare these patients with a group of healthy control subjects.

Subjects and Methods:

This cross-sectional study was conducted at Al-Hakim Hospital in Najaf City, Iraq. Ethical approval for the study was obtained from the Ethical Committee of the College of Dentistry, Baghdad University, under assigned project number 458722.

A total of 49 patients who had been diagnosed with major depressive disorder (MDD) and who had received treatment for a minimum of two weeks were included in the study. The control group comprised 34 healthy individuals without any indications or symptoms of systemic disease. The study group was diagnosed by psychiatric specialists at Al-Hakim Hospital in Najaf City in accordance with the criteria outlined in the fifth edition of the *Diagnostic and Statistical Manual of Mental Disorders* (DSM-5).

Individuals aged 18 years or older who had received a depression diagnosis from a qualified psychiatrist were considered for participation in this research. Exclusion criteria encompassed individuals seeking emergency medical attention, those unable to independently complete the questionnaire, pregnant individuals, individuals undergoing corticosteroid treatment, and individuals with a history of radiotherapy or chemotherapy. The examination of all patients involved the identification of oral manifestations, such as aphthous ulcers. Furthermore, patients were queried regarding the presence of burning mouth syndrome (BMS) and any alterations in taste perception. The data collection period spanned January 30th, 2021 to April 29th, 2022. Prior to the collection of samples, participants underwent a mouth rinse using distilled water. Detailed instructions were provided to all participants, directing them to hold saliva in their oral cavities for a period of 10 minutes without swallowing. After the designated time, participants expelled the accumulated saliva into a sterile plastic receptacle. During the collection process, the saliva samples were stored in a refrigerated environment to maintain their integrity. To minimize the formation of bubbles and foam, the samples underwent centrifugation at a rotational speed ranging from 3000 to 3500 revolutions per minute (RPM). Salivary alpha-amylase was assessed using the human AMY1 (Amylase Alpha 1, Salivary) ELISA kit, catalog number E-EL-H0320. The Statistical Package for the Social Sciences (SPSS), version 23, was utilized alongside Microsoft Excel for data insertion and analysis. Given that the data set encompasses both descriptive and quantitative data, it was imperative to assess the distribution of the variables in the research. The Chi-squared test and *t*-test were conducted to ascertain the presence of any correlations between the variables under investigation in this study. During the course of the investigation, the Kolmogorov-Smirnov test and correlation analysis emerged as two pivotal methodologies for determining the conformity of the quantitative data to a normal distribution.

Results

Age:

This study found that the individuals diagnosed with major depressive disorder (MDD) exhibited a broad age range of 23 to 66 years, while the control group, composed of individuals without MDD, ranged in age from 20 to 57 years. The mean age of individuals diagnosed with MDD was 44.3 years, with a standard deviation of ±10.19 years. In the control group, the mean age was 41.26 years, with a standard deviation of ±10.98 years. However, no statistically significant difference was observed between the two groups in terms of age. The group of patients diagnosed with MDD consisted of 26 males (53.1%) and 23 females (46.9%). In comparison, the control group, which consisted of healthy individuals, comprised 19 males (55.9%) and 15 females (44.1%), as shown in Table (1).

Table (1): Mean, range, and percentage of MDD patients and control subjects in different age groups

Age/year	Study Group		Age/ year	Control Group		*P*-value
	Freq.	%		Freq.	%	
35–23	12	24.5	26–20	9	26.5	
36–47	14	28.6	31–26.1	8	23.5	
48–50	13	26.5	41–31.1	10	29.4	0.133 (Ns)
51–66	10	20.4	57–41.1	7	20.6	
Total	49	100	Total	34	100	
Age range year	20–57		23–66			
Mean±SD	44.30±10.19		41.26±10.98			

NS: Non-significant.

Gender

In this study, among the individuals diagnosed with major depressive disorder (MDD), there were 26 males (53.1%) and 23 females (46.9%). In the control group comprising healthy subjects, there were 19 males (55.9%) and 15 females (44.1%). However, no significant difference was observed between the two groups in terms of gender distribution, as shown in Table (2) and Figure (1).

Table (2): The numbers and percentages of males and females in the MDD patient and control groups

Gender	Study Group		Control Group		*P*-value
	No.	%	No.	%	
Male	26	53.1	19	55.9	0.803 (NS)
Female	23	46.9	15	44.1	
Total	49	100	34	100	

NS: Non-significant

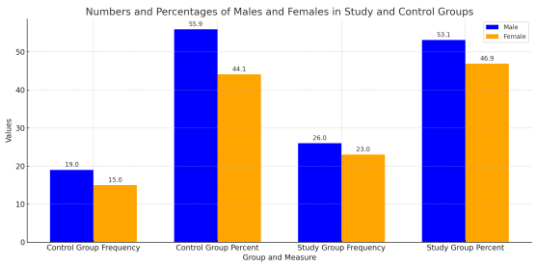

Figure (1): The numbers and percentages of males and females in the MDD patient and control subject groups.

1- Oral findings

1-Recurrent oral ulcerations

A total of 21 (42.9%) MDD patients reported that they do not have frequent oral ulcers, while 28 (57.1%) MDD patients confirmed that they have oral ulcers. In the control group, 28 (82.4%) individuals reported that they do not have a history of recurrent oral ulcers, while 6 (17.6%) reported a history of oral ulcers, as shown in Table (3). Statistical analysis showed a highly significant difference between MDD patients and control subjects ($P < 0.001$; Table 3).

Table (3): The numbers and percentages of subjects with oral ulceration in the MDD patient and control groups

Oral Ulceration	Study Group		Control Group		*P*-value
	No.	%	No.	%	
No	21	42.9	28	82.4	0.000 ** (Hs)
Yes	28	57.1	6	17.6	
Total	49	100	34	100	

****HS: Highly significant, p < 0.001**

Burning mouth syndrome

Within the study group, a total of 26 individuals (53.1%) did not exhibit symptoms of burning mouth syndrome (BMS), whereas 23 individuals (46.9%) had BMS.

In the control group, 29 (85.3%) individuals did not have BMS, while 5 (14.7%) individuals reported having BMS. A statistical analysis showed a significant difference between the study and control groups, as shown in Table (4).

A statistical analysis showed a highly significant difference between the MDD patients and control subjects ($P < 0.001$).

Table (4): The numbers and percentages of patients with BMS in the study and control groups

BMS	Study Group		Control Group		
No	No.	%	No.	%	*0.002 S
Yes	26	53.1	29	85.3	
	23	46.9	5	14.7	
Total	49	100	34	100	

***S: Significant, *P* < 0.05**

Altered taste

The results showed that 20 (40.8%) MDD patients did not have altered taste, while 29 (59.2%) patients had altered taste.

In the control group, 33 (97.1%) individuals did not have altered taste, while 1 (2.9%) individual reported having altered taste. A highly statistically significant difference was found between the study and control groups, as shown in Table (5).

Table (5): The numbers and percentages of subjects with altered taste in the MDD patient and control groups

Altered taste	Study Group		Control Group		P-value
No	No.	%	No.	%	
Yes	20	40.8	33	97.1	**0.000
Total	29	59.2	1	2.9	
No	49	100	34	100	(Hs)

**Hs: Highly significant, $P < 0.001$

Salivary alpha-amylase

\In the MDD patient group, the mean and standard deviation of salivary alpha-amylase was 1.37 ± 0.35 ng/ml, and the range was 0.5–2.25 ng/ml. In the control group, the mean±standard deviation was 1.19± 0.2 ng/ml, and the range was 0.76–1.83 ng/ml.

A *t*-test indicated a statistically significant difference in salivary alpha-amylase concentrations between the MDD patient group and the control group (p = 0.009), as shown in Table (6).

Salivary alpha-amylase was significantly higher ($P < 0.05$) in the MDD patients than in the control subjects.

Table (6): The mean, standard deviation and range of salivary alpha-amylase in the MDD patient and control groups

Group	No.	Mean ng/ ml	SD	Range (ng/ml)	*P*-value
MDD patients	49	1.37	0.35	0.5-2.25	*0.009 S
Control	34	1.19	0.20	0.76-1.83	

***S: Significant. *P* < 0.05).**

Discussion:

Oral findings

Recurrent oral ulcerations

The results of this study indicate that the number of MDD patients who have reported frequent oral ulceration is highly significant. The oral cavity is widely regarded as a reflection of overall systemic health, given that various physical and psychological disorders and systemic diseases can manifest in the oral mucosa (34,36). It is widely acknowledged that psychological factors, including anxiety, depression, and psychological stress, may influence a variety of oral lesions (37,40). Recurrent aphthous stomatitis (RAS) consists of painful ulcerations that typically manifest on non-keratinized mucosa of the oral cavity, exhibit a yellowish-white appearance, and are encircled by an erythematous halo. The prevalence of RAS in the overall populace ranges from 5% to 20%, with higher incidence rates observed in females than males. RAS is frequently observed in pediatric and young adult age groups. The exact etiology of recurrent aphthous stomatitis (RAS) remains unclear (41). Psychological stress, anxiety, and depression are regarded as common triggers in the occurrence and progression of RAS (42, 45). It is suggested that psychological disorders contribute to the onset and progression of oral health conditions. Several researchers have noticed that oral disorders commonly experience cycles of remission and exacerbation, which are often closely linked to the emotional state of the patients (46).

Given the great reactivity of oral tissues to psychological factors, it is typical for oral problems to emerge as psychosomatic symptoms. Psychological variables lead to changes in the markers of the neurological system. The oral disease is initiated and progresses due to the presence of catecholamines (adrenaline, noradrenaline, and dopamine), markers of the endocrine system (cortisol and aldosterone), and components of the immune system (T cells, B cells, natural killer cells, and immunoglobulins) (47).

Burning Mouth Syndrome

This study found a significant difference between the MDD and control groups in terms of the number of patients who reported burning mouth syndrome. This finding is consistent with previous studies (39,40,49,50). Multiple possible causative or precipitating factors of BMS have been suggested, including psychiatric disorders; these psychological factors could be a possible etiology of BMS (48,53).

Mental disorders like depression and anxiety play a critical role in the modulation of pain perception through various mechanisms that can alter the pain threshold, influence nerve transmission from peripheral pain receptors, and increase or decrease individual pain perception (54).

Altered taste

Studies investigating the association of major depression with altered taste are limited. This study found that 59.2% of patients with major depression report altered taste, which is higher than the results reported by an earlier study (55). The relatively high number of patients reporting altered taste could be explained by the recent Covid-19 outbreak, which can also cause altered taste in patients (56). A similar study using a questionnaire found a strong relationship between major depression and altered taste and smell dysfunction in adults in certain age groups in the general American population (57). One case-control study examining the correlation between taste perception and depression was identified in the existing literature. In a study conducted in 1969, it was observed that depressed patients exhibited a notably elevated threshold concentration in perceiving fundamental taste modalities (sweet, salty, sour, and bitter) compared to non-depressed patients. The study had a sample size of 39 individuals (58). Altered taste sensation could be a result of increased spontaneous firing rates of afferent taste fibers or efferent inhibition of other taste fibers. A change in salivary composition may also account for altered taste perception (59).

The link between taste irregularities and depression may be explained by the development of anhedonia, a key hallmark of depressive disorder. This can be observed in rat models through a reduced reaction to tasty food. A study showed that rats with anhedonia have lower levels of 5-HT1A receptors for serotonin in their taste cells. This suggests that changes in taste cells could play a role in the development of depressed symptoms (60).

Biochemical findings

Salivary alpha amylase

The findings of this study indicate that the levels of salivary alpha-amylase (sAA) among individuals diagnosed with major depressive disorder (MDD) are elevated to a statistically significant degree ($p < 0.05$) compared to the control group. Recent studies have found higher sAA levels in depressed patients and subjects with negative emotional states (61,62).

This finding is consistent with previous research that found that individuals diagnosed with major depressive disorder (MDD) exhibited significantly higher levels of alpha amylase compared to control subjects, both prior to and following electrical stimulation (63) Similarly, other researchers observed elevated levels of salivary alpha-amylase and cortisol in both unremitted and remitted depressed patients (64). It has been postulated that α-amylase could serve as an indicator of the activity of the sympathoadrenal medullary system (SAM) (65).

Elevated sAA levels have been observed in individuals diagnosed with major depressive disorder (MDD). Moreover, the administration of medications has the potential to decrease salivary alpha-amylase (sAA) levels and mitigate symptoms of depression. Other studies have concluded that the activation of the parasympathetic nervous system can also lead to the release of sAA (66).

Conclusions

MDD patients have much higher incidences of reported recurrent ulcerations, burning mouth syndrome, and altered taste than subjects without MDD, indicating the importance of psychological factors in these conditions. Salivary alpha-amylase levels are also higher in patients with MDD, suggesting that this is essential for the evaluation of MDD.

Authors' declaration:

We confirm that all the Figures and Tables in the manuscript belong to the current study. Besides, the Figures and images, which do not belong to the current study, have been given permission for re-publication attached to the manuscript.

Authors sign on ethical consideration's approval-Ethical Clearance: The project was approved by the local ethical committee in was conducted at Al Hakim Hospital in Najaf City, Iraq. Ethical approval for the study was obtained from the Ethical Committee of the College of Dentistry, Baghdad University, according to the code number 458722. The data collection period spanned January 30th, 2021 to April 29th, 2022.

Conflicts of Interest: None.

Funding: None

Authors' Contributions

The manuscript should mention the contribution of each author to the research done:

Study conception & design: Taghreed F. Zaidan. Literature search: Ameer A. Althabhawee, Mohammed Ali. Data acquisition: Ameer A. Althabhawee. Data analysis & interpretation: Taghreed F. Zaidan, Ameer A. Althabhawee. Manuscript preparation: Ameer A. Althabhawee Manuscript editing & review: Taghreed F. Zaidan

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النتائج الفموية وألفا الأميليز اللعابية في مرضى اضطراب الاكتئاب الشديد

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الخلاصة:
الخلفية: أصبح الانتشار العالمي المتزايد لاضطراب الاكتئاب الشديد (MDD) تحديًا مهمًا، مما أدى إلى زيادة الطلب على طب الفم في الدول المتقدمة. ينشأ هذا الطلب من الاعتراف بالارتباط بين الاضطرابات النفسية وغيرها من الحالات، بما في ذلك اضطرابات الألم الفموي الوجهي المختلفة.
الأهداف: أهداف هذه الدراسة هي تقييم حالات الفم مثل القرح القلاعية المتكررة، ومتلازمة الفم الحارق، وتغير الذوق وتقييم ألفا الأميليز اللعابي لدى الأفراد الذين تم تشخيص إصابتهم باضطراب اكتئابي كبير.
الطرق: يستخدم هذا البحث تصميم دراسة مقطعية يتضمن عينة من 49 مريضا تم تشخيص إصابتهم باضطراب اكتئابي كبير والذين خضعوا للعلاج لمدة أسبوعين على الأقل. تتكون المجموعة الضابطة من 34 شخصًا يتمتعون بصحة جيدة ولا تظهر عليهم أي علامات أو أعراض لأمراض جهازية. تم تشخيص مجموعة الدراسة في مدينة النجف وفق معايير الدليل التشخيصي والإحصائي للاضطرابات النفسية، الطبعة الخامسة .(DSM-5) وفيما يتعلق بالقرح القلاعية المتكررة، أظهرت نتائج هذه الدراسة أن نسبة المرضى الذين يعانون من تقرحات الفم أعلى بكثير من المجموعة الضابطة.
النتائج: أظهرت النتائج أيضا أن معدل انتشار متلازمة الفم الحارق (BMS) أعلى بكثير في المرضى الذين يعانون من MDD مقارنة بالأشخاص الأصحاء. تم العثور على فرق ذو دلالة إحصائية عالية بين مجموعة الدراسة والمجموعة الضابطة فيما يتعلق بتغير الذوق. هناك أيضًا اختلاف كبير في مستويات ألفا الأميليز اللعابية بين مجموعتي الدراسة والسيطرة (ع = 0.009). الاستنتاج: في الختام، مرضى MDD لديهم حالات أعلى بكثير من القرحات القلاعية المتكررة، ومتلازمة حرق الفم، وتغير الذوق من الأشخاص الأصحاء، مما يشير إلى أهمية العوامل النفسية في هذه الحالات. بالإضافة إلى ذلك، كانت مستويات ألفا الأميليز اللعابية أعلى في المرضى الذين يعانون من MDD مقارنة بالمجموعة الضابطة.
الكلمات المفتاحية: تغير الذوق، متلازمة الفم الحارق، اضطراب الاكتئاب الشديد، تقرحات الفم المتكررة، ألفا الأميليز اللعابي

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Research Article

Comparison of Pharmacokinetic Characteristics of Bilosomal Dispersion Versus Pure Solution of Oral Ropinirole Hydrochloride in Rats

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Abstract

Background: Ropinirole hydrochloride is a non-ergoline antiparkinson drug. It is a highly hydrophilic drug and classified as class III according to Biopharmaceutical Classification System with low absolute oral bioavailability of approximately 50% upon oral administration due to significant hepatic first-pass metabolism.

Objective: to compare the pharmacokinetic parameters of Ropinirole when administered orally in the form of an Ropinirole bilosomal dispersion in contrast to an oral Ropinirole solution.

Methods: This study involved the use of twelve male Wistar rats, with an average weight of 220±11 g, and these rats were divided into two groups, comprising six rats each. A 1.1 mg/kg doses of pure Ropinirole and Ropinirole bilosomes were administered orally through gavage after reconstituting in distilled water. Ropinirole was quantified in the rat's plasma using HPLC, subsequently establishing a spiked calibration curve with plasma samples and utilizing paracetamol as an internal standard. The statistics included mean values (± SD; n = 6) for pharmacokinetic parameters, with statistical significance assessed using a Student's *t*-test.

Results: For the oral bilosomes, the values were 9.4±0.11 µg /ml for Cmax, 3±0.00 h for Tmax, and 55.56±2.12 µg h/ml for AUC0-24. In contrast, for the oral solution, the corresponding values were 7.2±0.14 µg/ml for Cmax, 1.5±0.00 h for Tmax, and 23.70±2.23 µg h/ml for AUC0-24. These parameters were significantly higher ($P<0.05$) as compared with a pure drug solution. The comparative bioavailability of Ropinirole (AUC0-24 oral solution / AUC0-24 oral bilosomes) is equal to 42.66%, which indicates the bioavailability of the oral RH solution was less than that of *RH* bilosomal dispersion.

Conclusions: The use of nanovesicular carriers (bilosomes) shows significant potential as an effective delivery system for improving the oral bioavailability of ropinirole hydrochloride.

Keywords: Bilosomes; Bioavailability; nanovesicular carriers; Pharmacokinetics; Ropinirole hydrochloride.

Introduction:

Parkinson's disease is the second most prevalent neurodegenerative disorder, following Alzheimer's disease. Among movement disorders, it holds the distinction of being the most prevalent (1,2).

Ropinirole hydrochloride (RH) is classified as a non-ergoline antiparkinson drug and is also utilized for treating moderate-to-severe idiopathic restless leg syndrome (3). RH has a low absolute oral bioavailability of approximately 50% upon oral administration. The limited bioavailability is primarily attributed to significant hepatic first-pass metabolism (4). Furthermore, owing to its hydrophilic characteristics (BCS class III), RH might encounter challenges in traversing biological membranes, potentially restricting its permeation within the body. The existing conventional tablet formulations of RH face challenges in achieving satisfactory oral bioavailability (5, 6). RH peak plasma concentrations are typically attained within approximately 1.5 h after oral administration. The mean elimination half-life of RH has been reported to be approximately 6 h (7).

Bilosomes are nanovesicular carriers that incorporate bile salts into their vesicle bilayer, enhancing their flexibility and resistance to degradation within the GIT. While conventional nanocarriers like liposomes and niosomes provide limited protection against enzymatic degradation in the GIT, bilosomes offer a solution to this challenge (8). The nanoscale size and inherent stability of bilosomes render them a promising candidate for oral drug delivery. Numerous studies have demonstrated their safety and efficacy in this application (9). Nonionic surfactants (NSs) are commonly employed in the formulation of bilosomes due to their notable stability and compatibility compared to other surfactants, with minimal irritation to the body's cells. They are relatively less influenced by variations in pH and ionic strength. NSs play several key roles, such as increasing permeability, solubilizing substances, and emulsifying liquids. They are also effective inhibitors of P-gp, which can

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help to improve drug absorption and increase the targeted effect of the drug on specific tissues (10,11). Bile salts incorporated into bilosomes were thought to promote the penetration of drugs and enhance oral bioavailability associated with bilosomal drug delivery might arise from the improved uptake of drugs encapsulated within bilosomes by M-cells located in the Peyer's patches, along with enhanced transport through the lymphatic pathway (8, 12).

Pharmacokinetics (PK) refers to the changes over time in the concentrations of drugs and their metabolites in the body (13). Knowledge of drugs PK is essential for obtaining safe and effective drug products (14). In addition to a conventional method for determining the PK parameter, in recent years, there has been growing interest in physiologically-based pharmacokinetic (PBPK) modeling as a new method for determining pharmacokinetics after drug delivery using the software. PBPK modeling utilizes the physicochemical properties of the drug to predict its plasma concentration-time curves. To ensure the model's accuracy, it must be confirmed against existing established clinical pharmacokinetic data (15).

The study aimed to compare the pharmacokinetic properties of ropinirole when administered orally in the form of bilosomal dispersion in comparison with oral solution.

Materials and Methods:

Materials

Ropinirole hydrochloride (Wuhan Hanweishi Pharmchem, China), Cholesterol and sodium deoxycholate (Avonchem, UK), Acetonitrile -HPLC grade (Biosolve B V, France), Ammonium acetate and Mannitol (Thomas Baker, India), Chloroform, Diethyl ether, Span®60 and Tween®60 (Loba Chemie, India).

Preparation of Ropinirole bilosomal dispersion

Ropinirole bilosomal dispersion was prepared by the reverse-phase evaporation method (16). In 10 ml chloroform and diethyl ether mixture at a 1:1 ratio, a mixture of surfactants (tween® 60 and span® 60) and cholesterol were dissolved. RH and SDC were dissolved in 2 ml of deionized water; Next, the two phases were mixed using ultrasonic baths (LiebeWh, China) to form a stable white emulsion. The solvents were removed using a rotary evaporator operating at 150 rpm and 60°C for a duration of 20 min, forming a thin film. Subsequently, the formed film was hydrated using 10 ml of deionized water in a rotary evaporator set at 150 rpm and 60°C for 60 min, and the bilosomal dispersion was sonicated for 10 minutes. Many formulas were prepared and optimized to the composition of optimized bilosomal formula and then subjected to lyophilization using a freeze dryer (Labconco, Canada) (8). The composition of the optimized bilosomal formula is shown in Table 1.

Table (1): Composition of optimized bilosomal formula of RH

Ingredient	Amount
Ropinirole Hcl	50mg
Sodium deoxycholate (SDC)	5mg
Span®60	0.9% w/v
Tween®60	1.8% w/v
Cholesterol	0.9% w/v
Deionized water	10 ml

In-vivo pharmacokinetic study

Study design: The pharmacokinetic parameters were determined using male Wister rats (n=12) with an average weight (±SD) of approximately 220 ±11 g each.

Male Wister rats were obtained from the animal house at the College of Pharmacy, University of Baghdad. These rats were allowed to acclimatize for a minimum of one week under standard conditions of room temperature (25± 3ºC). All experimental procedures were approved by the Institutional Animal Ethical Committee at the College of Pharmacy, University of Baghdad (Approval No: REACUBCPS32023A).

The rats were divided into two groups, each consisting of six rats. In Group 1, the rats were orally administered 1.1 mg/kg of pure RH solution. In Group 2, the rats were orally administered the same dose of the optimized bilosomal formula (17). Before the administration, the rats were fasted overnight but had free access to water throughout the study. The lyophilized optimized formula and pure RH powder were dissolved in distilled water based on the amount of RH in the formulation and then orally administered to the rats using a gavage tube. This method of administration ensures precise dosing of the drugs to the rats.

The blood samples were collected from the Retro-orbital venous plexus before administration of the dose to obtain a blank (zero-time point) and after administration at different time intervals at (0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, and 24h), in EDTA vacuum glass tubes. The plasma was separated by centrifugation at 4500 rpm for 15 min and stored at −25°C until analysis (18, 19).

Analytical method

The RP-HPLC system used in this study consisted of a Sykam HPLC equipped with an S 3250 UV/Vis detector. The chromatographic separation was performed using an isocratic technique on an analytical grade C18 column with the following specifications: (150× 4.6 mm, 5 µm). A mixture of HPLC-grade acetonitrile and 0.05 M ammonium acetate buffer at pH 2.5 in a ratio of 25:75(v:v) was used as the mobile phase. Before introducing the system, the mobile phase was meticulously filtered using a 0.45µm micro-filter and degassed using bath sonication. Throughout the analysis, the eluent was consistently monitored at a 254 nm wavelength while being pumped in isocratic mode at a steady flow rate of 1ml/min. Various dilutions of stock solutions of

RH were prepared using the mobile phase. Working standard solutions of RH were prepared within a concentration range from 2.5 to 20µg/ml. An aliquot of 10µL of working standards was spiked to 100 µL of plasma separately to get concentrations of 2.5, 5, 7.5, 10, and 20µg/ml. Internal standard (IS) paracetamol (5µg/ ml) was added, followed by the above sampling procedure. 20µL of these solutions were injected for analysis. Then the standard curve was plotted by taking a concentration on X- the axis and the ratio of peak area of drug/IS on the Y-axis. Each trial was replicated three times, and the average values were reported. The sample's RH concentration was calculated from the standard calibration curve. This calibration curve determined RH plasma concentration during the *in-vivo* study (18, 20).

The validation parameters, including specificity, linearity, accuracy, precision, LLOD, and LLOQ, in rat plasma were conducted following the guidelines outlined by the US FDA for industry, specifically the guidance for bioanalytical method development and validation (21, 22).

In this study, plasma samples were subjected to processing using tert-butyl-methyl-ether as a precipitating agent for extraction. Initially, 100 µL of plasma and 100µL of IS, were mixed for 3 min. Subsequently, 500 µL of tert-butyl-methyl-ether was added to the mixture and stirred for 5 min. After that, the sample was centrifuged at 5000rpm for 15 min at 25°C, separating the supernatant organic layer. This process was repeated twice to ensure thorough extraction. The obtained organic phase was dried, and the residue was dissolved in 100µL of the mobile phase.

Additionally, control blank plasma samples (free from the drug) with and without the IS were prepared using the same procedure. Finally, the solution samples were directly injected into the HPLC column (18). Using HPLC measurements, the unknown concentration of RH could be derived based on the relative peak area. To determine primary pharmacokinetic parameters, non-compartmental analysis was implemented using PK-SOLVER. RH plasma concentrations were measured over time for both groups. Parameters such as the maximum plasma concentration of the drug (Cmax) and the corresponding time (Tmax) were ascertained. The area under the plasma concentration-time curve from 0 to 24 hours (AUC0-24) was also calculated(19, 23,24).

Statistical analysis

All the pharmacokinetic parameter values obtained were presented as mean results of the study (± SD; n = 6). A statistically significant difference was considered when the *P*-value was less than 0.05. The pharmacokinetic parameters (Cmax, Tmax, and AUC0-24), were subjected to statistical analysis using the Student's *t*-test (25).

Results:

Calibration curve of spiked plasma samples

The calibration curve was established by implementing the recommended procedure involving the addition of a known concentration standard solution of RH to spiked plasma samples; the outcomes of HPLC analysis indicated the absence of endogenous components that could interfere with the chromatogram of blank plasma. The method demonstrated precision, specificity, and sensitivity in determining RH concentrations in standard mobile phase solutions and spiked plasma samples. The chromatogram of the spiked plasma displayed complete separation between RH and the IS, with RH exhibiting a retention time (Rt) of approximately (2 ± 0.15 min) and the IS (paracetamol) exhibiting a peak at around (6 ± 0.25 min), as depicted in figures 1 and 2.

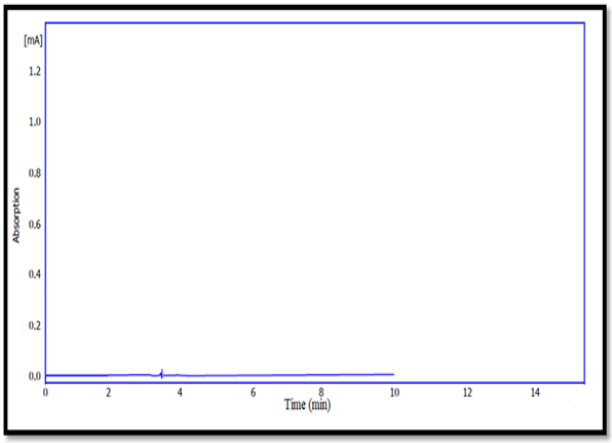

Figure (1): HPLC Chromatograms of blank rat plasma.

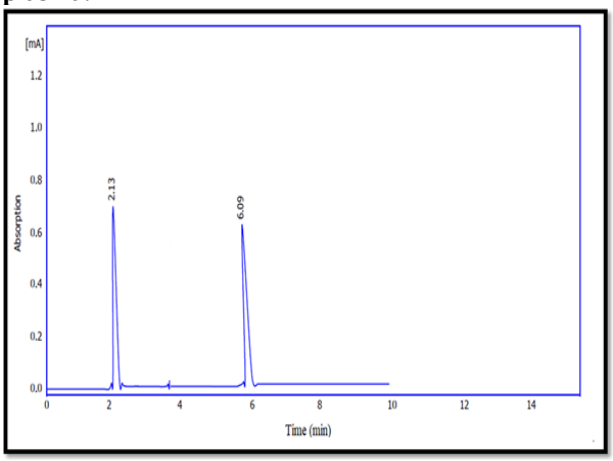

Figure (2): HPLC Chromatograms of RH in plasma spiked with IS.

Figure 3 displays the calibration curves of RH, obtained by plotting the ratio of the peak area of the drug/IS against the drug concentration. These curves exhibit a straight line and a high correlation coefficient of 1, indicating that they follow a linear relationship within the concentration range used in the experiment. (18,20).

The precision and accuracy of the method were assessed by analyzing replicates of samples covering the linearity range at three concentrations on three different days. The coefficients of variation (CV) for

intra-day and inter-day precision were 0.96-1.57 % and 1.21- 1.47%, respectively, with a low % CV (<2%), indicating high precision. The intra-day and inter-day accuracies for RH were 95.40-104.0% and 95.20- 103.4% respectively. The results presented in table 2, demonstrated that the method was both accurate and precise (26,27). In this study, the LLOD was determined to be 0.024 µg/mL, while the LLOQ was found to be 0.075 µg/mL, with a standard error of the intercept (2.514) and a standard deviation of the intercept (6.636). All validation parameters were within acceptable criteria (22).

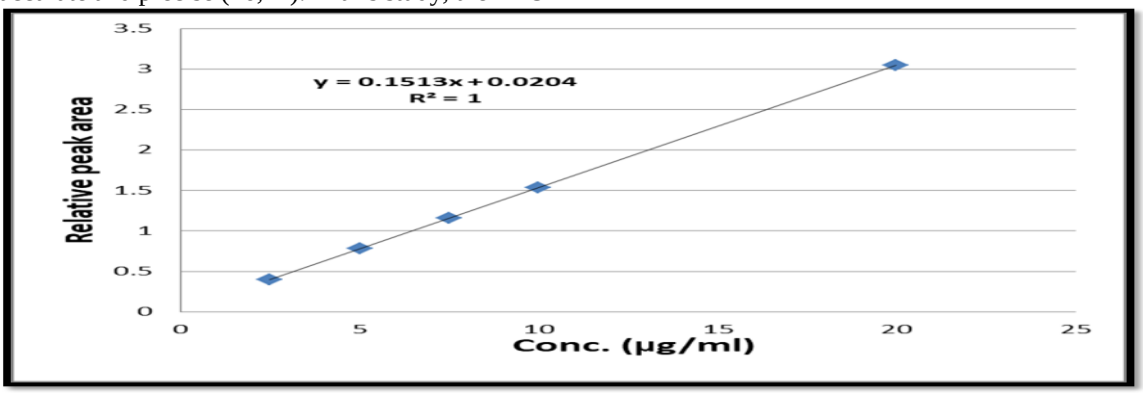

Figure (3): Calibration curve for the estimation of RH in rat plasma.

Table (2): Precision and Accuracy of the HPLC Method in Rat Plasma for RH

RH concentration (µg/ml)			CV %		Accuracy %	
	Intra-day*	Inter-day*	Intra-day	Inter-day	Intra-day	Inter-day
5	4.77	4.76	1.22	1.21	95.40	95.20
7.5	7.34	7.40	1.57	1.35	97.86	98.70
10	10.40	10.34	0.96	1.47	104.0	103.4

*Results expressed as mean ± SD, n=3

Ropinirole bilosomes pharmacokinetics: The pharmacokinetic evaluation of the optimized bilosomal formula was conducted after oral administration and compared with the oral solutions of the pure drug. Throughout the study, the test products were well tolerated by the rats, as there were no observed adverse effects or allergic reactions. Figure 4 shows the RH oral solution's plasma concentration versus time profiles and optimized bilosomal formula. Additionally, the pharmacokinetic parameters of the oral solution of RH and optimized bilosomes formula were presented in Table 3.

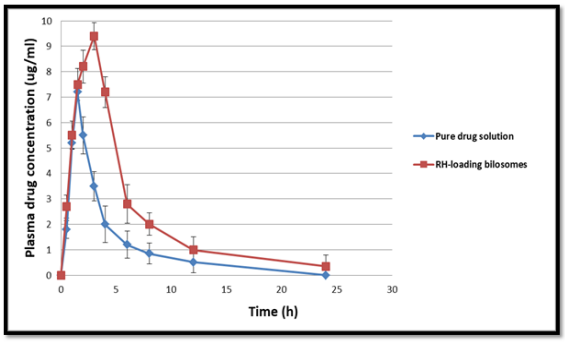

Figure (4): Plasma Concentration (µg/ml) of optimized bilosomal formula and Pure RH solution vs. time (h).

Table (3): Pharmacokinetic parameters of Optimized Bilosomal Formula and Pure RH Solution

Results expressed as mean ± SD, n=6

Parameter	Unit	Pure RH solution	Optimized bilosomal formula
Cmax	µg/ml	7.2±0.35	9.4±0.24
Tmax	h	1.5±0.00	3±0.00
AUC 0-24	µg. h/ml	23.70±1.02	55.56±2.97

The pharmacokinetic parameters of Ropinirole Hydrochloride (RH) were evaluated for both oral solution and oral optimized bilosomal formula. The maximum plasma concentration (Cmax) of RH was determined to be (7.2±0.14 µg/ml) for the oral solution and (9.41±0.11 µg/ml) for oral bilosomes, indicating an approximately 1.3-fold increase in Cmax for the optimized bilosomal formula compared to the RH oral solution.

Additionally, the area under the concentration-time curve from 0 to 24 hours (AUC0-24) value of the optimized bilosomal formula was significantly higher ($p < 0.05$) compared to the AUC0-24 value of the RH oral solution.

The AUC 0-24 value of RH bilosomes was (55.56±2.12 µg h/ml), while those of the RH solution was (23.70±2.23 µg h/ml). The comparative bioavailability of RH (AUC0-24 oral solution / AUC0-24 oral bilosomes) was equal to 42.66%.

Discussion:

The results demonstrate the successful establishment of a calibration curve for RH quantification using HPLC in plasma samples. Notably, the analytical method showed precision, specificity, and sensitivity

in measuring RH concentrations in both standard mobile phase solutions and spiked plasma samples. These findings indicated a reliable and accurate analytical method for RH analysis in plasma.

The significantly higher Cmax of the optimized bilosomal formula suggests an improved drug delivery profile via bilosomes, overcoming the limitations of poor delivery observed in the oral solution. Moreover, the prolonged release of RH from the vesicles in the bilosomes contributes to a more prominent time to reach maximum concentration (Tmax) for the optimized bilosomal formula compared to the oral RH solution (28,29).

The study shows that the bioavailability of oral RH solution was less than the RH bilosomal dispersion. However, This indicates an enhancement in the bioavailability of optimized bilosomal formula compared to the RH oral solution, attributed to enhanced permeation across the GIT (29- 32).

The higher Cmax and AUC 0-24 values observed for RH bilosomes can be attributed to various factors, including improved drug retention due to enhanced entrapment within the bilosome vesicles, sustained release properties, smaller vesicle size facilitating GIT absorption, and avoid of first-pass metabolism. These factors collectively contribute to the promising pharmacokinetic profile of optimized bilosomal formula (33, 34).

The enhanced uptake of intact RH bilosomal vesicles by the M cells in the Peyer's patch of the intestinal part in the GIT and their absorption via carrier-mediated transport are believed to be crucial factors contributing to the significantly higher comparative bioavailability compared to RH solution ($P < 0.05$) (28,35).

This study suggests that the optimized bilosomal formulation offers an alternative to the traditional dosage form of RH, with promising pharmacokinetic characteristics. The absence of adverse effects of optimized bilosomal formulation in rats and the pharmacokinetic data support the potential benefits of the bilosomal formulation for drug delivery and therapeutic effectiveness.

Conclusions:

The pharmacokinetic parameters (Cmax, Tmax and AUC0-24) of bilosomal dispersion of oral ropinirole hydrochloride were better than those of its pure oral solution which might indicate that bilosomal preparations can enhance bioavailabilty of orally-administered highly hydrophilic drugs.

Limitation:

The study has no any limitation

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Authors' Declaration:

We confirm that all the figures and tables in the manuscript are mine/ ours. Authors sign on ethical consideration's approval-Ethical Clearance: The project was approved by the college's in-animal Ethical committee of Pharmacy, University of Baghdad. According to the code number (Approval No: REACUBCPS32023A).

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Authors Contributions:

Study conception & design: Entidhar J. Al-Akkam1). Literature search: (Entidhar J. Al-Akkam1& Samer K. Ali). Data acquisition: (Samer K. Ali). Data analysis & interpretation: (Samer K. Ali). Manuscript preparation: (Entidhar J. Al-Akkam1& Samer K. Ali). Manuscript editing & review: (Entidhar J. Al-Akkam1& Samer K. Ali).

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مقارنة الخصائص الحرائك الدوائية لمنتشر بيليوسومال مقابل المحلول النقي لروبينيرول هيدروكلوريد عن طريق الفم في الجرذان

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خلفية البحث : روبينيرول هيدروكلوريد هو دواء مضاد للباركنسون غير إرجولين. وهو دواء محب للماء للغاية ويصنف ضمن الفئة الثالثة وفقًا لنظام تصنيف الأدوية الحيوية مع توافر حيوي عن طريق الفم منخفض بنسبة 50٪ تقريبًا عند تناوله عن طريق الفم بسبب استقلاب الكبد الأول.
الاهداف: مقارنة الخصائص الحركية الدوائية الصيدلانية للروبينيرول عند إعطائه عن طريق الفم في شكل منتشر بيليوسومال للروبينيرول مقابل محلول فمي للروبينيرول.
طرق العمل : شملت هذه الدراسة استخدام اثني عشر جرذ ويستار ،بمتوسط وزن كل منها حوالي 220 ± 11 جم ، وتم تقسيم هذه الجرذان إلى مجموعتين ، تضم كل مجموعة ستة جرذان. تم إعطاء جرعة
1.1 مجم / كجم من وزن الفارعن محلول الدواء النقي والروبينيرول بشكل بلبيليوسومات عن طريق الفم من خلال الانبوب بعد إعادة تكوينها في ماء مقطر. بعد إعداد منحنى المعايرة باستخدامHPLC تم قياس كمية الدواء في عينات البلازما وذلك باستخدام مركب الباراسيتامول كمعيار داخلي. وتضمنت الإحصائيات القيم المتوسطةSD ±) ؛ (n = 6 لمعلمات الحرائك الدوائية، مع تقييم الدلالة الإحصائية باستخدام اختبار الطالب
Cmax , Tmax, AUC0-24
النتائج: بالنسبة للبيليوسومات الفموية، كانت القيم 9.4 ± 0.11 ميكروغرام / مل لـCmax ، و3 ± 0.00 ساعة لـTmax ، و55.56 ± 2.12 ميكروغرام / مل لـ .AUC0-24 في المقابل، بالنسبة للمحلول الفموي، كانت القيم المقابلة 7.2 ± 0.14 ميكروغرام / مل لـCmax ، و1.5 ± 0.00 ساعة لـTmax ، و23.70 ± 2.23 ميكروغرام / مل لـ .AUC0-24 كانت هذه المعلمات أعلى بكثير (P <0.05) مقارنة بالمحلول الدوائي النقي. التوافر الحيوي المقارن لروبينيرول AUC0-24) محلول فموي AUC0-24 / بيلوسومات فموية) يساوي 42.66%، مما يشير إلى أن التوافر الحيوي لمحلول RH الفموي كان أقل من منتشر البيليوسومال لـRH .
الاستنتاجات: ان استخدام الناقلات الحويصلات النانوية البيليوسومات تظهر امكانيات كبيرة كنظام توصيل فعال لتحسين التوافر الحيوي لدواء روبينيرول هيدروكلوريد.
الكلمات المفتاحية : البيليوسومات , التوافر الحيوي , الناقلات الحويصلية النانوية, الحرائك الدوائية , روبينيرول هيدروكلوريد .

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Research Article

Proportion and Potential Risk Factors of Poor Glycemic Control among Patients with Type 2 Diabetes Mellitus: Experience of a Tertiary Center in Baghdad, Iraq, 2020

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Abstract

Background: Diabetes Mellitus is the most prevalent metabolic disorder worldwide. Effective diabetes self-management and keeping the Glycosylated hemoglobin level within the normal range could decrease the burden on the health system by reducing hospital admissions and diabetic complications, lowering the financial strain on the health system.

Objective: To recognize the potential risk factors of poor glycemic control in patients having type 2 Diabetes Mellitus in Baghdad, Iraq.

Methods: This cross-sectional study was conducted in the Diabetes and Endocrine Center at Al-Kindy Teaching Hospital, Baghdad, Iraq, from June to November 2020. The total number of the study's participants was 234. Based on the cutoff point of glycosylated hemoglobin of 7, the patients with glycosylated hemoglobin of ≥7 were considered uncontrolled, and those with glycosylated hemoglobin of <7 were considered controlled.

Results: The proportion of cases with uncontrolled diabetes was 68.4% (160). The remaining 74 patients had controlled diabetes. Age, sex, marital status, and employment status were not significantly associated with the control status (P>0.05). The binary analysis showed a significant association observed between the control status and level of education (P=0.001), income (P=0.001), presence of comorbidities (P=0.028), positive family history (P=0.03), dyslipidemia (P=0.001), cholesterol level (P=0.002), high triglyceride level (P=0.001), and low-density lipoprotein-cholesterol level (P=0.025). The smoking status, body mass index, and high-density lipoprotein-cholesterol level were not significantly associated with the control status (P>0.05). The disease characteristics, including the disease duration, fasting blood glucose, type of medication, self-monitoring, healthy diet, physical activity, and medication adherence, were significant factors (P<0.001). The multivariate regression method showed that dyslipidemia, FBS≥130, physical inactivity, and poor medication adherence were significant predictors for uncontrolled DM (P-value = 0.03, 0.001, 0.03, and 0.043, respectively).

Conclusion: The most important potentially modifiable risk factors for poor diabetes control were dyslipidemia, physical inactivity, and poor adherence to the management protocol. Enhancing the education of patients and their healthcare providers on these factors is crucial to improving the patients' control status.

Keywords: Risk Factors; Glycemic Control; Type 2 Diabetes Mellitus.

Introduction

Diabetes Mellitus (DM) is the most prevalent metabolic disorder worldwide and is one of the most prevalent non-communicable chronic degenerative disorders worldwide. It is characterized by chronic elevation of blood glucose due to various causes including abnormalities in insulin secretion, action, or both. (1, 2). It is estimated that 5–10% of the population suffers from DM. This prevalence is estimated to continually rise globally, with multiple implications for social, financial, and health systems. (2) Effective diabetes self-management through keeping the glycosylated hemoglobin A1C (HbA1C) level within the normal range could decrease the burden on the health system by reducing diabetic complications and hospital admissions, minimizing the finical strain on the health system (3) Given the importance of self-management, literature has identified factors like age, ethnicity, socioeconomic status, disease duration, use of medications, comorbidities, body mass index, understanding of nutrition, level of empowerment, and self-efficacy could play an essential role in controlling diabetes. (4, 5) Increasing confirmations on good control of diabetes significantly impact patients and the health system. Despite the availability of a large number of studies which had examined the variables influencing glycemic control in people with type 1 or type 2 diabetes (6), they still have to be documented in populations with its etiological characteristics (7–9)

Diabetes has a significant global and national burden. The disease is reported in 10.5% of the adult population (20-79 years) globally, with almost half of the patients being unaware that they are living with the condition (10,11). The mortality rate due to diabetes reached 10.7% in adult patients (20-79

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years) worldwide. In the Middle East and North Africa Region, diabetes has accounted for more than 350000 deaths with about half of these deaths occurring in patients younger than 60 years (10). According to the last STEP survey in Iraq, more than 2 million Iraqis adults (18+) were hyperglycemic with an overall prevalence of 13.9% (12).

Considering the increasing prevalence of DM and the high proportion of uncontrolled DM in the country, this study examined a sample of type 2 DM patients to determine the risk factors for poor glycemic control in Baghdad, Iraq, 2020.

Patients and methods

Study design, setting, sampling, and definition of variables: This cross-sectional study was conducted in the Diabetes and Endocrine Center in Al-Kindy Teaching Hospital, Baghdad, Iraq, from June to November 2020. The included cases were patients with type 2 DM for a minimum of one year and whose age was greater than 18 years. Patients who were mentally unstable, critically ill, or unable to respond were excluded. The included patients were classified into two groups according to their HbA1c status. The patients with HbA1C of ≥7 were considered uncontrolled, and those with HbA1C of <7 were considered controlled. The cases were selected as a consecutive sample.

Data Collection Tool: A questionnaire was developed and filled out through direct patient interviews. The questionnaire included sections on demographics (age, gender, educational level, marital status, smoking status, employment status, average family income, alcohol drinking, and body mass index), lipid profile (total cholesterol**,** triglycerides, high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C)), and disease characteristics (comorbidities, duration of DM, fasting blood sugar (FBS), HbA1C level, medications used, self-monitoring of blood sugar, physical activity classified as sedentary, occasional, or regular physical activity, healthy diet, and medication adherence using the Morisky Medication Adherence Scale-8 (MMAS-8)).

Statistical Analysis: Version 22 of the Statistical Package for Social Sciences (SPSS) was used to analyze the data. For the presentation of categorical data, frequencies, and percentages were used. Pearson's Chi-square test assessed the statistical association between categorical variables. Logistic regression analysis was carried out to identify the significant unconfounded factors connected to the control status of DM. A P-value of <0.05 was regarded as statistically significant.

Ethical and official approval: The study's details were verbally explained to each patient, and their permission was granted before conducting the interview. Anonymized personal data was respected, and the data was only used for this study's purposes. Official approval was granted from the Iraqi Council of Medical Specializations and the Diabetes and Endocrine Center in Al-Kindy Teaching Hospital.

Results

In this investigation, 243 patients were included. The number of patients with uncontrolled DM was 160 (68.4%), and those with controlled DM were 74 (31.6%). The two groups were distributed according to certain sociodemographic and disease variables (table 1). A significant association between controls status and educational level was found (P=0.001), inadequate income (P<0.001), presence of comorbidities (P=0.021), and positive family history (P=0.023). Other variables, namely gender, age, marital status, occupation, smoking, and BMI, were not significantly associated with DM control status (P>0.05

Table 1: Distribution of the two study groups by demographic and disease characteristics

Variable	Categories	Uncontrolled DM No. 160 (%)	Controlled DM No. 74 (%)	Total No. 234	P-value	Odds ratio	95% CI
Gender	Female	101 (63.1)	38 (51.4)	139	0.059	0.616	0.3-1.07
	Male	59 (36.9)	36 (48.6)	95			
Age Group (Years)	<50	48 (30.0)	20 (27.0)	68	0.37	1.37	0.6–2.7
	50 – 60	65 (40.6)	27 (36.5)	92		1.38	0.7–2.6
	>60	47 (29.4)	27 (36.5)	74			
Education	Illiterate / Primary	89 (55.6)	30 (40.5)	119	0.001	4.6	2.1-10.2
	Secondary	57 (35.6)	22 (29.7)	79		4.0	1.7-9.3
	University	14 (8.8)	22 (29.7)	36			
Marital Status	Single	5 (3.1)	0	5	0.14	0.47	1.3-1.6*
	Married	155 (96.9)	74 (100)	229			
Employment	Employed	27 (16.9)	21 (28.4)	48	0.066	0.44	0.2-1
	Retired	20 (12.5)	14 (18.9)	34		0.49	0.2-1.2
	Freelance	67 (41.9)	23 (31.1)	90		1.01	0.4-2.1
	Housewife	46 (28.7)	16 (21.6)	62			
Income	Adequate	42 (26.3)	43 (58.1)	85	<0.001	3.89	2.1 – 6.9
	Inadequate	118 (73.8)	31 (41.9)	149			
Comorbidities	Yes	116 (72.5)	43 (58.1)	159	0.021	0.52	1.06 – 3.38
	No	44 (27.5)	31 (41.9)	75			
Family history of DM	Yes	127 (79.4)	49 (66.2)	179	0.023	0.5	0.27 – 0.94
	No	33 (20.6)	25 (33.8)	58			
Smoking History	Ever use	36 (22.5)	13 (17.6)	49	0.24	0.73	0.3-1.4
	Never use	124 (77.5)	61 (82.4)	185			
BMI	<25	28 (17.5)	13 (17.5)	41	0.31	0.71	0.3-1.6
	25 – 29.9	78 (48.7)	43 (58.1)	121		0.6	0.3-1.1
	>30	54 (33.7)	18 (24.3)	72			

The association between DM management and lipid profile is shown in Table 2. The control status and dyslipidemia had a very strong association (P< 0.001), including cholesterol level, TG level, and LDL level (P=0.002, <0.001, and 0.038, respectively). The HDL level did not show a significant association with DM control.

Table 2: Distribution of the study groups by lipid profile

Variable	Categories	Uncontrolled DM No. 160 (%)	Controlled DM No. 74 (%)	Total No. 234	P-value	Odds ratio	95% CI
Dyslipidemia	Yes	121 (75.6)	39 (52.7)	160	<0.001	0.35	0.2 – 0.6
	No	39 (24.4)	35 (47.3)	74			
Cholesterol	≥200 mg/dl	56 (35.0)	11 (14.9)	67	0.002	3.08	1.5 – 6.3
	<200 mg/dl	104 (65.0)	63 (85.1)	167			
TG	≥150 mg/dl	81 (50.6)	11 (14.9)	92	<0.001	5.8	2.8 – 11.9
	<150 mg/dl	79 (49.4)	63 (85.1)	142			
LDL	≥100 mg/dl	40 (25.0)	9 (12.2)	49	0.038	2.4	1.1 – 5.2
	<100 mg/dl	120 (75.0)	65 (87.8)	185			
HDL	High	76 (47.5)	32 (43.2)	108	0.63	0.84	0.4 – 1.4
	Low	84 (52.5)	42 (56.8)	126			

The association between DM control and disease characteristics is shown in Table 3. All studied disease characteristics variables (Disease duration, FBS, medications used, physical activity, self-monitoring, healthy diet, adherence to medications, and other factors) were substantially linked to the control status (P<0.001).

To approach variables associated with poor control of DM, a multivariate regression analysis was conducted to identify the significant unconfounded factors correlated with the uncontrolled status of DM. The following variables were significant predictors: dyslipidemia, FBS≥130, inactivity, and poor adherence only (p-value= 0.03, 0.001, 0.03, and 0.043, respectively).

Table 3: Distribution of the study groups by DM related variables

Variable	Categories	Cases No. 160 (%)	Control No. 74 (%)	Total No. 234	P-value	Odds ratio	95% CI
Disease duration	<7 years ≥7 years	42 (26.3) 118 (73.8)	41 (55.4) 33 (44.6)	83 151	<0.001	3.49	1.9 – 6.2
FBS	<130 mg/dl ≥130 mg/dl	15 (9.4) 145 (90.6)	48 (64.9) 26 (35.1)	63 171	<0.001	17.8	8.7 – 36.4
Medication	OHA OHA & Insulin Insulin	45 (28.1) 85 (53.1) 30 (18.8)	56 (75.7) 13 (17.6) 5 (6.8)	101 98 35	<0.001	1.3 1	0.1-0.3 0.3-3.3
Physical activity	Sedentary Occasional Regular	96 (60.0) 48 (30.0) 16 (10.0)	20 (27.0) 26 (35.1) 28 (37.8)	116 74 44	<0.001	8.4 3.2	3.8-18 1.4-7
Self-monitoring	Yes No	80 (50.0) 80 (50.0)	64 (86.5) 10 (13.5)	144 90	<0.001	6.4	3-13.3
Healthy diet	Yes No	44 (27.5) 116 (72.5)	54 (73.0) 20 (27.0)	98 136	<0.001	7.1	3.8-13
Adherence/medication	Low/Moderate High	78 (48.8) 82 (51.2)	6 (8.1) 68 (91.9)	84 150	<0.001	0.09	0.2-0.3

Logistic regression analysis was used to identify the significant unconfounded potential risk factors. The model included all the variables that proved significant in the binary analysis. Only four factors were found statistically significant: Dyslipidemia [OR=11.3, 95%CI:1.7-108.8), P=0.03], high fasting blood sugar [OR=6.23, 95%CI:2.02-19.15), P=0.001], absence of exercise [OR=2.06, 95%CI:2.0-21.1), P=0.03], and poor adherence to the medications [OR=22.5, 95%CI:1.6-46.1), P=0.043].

Discussion

Identifying the proportion and the potential risk factors for patients with type 2 DM who have inadequate glycemic control [HbA1c level >7% (>53mmol/mol) (13)] will help physicians and patients overcome those factors and try to control them as early as possible to decrease the burden on patients and the health system.

The finding that more than two-thirds of the studied DM patients had uncontrolled DM can be attributed to the study setting where the patients are referred because of DM complications or uncontrolled status.

The under-privileged social conditions such as low education and low income were both associated with bad control of DM as indicated higher HbA1C level. (14, 15) Many studies demonstrated that income might contribute to good quality of medication, and in Western countries, income will determine the type of insurance that may affect the glycemic control.(16–18) Managing diabetes with substantial comorbidities is always challenging (19) and the presence of comorbidities was proved to be associated with inadequate glycemic control.(16)

The binary analysis in this study revealed an association between poor control and a positive family history of DM, which was in line with other studies (20). However, another study showed no association between positive family history and poor control.(21) The variability between these results remains inexplicable.

Despite establishing associations between age, BMI, gender, and smoking with control of T2DM by other studies, our results did not show such associations. Abdelmoneim and Al-Homrany had reviewed the files of 198 diabetic patients attending a diabetes clinic in Abha, Saudi Arabia, and reported that type 2 diabetes in younger adults (20–40 years old) was linked to poor glycemic control.(21) Other studies found a rise in the prevalence of DM and poor glycemic control in older (60–74 years) and middle-aged (40–50 years) patients.(22) Due to these inconsistent findings regarding age, it has not been possible to establish a definitive link between age and glycemic control. Glycemic control of DM patients with higher BMI (BMI; ≥25 kg/m^2) tends to be harder to achieve.(23) However, some studies suggested that lower BMI may be present in patients with poor glycemic control (24). This may be related to the frequent irregular meal consumption by obese diabetic patients, resulting in decreased insulin sensitivity and poorer glycemic control. (22)

Dyslipidemia including high cholesterol, TG, and LDL levels was found to be associated with poor control, while this was not the case for HDL. As dyslipidemia is associated with poor control,(25) the metabolic pathogenesis of DM could play an essential role in developing dyslipidemia in DM patients.(26,27)

The disease characteristics investigated in this study, were significantly associated with DM control. Having DM for a long duration is known to be associated with more insulin resistance which may contribute to its association with poor control.(24,28) On the other hand, Type 2 DM patients are frequently in denial and refuse to alter their routines and lifestyles, which prevents successful glycemic control.(21,25)

Since medications are given chronologically, i.e., patients with new-onset DM are given oral medicine first and tend to increase the dose or the frequency of the medication later. Patients with insulin or

combined therapy expect resistance to previous glycemic control medicines that could not control their DM. This was demonstrated in a national study(28), which found that treatment with insulin is associated with poor glycemic control. Also, poor insulin self-management due to a lack of information or skills contributes to inadequate glycemic control among patients on Insulin therapy.(28) Other studies indicated that a lack of diabetes-related knowledge affects one's ability to follow instructions for taking medications and monitoring one's blood sugar levels.(21)

A healthy diet and adherence to medications play an essential role in DM control. A healthy diet improves by improving the knowledge, attitude, and practices that eventually lead to better disease control.(29) Overall, persons with type 2 DM who followed diets based on carb counting showed improvements in their HbA1c and fasting blood glucose levels.(26) Adopting an intervention program to increase medication adherence can enhance glycemic control.(30)

Dyslipidemia has been shown to play a part in managing diabetes mellitus. (20,22), but the specific mechanism of dyslipidemia on DM control is not fully clear. However, insulin resistance has a major impact on the pathogenesis of dyslipidemia in diabetics.(23) The importance of dyslipidemia in DM patients increases the risk of coronary heart diseases.(20) The American Diabetes Association's 2019 guidelines advise using a high-intensity statin to treat all DM patients with dyslipidemia. (22)

Also, adherence to medications has a strong correlation with inadequate glycemic control.(21) The medical condition may worsen due to patients' non-adherence to their prescription diabetic treatment, linked to poor glucose control and subpar effects from the drug, developing comorbidities, reducing the quality of life, elevating healthcare costs, and increasing mortality.(17) Educational programs will positively impact adherence to DM medication and control by lowering the HbA1C.(18)

Physical activity has an essential role in controlling HbA1C(20). Patients with DM experienced a decrease in HbA1C after adapting to a program for physical activity.(31) Given that fact, an educational program for physical activity and adherence will increase the control of DM patients. Finally, and as expected, DM control directly affects FBS; patients with high HbA1C have a higher level of FBS than controlled ones.

While disease characteristics (Duration, Medication, self-monitoring, and healthy diet) were associated with the DM control status in the binary analysis, they failed to show such an association using the logistic regression analysis. These variables might be confounded by other factors that render them insignificant in this study.

The limited size of the well-controlled diabetics is one of the study's shortcomings. This may reflect the general status of the DM population in the country where uncontrolled DM is predominant.

In conclusion, the most important potential modifiable determinants affecting DM's control status were dyslipidemia, high FBS, physical inactivity, and poor medication adherence. Enhancement of education of the patients and their healthcare providers on these factors is crucial to improve the patients' control status.

Authors' declaration:

We confirm that all the Figures and Tables in the manuscript belong to the current study. Besides, the figures and images that do not belong to the current study have been permitted to be re-published in the attached manuscript. Authors sign on ethical consideration's approval-Ethical Clearance: The project was approved by the local ethical committee, the Iraqi Council of Medical Specializations, and the Diabetes and Endocrine Center in Al-Kindy Teaching Hospital according to code number (212) on (25/ 06/ 2020).

Conflicts of Interest: Dr. Faris Lami is an Editor-in-chief of the journal but did not participate in the peer review process other than as an author.

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نسبة وعوامل الإختطار المحتملة لضعف السيطرة على نسبة السكر في الدم بين مرضى السكري من النوع الثاني: تجربة مستشفى ثالثي في بغداد، العراق، 2020

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الخلاصة

الخلفية: داء السكري هو أكثر حالات إضطراب التمثيل الغذائي إنتشارًا في جميع أنحاء العالم. يمكن أن تؤدي الإدارة الذاتية الفعالة لمرض السكري والحفاظ على مستوى الهيموجلوبين الغليكوزيلاتي ضمن المعدل الطبيعي إلى تقليل العبء وتقليل الضغط المالي على النظام الصحي عن طريق تقليل الرقود في المستشفى ومضاعفات مرض السكري.

الهدف: التعرف على عوامل الإختطار المحتملة لضعف السيطرة على نسبة السكر في الدم لدى المرضى المصابين بداء السكري من النوع 2 في بغداد، العراق.

الطريقة: أجريت هذه الدراسة المقطعية في مركز السكري والغدد الصماء في مستشفى الكندي التعليمي، بغداد، العراق. بلغ العدد الإجمالي للمشاركين في الدراسة 234. وبناءً على نسبة الهيموجلوبين الغليكوزيلاتي لـ 7، تم إعتبار المرض عند المرضى الذين تبلغ نسبة الهيموغلوبين الغليكوزيلاتي ≤ 7 غير مسيطر عليه، وأولئك الذين لديهم هيموجلوبين غليكوزيلاتي أقل من 7 يعتبر المرض مسيطر عليه.

النتائج: بلغت نسبة مرضى السكري غير المنضبط 68.4٪ (160). أما الـ 74 مريضا الباقون فكانوا يسيطرون على مرض السكري. لم يظهر العمر والجنس والحالة الإجتماعية والمهنة تمايزاً إحصائيا بين المجموعتين (P> 0.05). أظهر التحليل الثنائي إرتباطًا معنويًا بين حالة السيطرة على السكري ودرجة التعليم (P = 0.001)، والدخل (P = 0.001)، ووجود الأمراض المصاحبة (P = 0.028)، والتاريخ العائلي الإيجابي (P = 0.03)، وخلل شحميات الدم (P = 0.001)، مستوى الكوليسترول (P = 0.002)، ارتفاع مستوى الدهون الثلاثية (P = 0.001) ، ومستوى البروتين الدهني منخفض الكثافة (P = 0.025). لم تختلف حالة التدخين ومؤشر كتلة الجسم ومستوى البروتين الدهني عالي الكثافة بشكل كبير عن حالة السيطرة على السكري (P> 0.05). كانت خصائص المرض، مثل مدة المرض، ونسبة الجلوكوز في الدم، ونوع الدواء، والمراقبة الذاتية، والنظام الغذائي الصحي، والنشاط البدني، والإلتزام بالأدوية، من العوامل المهمة (P <0.001). أظهرت طريقة الإنحدار متعدد المتغيرات أن عسر شحميات الدم، FBS≥130، قلة النشاط البدني، وضعف الالتزام بالعلاج كانت محددات مهمة لعدم السيطرة على السكري (القيمة P = 0.03، 0.001، 0.03، و0.043، على التوالي).

الإستنتاج: كانت أهم عوامل الإختطار التي يمكن تعديلها لضعف السيطرة على مرض السكري هي عسر شحميات الدم، وقلة النشاط البدني، وضعف الإلتزام بالبروتوكول العلاجي. يعد تعزيز تثقيف المرضى ومقدمي الرعاية الصحية حول هذه العوامل أمرًا بالغ الأهمية لتحسين حالة السيطرة على مرض السكري.

الكلمات المفتاحية: عوامل الإختطار، السيطرة على نسبة السكر في الدم، السكري من النوع الثاني

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Research Article

Dental Occlusion as A Health Risk for Visual Acuity in Relation to Salivary Transforming Growth Factor Beta-1 (TGF-B1) among Students Aged 8-10 Years

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Abstract:

Background: Correct mouth function is one of the functional matrices that contribute to the growth of the maxilla, which is connected to the growth of orbit, and vice versa.

Objective: to ascertain how dental malocclusion affects the visual acuity related salivary transforming growth factor beta-1 (TGF-β1).

Methods: This is a cross-sectional study on 653students, 8–10 years of age from elementary schools in the governorate of Al-Diwaniyah during the period from 1st of November 2022lto 30th March 2023. Using the Snellen E chart and Angle's classification to identify malocclusion, they were subjected to visual-capacity testing for refractive disorders. Sub-samples were selected from the normal and visually impaired groups for salivary transforming growth factor beta-1 salivary analysis measurement.

Results: Only 70 out of 653students were found to have reduced visual acuity. When compared to students with normal visual acuity, they had significantly higher levels of salivary transforming growth factor beta-1. Those who with reduced visual acuity were found to have a much higher occurrence of dental malocclusion utilizing molar's relation of Angle's categorization.

Conclusion: The students with impaired visual acuity were more likely to have dental malocclusions and increased levels of TGF-β1.

Keywords: Dental occlusion; health risk; visual acuity; TGF-β1; visual impairment.

Introduction:

Visual acuity (VA) is a measurement of how well the eye can distinguish between small features; it is the sharpness or clarity of vision. Visual disabilities can also affect how well children perform in school and their chances of finding employment in the future 1. Malocclusion is described as an occlusion in which the relationship between the dental arches is abnormal in any plane of space or in which the position of the teeth deviates significantly from normal 2,3.

The continuity of anatomy between the oral and visual organs has been the focus of some investigations. The eye, nasal cavity, maxilla and external ear act as functional matrices, influencing each other under a sophisticated mechanism. Due to the maxilla's interface with every orbital bone, untreated malocclusions can cause visual issues that in turn can change the posture of the mandible 4. Based on its composition, saliva serves several functions- the features of saliva that determine its composition, such as pH, viscosity, flow rate, and buffering capability, are linked to the efficiency of these processes 5.

One of the most significant ligands involved in regulating cell behavior in ocular tissues is the multifunctional growth factor transforming growth factor beta-1 (TGF-β1). This includes regulating cell migration and proliferation, cell death, and protein synthesis during development, tissue repair, and other physiological or pathological processes. TGF-β1 typically increases the production of extracellular matrix and inhibits cell proliferation 6.

In the initial phase of dental occlusion, there is a predominant loss of subchondral bone in the TMJ condyles 7. Compressive forces applied to the TMJ could enhance the expressions of TGF-β-1 8. So far, there has been no previous Iraqi study concerning the effect of dental occlusion on visual acuity related to salivary TGF-β1.

Therefore, this study was conducted in order to assess these relationships under the hypothesis that there was no effect of malocclusion on visual acuity related to salivary TGF-β1.

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Patients and Methods

This study is a cross-sectional study on 110 normal subjects and 70 subjects with visual impairment. These two groups were selected after examining 653 students, 8–10- years of age, recruited from elementary schools in the Governorate of Al-Diwaniyah during the period from 1st of November 2022 to 30 th March 2023. Approvals to conduct the study were obtained through the College of Dentistry's Scientific Committee at the University of Baghdad, number: 726322. Parents' consent for the complete full participation of their children in the study were obtained through a specific consent form sent to the parents prior to the commencement of the data-collecting process. The students enrolled in the study were healthy without any systemic disorders. Students who used eye-glasses, had systemic disorders, or had their first molars extracted were excluded from study.

Snellen E chart and auto-refractometer device were used in the optometrist's examination 9. A Snellen E chart was carried out to assess visual acuity at a distance of 6 meters. The students are asked to point at the letters on the various lines of the chart with each optotype's direction should to be described "up, down, to the door, or to the window". When a student can no longer discern at least half of the letters on a line that is thought to be the smallest line that they can see, both of their eyes are alternately examined. The worse eye level of reduced VA is noted. The distance at which the student starts to read the chart's largest letters is entered as the top number. If the student was not able to read the chart's largest letters he/she was excluded from the study 10,11.

Using the dental relationships of Angle's classification, dental malocclusion was assessed when the teeth were in occlusion. According to Angle's classification of the anteroposterior position of 1st molar, the following is how the upper and lower molars relate to anteroposterior:

- Class I molar relationship: The upper first permanent mesial cusp tooth occludes with the fossa of the lower first permanent molar, which denotes a typical anteroposterior connection.
- Class II molar relationships represent a retrognathic skeletal structure and imply that the lower first permanent molar and upper first permanent molar are disto-occluded.
- Class III molar relationships represent a prognathic jaw and imply a mesially positioned lower first permanent molar 12.

To assess transforming growth factor beta-1 (TGF-β1) salivary analysis was used for 90 participants, including 45 students with normal visual acuity and 45 visually impaired students who were selected randomly from the sample. On a specific case sheet, information on the student's age, gender, Angle classification, visual acuity measurements, salivary flow rate, and TGF-β1 were all recorded.

For the measurement of salivary (TGF-β1) in the morning, an unstimulated saliva sample was taken from drooling passively into a disposable gathering tube 13. An enzyme-linked immunosorbent test (ELISA) was used to identify the salivary TGF-β1.

Statistical analysis:

SPSS version 22, a statistical package for social sciences, was used for data description, analysis, and presentation. The means and standard deviations (SD) were used for quantitative variables. For qualitative variables, frequencies and percentages were utilized. The independent T-test was used to compare the means of the two groups. The Pearson correlation was used to test the correlation between two quantitative variables. $P \leq 0.05$ was used as the levels of significance.

Results

The distribution of the 653 students by the eye sides where visual acuity was tested (left and right) is shown in table 1. A significant association between visual impairment and the sides of the eyes. Eight students (1.2%) had visual acuity impairment in the right eye, seven (1.1%) in the left eye, and 55 (8.4%) in both eyes. The findings revealed an association between malocclusion on the right and left sides and the degree of visual acuity. ClassI students were found to have a higher occurrence of malocclusion than those with normal visual acuity on both sides as shown in table (2). The result in table (3) provides additional evaluations of the relationship between malocclusion and visual acuity at the same sites. This table also demonstrates a strong association on both sides. Table (4) shows that students with visual impairments have significantly higher levels of salivary TGF-β1 than students with normal vision.

The descriptive statistics and statistical differences affecting malocclusion of the molar teeth and visual acuity to transform growth factor beta-1 is shown in table (5). This table shows that while the level of transforming growth factor beta-1 was greater among students with vision impairment, the changes were only appreciable among students with class II on the right side and class III on the left side.

Table 1: Visual acuity status in relation to side affected

		Right eye		P- value
		VA Normal	VA Impairment	
Left eye	VA Normal	583 (89.3%)	8 (1.2%)	0.000
	VA Impairment	7 (1.1%)	55 (8.4%)	

*Significant P≤0.05

Table 2: Distribution of the students by Angle's classification by side and visual acuity

Angle's	classification	Visual Acuity				P-value
		VA Normal		VA Impairment		
		N	%	N	%	
Right	CL Ⅰ	76	69.1	27	38.6	0.000
	CL Ⅱ	23	20.9	23	32.9	
	CL Ⅲ	11	10.0	20	28.6	
Total		110	100.0	70	100.0	
Left	CL Ⅰ	73	66.4	27	38.6	0.001
	CL Ⅱ	23	20.9	22	31.4	
	CL Ⅲ	14	12.7	21	30.0	
Total		110	100.0	70	100.0	

*Significant P≤0.05

Table 3: Distribution of the students by visual acuity by side and Angle's classification

Angle's Classification		VA Right eye				P-value	VA Left eye				P-value
		VA Normal		VA Impairment			VA Normal		VA Impairment		
		N	%	N	%		N	%	N	%	
Right	CL Ⅰ	77	65.8	26	41.3	0.004	79	66.9	24	38.7	0.001
	CL Ⅱ	26	22.2	20	31.7		26	22.0	20	32.3	
	CL Ⅲ	14	12.0	17	27.0		13	11.0	18	29.0	
Total		117	100.0	63	100.0		118	100.0	62	100.0	
Left	CL Ⅰ	74	63.2	26	41.3	0.013	77	65.3	23	37.1	0.001
	CL Ⅱ	26	22.2	19	30.2		25	21.2	20	32.3	
	CL Ⅲ	17	14.5	18	28.6		16	13.6	19	30.6	
Total		117	100.0	63	100.0		118	100.0	62	100.0	

*Significant P≤0.05

Table 4: Salivary transforming growth factor beta-1 (ng/l) (Mean±SE) in relation to visual acuity

Angle's Classification		Salivary Status	Visual Acuity				T-test	P-value
			VA Normal		VA Impairment			
			Mean	±SE	Mean	±SE		
Right	CL Ⅰ	TGF-β1	217.03	14.02	261.81	18.54	0.141*	0.057
	CL Ⅱ		210.43	25.23	314.97	29.58	0.230*	0.021
	CL Ⅲ		213.33	26.79	356.39	53.08	0.98	0.106
Left	CL Ⅰ		221.92	13.19	260.35	18.32	0.211	0.088
	CL Ⅱ		214.19	27.93	294.14	31.28	0.338	0.088
	CL Ⅲ		190.41	28.99	360.28	49.56	0.776*	0.027

*Significant P≤0.05

Table 5: Salivary transforming growth factor beta-1 (Mean±SE) according to Angle's classification by visual acuity status

Salivary Variables	Visual acuity				T-test	P-value
	VA normal		VA impairment			
	Mean	±SE	Mean	±SE		
TGF-β1	215.06	11.04	302.89	21.84	2.402 *	0.000

*Significant P≤0.05

Discussion

In the current study, among the 653 primary school students investigated, the prevalence rate of decreased VA was 10.7%. Differences in sampling techniques, operational definitions, testing population sizes, and geographic regions may be responsible for the variations in prevalence found in different studies. This prevalence was lower than found in northern Iraq 14, and those detected in other countries such as Al Hassa Region/ Saudi Arabia 15, Qassim Regions/ Saudi Arabia 16, and Qatar 17. The overall prevalence of visual impairment was higher than that found in South Africa 18, Iran 19, Malaysia 20, and Nepal 21 considerably

The differences in the percentages of unilateral versus bilateral VA impairment found in the current study are in line with earlier research 22.

In the current investigation, malocclusion was expressed using the same classification system as in an earlier study conducted at Iraq 23. Depending on the Angle's classification, the data shows that children who have poor visual acuity are more likely to suffer malocclusions. with an important association on both sides. This outcome is consistent with a study that indicated that patients with vision impairment had higher percentages of malocclusion 4.

Drooling saliva was collected using the same technique as in earlier investigations conducted in Iraq 24-29. According to a prior study, TGF-β1 activity generally increases in ocular disorders 6. Additionally, in the current investigation, children with visual acuity impairment had significantly higher levels of TGF-β1 than children with normal visual acuity. One of the elements that affect how strong the inflammatory response of this cytokine is, maybe that TGF-β1 affects host cells in a pro- and anti-inflammatory manner during the genesis and development of illness. It is a crucial mediator in the fight against inflammation, suggesting long-lasting wound healing and chronic inflammation during host responses 30. Some or all of these TGF-β1 dependent mechanisms may be involved in the start and control of tissue degradation and inflammation throughout disease processes 30-32. Due to the association between malocclusions with many factors 33 including visual acuity impairment, it is advised that visual tests be incorporated into the assessment techniques applied to the examination of the stomatognathic system.

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Conclusions

A considerably higher prevalence of visual impairment was found among students affected by dental malocclusion and increased in salivary TGF-β1.

Authors' declaration:

We confirm that all the Figures and Tables in the manuscript belong to the current study. Besides, the Figures and images, which do not belong to it have been given permission for re-publication attached to the manuscript. Authors sign on ethical consideration's approval-

Ethical Clearance: The project was approved by the local ethical committee in the University of Baghdad's internal ethics committee for dentistry according to the code number (726322),) on (Date/ Month/ Year).

Conflict of interest: None.

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Author Contributions:

Study conception & design: (Ban S. Diab). Literature search: (Noor A. Ajeej, Ban S. Diab). Data acquisition: (Noor A. Ajeej). Data analysis & interpretation: (Noor A. Ajeej). Manuscript preparation: (Noor A. Ajeej, Ban S. Diab). Manuscript editing & review: (Noor A. Ajeej, Ban S. Diab).

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1- إطباق الأسنان عامل خطورة على حدة البصر وعلاقته بعامل النمو المحول اللعابي بيتا (TGF- β1)بين الطلاب الذين تتراوح أعمارهم بين 8-10 سنوات

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الخلاصة
الخلفية: وظيفة الفم الصحيحة هي واحدة من المصفوفات الوظيفية التي تساهم في نمو الفك العلوي ، والتي ترتبط بنمو محجر العين، والعكس صحيح.
الأهداف: التأكد من تأثير سوء إطباق الأسنان على حدة البصر المرتبط بعامل النمو المحول اللعابي بيتا -1(TGF-β1) .
الحالات والمنهجية: هذه دراسة مقطعية على 653 طالباً ، تتراوح أعمارهم بين 8-10 سنوات من المدارس الإبتدائية في محافظة الديوانية خلال الفترة من 1 نوفمبر 2022 إلى 30 مارس 2023، وبمساعدة مخطط Snellen E وتصنيف Angle لتحديد سوء الإطباق، تم إخضاعهم لإختبار القدرات البصرية للإضطرابات الإنكسارية. تم إختيار عينات فرعية من المجموعات العادية وضعاف البصر لتحويل قياس تحليل عامل النمو المتحول بيتا -1 اللعابي.
النتائج: بينت الدراسة الحالية أن 70 فقط من أصل 653 طالباً لديهم إنخفاض في حدة البصر. عند مقارنتها بالأفراد العاديين الذين لديهم مستويات أعلى بكثير من عامل النمو المتحول بيتا -1 اللعابي ، وجد أن أولئك الذين لديهم إنخفاض في حدة البصر لديهم نسبة أعلى بكثير من سوء إطباق الأسنان بإستخدام علاقة الضرس بتصنيف الزاوية.
الإستنتاجات: أن الطلاب الذين يعانون من ضعف حدة البصر كانوا أكثر عرضة للإصابة بسوء إطباق الأسنان مع زيادة مستويات TGF-β1.
الكلمات المفتاحية: إطباق الأسنان، المخاطر الصحية، حدة البصر،TGF-β1، ضعف البصر.

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Research Article

The Effect of Emotional Abuse on Periodontal Status and Salivary Resistin

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Abstract:

Background: Emotional abuse is a widespread form of child abuse. It is suggested that the adverse outcomes from all forms of abuse mostly result from the emotional impacts. Resistin, a polypeptide high in cysteine, can serve as a valuable biochemical indicator of periodontal tissue damage when detected in the saliva.

Objective: To assess the relationship of emotional abuse with periodontal status and salivary resistin levels.

Methods: This cross-sectional comparative study included 522 intermediate school students aged 13-15 years in Kirkuk/Iraq. Information on emotional abuse was obtained using Glaser criteria which is a self-administered, structured, questionnaire form. The levels of emotional abuse were categorized into mild (score between 24-26), moderate (score between 27-42), and severe (score between 43-72). Periodontal health was assessed by using the community periodontal index (CPI). Non-stimulated saliva was collected and salivary resistin levels were evaluated using an enzyme-linked immunosorbent assay (ELISA).

Results: The most common level of abuse was moderate. The largest percentages of participants with dental calculus were observed in moderate (48.2%) and severe (57.5%) abuse groups and the highest mean number of sextants with gingival bleeding was observed in participants who experienced a moderate level of abuse (0.34). While the level of resistin was higher in the mild abuse group (237.06), all of its relationships with the periodontal condition were non-significant.

Conclusion: Emotional abuse affects salivary resistin levels but has no obvious effect on periodontal status. Non-significant correlations were observed between salivary resistin levels and the levels of abuse with periodontal conditions.

Keywords: Emotional abuse; Child abuse; Periodontal disease Saliva; Resistin.

Introduction:

All forms of child abuse, including physical and sexual abuse as well as neglect, occur simultaneously with emotional abuse (1). It is one of the most harmful forms of abuse, but it is also one of the least recognized causes of trauma. Because of its insidious nature, emotional abuse can go undetected for a long time even when it has severe consequences for an adult victim (2). Emotional abuse is frequently disregarded due to the absence of a globally recognized term. Researchers may define "emotional abuse" based on either the behaviors of victims or the effects, depending on their specific area of study (3). Due to the broad scope of the term "abuse," scholars have employed many alternative names such as "emotional abuse," "psychological maltreatment," "psychological abuse," and "verbal abuse." Glaser (4) presents an alternative framework for assessing emotional abuse, excluding any consideration of parental behavior or relationships between parents and children. Periodontal disease, including gingivitis and periodontitis, is one of widespread diseases in the world 5, and its prevalence varies by gender, ethnicity, geographic area, and socioeconomic status. Some conditions such as nutritional insufficiency can predispose an individual to develop periodontitis or exacerbate existing periodontitis (6). Several studies have examined the relationships between psychological and social factors and the development of periodontal disease, and have reported that patients who have inadequate coping strategies and particular emotional behaviors (e.g., anger) are at a greater risk of developing severe periodontal diseases (7). Other studies have found associations between exposure to abuse and poor oral health behaviors and outcomes (8). For instance, when parents show a lack of motivation and interest in preserving the oral health of child victims of abuse, and when the children themselves have low self-esteem, it can lead to elevated levels of gingivitis in comparison to children who have not experienced violence (9). Minhas et al 8 reported that exposure to psychological and domestic abuse is linked to a higher likelihood of having periodontal disease, which includes periodontitis and/ or gingivitis. Others have reported that immunity can be greatly impaired by psychological abuse which can promote the progression of periodontal infections (10).
Depression is also biologically linked to oral health and overall quality of life, and researchers have found

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that stressful circumstances can negatively affect gingival tissue (11,12,13). The effects of stress on periodontal tissues involve reduced salivary flow, which can enhance dental plaque formation (7). Decreased stressors in the social environment are connected with positive attitudes to health in general including oral and thus gingival health (14).

Saliva is an ideal biological fluid for medical diagnosis as it is inexpensive to sample, and collection is non-invasive, painless, and easy for individuals of all ages, including small children and the elderly (15). The composition of different compounds in the saliva serves as an indicator of an individual's overall health and can be utilized to promptly and effortlessly identify pathological alterations in humans (16).

Resistin is a polypeptide rich in cysteine, that is commonly referred to as a secretory factor particular to adipose tissue (17,18). The typical serum concentration of human resistin falls within the range of 7 to 22 ng/mL (19). Resistin is mostly released by immunological mononuclear cells in humans, which are responsible for various pro-inflammatory effects 20. This peptide is minimally expressed, if at all, in human adipose cells, but it is abundantly expressed in mononuclear leukocytes, macrophages, spleen, and bone marrow cells (21). The expression of resistin in human peripheral blood mononuclear cells is increased by pro-inflammatory cytokines such as IL-1, IL-6, and TNF-α (22). Thus, resistin likely plays a significant function in inflammation in humans.

A study was carried out by Abdalla et al (23); on measuring resistin levels in the saliva and examining the correlation between its levels in saliva and blood serum using a commercially available enzyme immunoassay technique. They found a strong positive link between the levels of resistin in the blood and saliva, with no notable correlation observed between these levels and age, body fat percentage, or BMI. The serum-to-salivary ratio of resistin was 0.2. This correlation between salivary and serum levels of resistin suggests that resistin is transferred from the blood to the saliva. This finding supports the potential use of salivary resistin levels rather than serum levels for early detection of disease. Sabir and Ahmed (24) have reported that salivary resistin could serve as a valuable biochemical indicator of periodontal tissue damage. Indeed, others have found that salivary resistin levels are significantly higher among individuals with chronic periodontitis, as compared to those with gingivitis and individuals in a healthy control group (25).

Individuals who have experienced childhood abuse may have lasting negative physiological impacts as a result of the very stressful early-life circumstances they have endured. They are significantly more susceptible to illnesses such as obesity and metabolic disorders (26). Childhood abuse, as well as other diseases including depression and psychological stress, is marked by a significant pro-inflammatory state (27). Researchers have investigated whether individuals who have experienced childhood abuse exhibit elevated levels of resistin, in addition to the previously observed inflammatory abnormalities. Lehto et al. (28); have documented a direct association between levels of resistin and childhood maltreatment. However, it has been suggested that this association is due to low concentrations of the anti-inflammatory biomarker adiponectin. Reduced levels of adiponectin can result in decreasing the ability to counteract inflammation, hence increasing the vulnerability to conditions that exhibit significant pro-inflammatory characteristics, both physically and psychologically.

To the best of our knowledge, no Iraqi study has assessed the emotional abuse effect on periodontal status or assessed its correlation to salivary resistin levels. For that reason, this study aimed to detect the influence of emotional abuse on periodontal status and salivary resistin levels.

Participants and methods

A cross-sectional comparative study was conducted on 522 female intermediate school students aged 13-15 years in Kirkuk, Iraq, who were from different socioeconomic backgrounds. The study was conducted from November 2022 to February 2023. Approval to conduct the study was obtained from Kirkuk Directorate of Education which helped to ensure the cooperation of school staff.

Assessment of emotional abuse: The Glaser criteria 4, were used to assess emotional abuse. This tool consists of 44 questions answered on a Likert scale. The original English version of the emotional abuse questionnaire was translated by experts into the Arabic language and the number of questions was reduced to (30); using input from psychologists and psychiatrists. As the reliability and validity of this questionnaire needed to be assessed, it was initially administered to (150 female students) and the answers were sent to a group of experts (psychologists and psychiatrists) in the College of Educational Psychology / Department of Psychological Research at the University of Baghdad for revision and assessment. These experts adjusted the questions to improve compatibility with the Iraqi community and to improve the level of students' understanding of the questions. Ultimately, the number of questions was reduced to (24).

Before collecting the data, the students were informed of the nature of the study, were told that their data would be kept confidential, only used for scientific research purposes, and that it would not affect their academic evaluations. The students were also allowed to ask any questions at this stage. All students were required to have a consent form signed by their parents, before completing the questionnaire. After calculating the score for each case sheet that the students filled out, certain answers received a reverse degree and then the final score was determined for each student. The levels of emotional abuse were categorized into:

- Mild if the score on the questionnaire was 24-26.

- Moderate if the score was 27-42.
- Severe if the score was 43-72.

The maximum possible score on the questionnaire was (72) degrees for each paper of the questionnaire. A subsample of 88 students from the total of 522 was chosen for saliva collection (44 experiencing mild abuse and 44 experiencing severe abuse).

Assessment of periodontal health

Periodontal health was assessed using the community periodontal index (CPI) which was modified by the WHO in 1997 29. The CPI probe is unique in that it is light-weighted, has a 0.5 mm ball at the edge, and is graded, with a ring of black separating 3.5 and 5.5 mm and circles 8.5 and 11.5 mm from the edge 30. For subjects under the age of 20 years, only six index teeth 16, 11, 26, 36, 31 and 46 (maxillary right first molar, maxillary right central incisor, maxillary left first molar, mandibular left first molar, mandibular left central incisor, mandibular right first molar) are examined. This modification is made to avoid scoring the deepened sulci associated with eruption as a periodontal pocket. For the same reason, when children under the age of 15 are examined, the periodontal pocket should not be recorded, i.e. only bleeding and calculus should be considered. Periodontal health status was coded according to the following criteria Table (1):

Table1: Periodontal health status code

Code	Definition
0	Healthy (no bleeding, no calculus)
1	Immediate bleeding was noted following probing
2	Calculus was discovered during the probing, but the entire black band on the probe was visible

After examination, the participants received instructions about how to keep their mouth clean and were informed about the importance of teeth brushing to prevent the formation of dental plaque that causes gingivitis and periodontitis.

Salivary resistin measurement: Students were directed to rinse their mouths with water approximately 5 minutes before delivering the saliva sample. Non-stimulated saliva was obtained by allowing it to passively accumulate in a disposable collection cup, following the saliva collection procedures established by the University of South California School of Dentistry31 as used in a prior study 32. Following the collection process, the salivary samples underwent centrifugation at a speed of 2000-3000 rpm for 20 minutes. The resulting liquid portion, known as the supernatant, was carefully transferred into sterile Eppendorf tubes. These tubes were then labeled with unique codes assigned to each student and stored at a temperature of -20°C until they could be subjected to additional analysis. The measurement of human resistin was conducted using an enzyme-linked immunosorbent assay (ELISA) by the instructions provided by the manufacturer.

Statistical analysis

Data analyses were performed using the statistical package for social sciences SPSS v.22 (Chicago, Illinois, USA). For quantitative variables, the means, and standard errors (SE) were calculated, while frequencies and percentages were used for categorical variables. Independent sample t-tests were used for comparisons between two means. Pearson's correlation was used to determine the linear correlation between two quantitative variables. ANOVA test was used for a quantitative dependent variable by a single factor (independent) variable. The Chi-square was used for analyses of contingency tables between two categorical data when the sample size was large. A P value of ≤ 0.05 was considered statistically significant.

Results

The levels of abuse across ages are shown in Table 2. For the total sample (n = 522), the highest reported level of abuse was moderate (75.1%) followed by severe (15.3%) and mild (9.6%). The same pattern was seen across all age groups with no significant association between age and abuse level.

Table 2. Distribution of the levels of abuse across ages

Abuse level	Age (Years)								Chi square	p value
	13		14		15		Total			
		%	n	%	n	%	n	%		
Mild	20	8.1	18	13.1	12	8.7	50	9.6	5.941	0.204
Moderate	195	79.0	98	71.5	99	71.7	392	75.1		
Severe	32	13.0	21	15.3	27	19.6	80	15.3		
Total	247	47.3	137	26.3	138	26.4	522	100.0		

The CPI scores across the abuse levels are shown in Table 3. Generally, across all abuse levels, the most common
CPI was score 2 (dental calculus) followed by score 0 (healthy gingiva), and score 1 (bleeding gingiva). There was no significant association between CPI scores and the levels of abuse.

'Table 3. Distribution of CPI scores across abuse levels

CPI score	Abuse level						Chi square	p-value
	Mild		Moderate		Severe		6.324	0.176
	n	%	n	%	n	%		
0	19	38.0	164	41.8	27	33.8		
1	1	2.0	39	10,0	7	8.8		
2	30	60.0	189	48.2	46	57.5		

Table 4 shows that the mean number of sextants with CPI 0 (healthy gingiva) was higher among individuals experiencing a moderate level of abuse. This pattern of results was also observed for the mean number of

sextants with CPI 1 (bleeding gingiva), while for CPI 2 (dental calculus) the mean number of sextants was higher for the mild level of abuse. However, the mean number of sextants did not differ significantly across the levels of abuse.

Table 4. Mean values of sextants at each CPI score across abuse levels

CPI score	Abuse level						F	P-value
	Mild		Moderate		Severe			
	Mean	SE	Mean	SE	Mean	SE		
CPI 0	4.72	0.216	4.84	0.068	4.44	0.117	0.317	0.728
CPI 1	0.18	0.062	0.34	0.036	0.23	0.056	1.881	0.153
CPI 2	1.14	0.090	0.85	0.056	1.04	0.035	2.012	0.135

Table 5 displays the differences in resistin levels between the groups with mild and severe abuse. Mild abuse was associated with greater mean salivary resistin levels than severe abuse (p=0.018).

Table 5. Mean salivary resistin levels in mild and severe abuse groups

Saliva Sample	Abuse					
	Mild		Severe			
	Mean	SE	Mean	SE	t	p-value
Resistin (ng/L)	237.06	20.518	178.78	13.123	2.415	0.018

The correlations between salivary resistin levels with periodontal health status across mild and severe abuse groups are displayed in Table 6. Salivary resistin levels were negatively correlated with CPI 1 (gingival bleeding) and CPI 2 (dental calculus) at the mild level of abuse and positively correlated with CPI 0 (healthy gingiva) while, at a severe level of abuse they were negatively correlated with CPI 0 and CPI 1 and positively correlated with CPI 2. However, all correlations were non-significant.

Table 6. Correlation of salivary resistin levels with periodontal parameters across the abuse level

Abuse	CPI	Resistin	
		r	p
Mild	CPI 0	0.101	0.519
	CPI 1	-0.058	0.712
	CPI 2	-0.005	0.975
Severe	CPI 0	-0.031	0.838
	CPI 1	-0.064	0.677
	CPI 2	0.067	0.660

Discussion:

In the present study, a moderate level of emotional abuse was the most commonly observed type among Iraqi children in Kirkuk. These results are in disagreement with a previous Iraqi study 33 which reported that the percentage of children experiencing severe abuse was higher than that for mild abuse. This may be attributed to life difficulties and stress making parents psychologically tense, which is reflected on children and can affect their mental health (34,35). A study conducted by Dye in 2020 reported that emotional abuse has moderate positive correlations with depression, anxiety, stress, and neurotic personality (36).

The results of the current study with the highest percentages of CPI 2 (dental calculus) found in the moderate and severe levels, and the highest mean number of sextants with CPI 1 (gingival bleeding) being in moderate levels of abuse are in agreement with those of previous studies (37,38). The effects might be ascribed to the emotional repercussions and diminished self-worth endured by abuse victims, leading them to disregard their overall and dental healthcare. Parents of abused children may be less inclined to prioritize the oral health of their children and have a reduced inclination to seek dental care (39). In addition, many abused children may fear dental treatment as a result of loneliness or a lack of emotional support from parents, which can lead to a failure to return to the dentist and even rejection of a proposed treatment plan (9). These same arguments were brought up in an earlier systematic analysis that analyzed the causes and manifestations of dental neglect (37,38).

The current study revealed that resistin levels were higher at the mild level of abuse compared to the severe level, suggesting that emotional abuse decreased the levels of resistin. This contradicts the findings of Lehto (28), who concluded that there was an association between low levels of adiponectin and childhood abuse, while serum resistin levels showed no such association. Nevertheless, this outcome aligns with the findings of Levandowski et al 40, who found that those who experienced childhood maltreatment exhibited lower plasma levels of resistin compared to those who did not. Other research also reported that individuals who have experienced childhood maltreatment or are currently experiencing psychiatric symptoms like depression exhibit an abnormal release of resistin (41,42).

In the present study, no significant relationships were observed between periodontal condition and abuse scores with salivary resistin levels. The findings of this study contrast a recent publication that found a significant positive correlation between salivary resistin levels and gingival crevicular fluid levels, mean periodontal pocket depth, clinical attachment loss, and the extent of the periodontal inflamed surface area. According to the findings of other studies, high concentrations of resistin in the saliva are associated with severe local inflammation in the periodontium. Furthermore, these findings revealed that resistin seeps from the gingival crevicular fluid into the oral fluid, and it is produced by immune cells that respond to periodontopathic bacteria (43).

Conclusion:

Emotional abuse affects salivary resistin levels but has no obvious effect on periodontal status. Non-significant correlations were observed between

salivary resistin levels and the levels of abuse with periodontal conditions.

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Authors' declaration:
We affirm that all the tables included in the manuscript belong to us. The approval of ethical considerations is signed by the authors. Ethical Clearance: According to the code number (project No. 684322), the project was accepted by the University of Baghdad's internal ethics committee for dentistry.
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Study conception & design: (Ban S. Diab). Literature search: (*Qabas F. Sami*, Ban S. Diab). Data acquisition: (*Qabas F. Sami*). Data analysis & interpretation: (*Qabas F. Sami*). Manuscript preparation: (*Qabas F. Sami*& Ban S. Diab). Manuscript editing & review: (*Qabas F. Sami*& Ban S. Diab).

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تأثير الإساءة العاطفية على حالة اللثة وعلاقتها بمقاومة اللعاب

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الخلاصة
الخلفية: الإساءة العاطفية هي شكل شائع جدًا من أشكال إساءة معاملة الأطفال. يقترح أن النتائج السلبية الناجمة عن جميع أشكال سوء المعاملة تنجم في الغالب عن التأثيرات العاطفية. الريسيستين هو متعدد الببتيد غني بالسيستين وقد تكون مستوياته في اللعاب علامة كيميائية حيوية مفيدة لتدمير أنسجة اللثة.
الهدف: تقييم آثار الإساءة العاطفية على حالة اللثة فيما يتعلق بمستويات الريسيستين في اللعاب.
المواد والطرق: شملت هذه الدراسة المقارنة 522 طالبة تتراوح أعمارهن بين 13-15 سنة من المدارس المتوسطة في كركوك / العراق. تم الحصول على معلومات عن الإساءة العاطفية باستخدام إستبيان منظم ذاتي الإدارة وتم تقييم صحة اللثة باستخدام مؤشر اللثة المجتمعي **(CPI)**. تم جمع اللعاب غير المحفز وقياس الريسيستين في اللعاب باستخدام مقايسة الماصة المناعية المرتبطة بالإنزيم.
النتائج: كان مستوى سوء المعاملة الأكثر شيوعا هو المستوى المتوسط. وقد لوحظت أكبر النسب المئوية للمشاركات اللواتي يعانين من التكلسات على الاسنان في مجموعات سوء المعاملة المعتدلة والشديدة ولوحظ أعلى عدد متوسط من السدسية مع نزيف اللثة في المشاركات اللواتي عانين من مستوى معتدل من سوء المعاملة. في حين أن مستوى الريسيستين كان أعلى في مجموعة سوء المعاملة الخفيفة، فإن جميع علاقاته بحالة اللثة كانت غير معنوية.
الإستنتاج: يؤثر الإيذاء العاطفي على مستويات مقاومة اللعاب ولكن ليس له تأثير واضح على حالة اللثة. وقد لوحظت ارتباطات غير هامة بين مستويات ريسيستين اللعاب ومستويات سوء المعاملة مع أمراض اللثة.
الكلمات المفتاحية: الإساءة العاطفية، إساءة الأطفال، أمراض اللثة، اللعاب، الريسيستين

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Research Article

Role of Ramadan Fasting on Secretory IgA and Statherin Levels in Individuals with Dental Caries

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Abstract

Background:
Tooth decay happens when bacteria in the mouth create acid from carbohydrates that can be fermented. Along with factors related to the person (like genetics or oral hygiene habits) and saliva, this process leads to cavities. During Ramadan fasting, when eating habits change, there could be effects on health, particularly on the salivary biomarkers that are important for dental well-being.

Objectives: This cross-sectional study aimed to detect the activity and concentrations of salivary levels of secretory immunoglobulin A and Statherin during and after Ramadan fasting and to evaluate the association between these biomarkers with dental caries.

Methods: The study comprised 40 individuals, aged 20-25 years, diagnosed with dental caries. Participants were assessed for periodontal parameters using the Plaque Index and the Gingival Index. Saliva samples were collected during the fourth week of Ramadan fasting and two weeks after Ramadan fasting. The concentrations of both secretory IgA and Statherin were measured in salivary samples using Enzyme-Linked Immunosorbent Assay.

Results: A significant decrease in the mean concentration of secretory immunoglobulin A was observed during Ramadan fasting (2.14±0.21 ng/L) compared to post-fasting (3.34±0.35 ng/L) (p=0.001). However, there was a non-significant difference (p=0.05) in slathering levels between the fasting state (2.25±0.18 ng/L) and the post-fasting state (2.85±0.22 ng/L). No statistically significant difference was found concerning both the Plaque Index and the Gingival Index within fasting and post-fasting states.

Conclusion: Low concentration of sIgA and Statherin during Ramadan fasting may indicate altered salivary gland activity or systemic response to fasting, potentially affecting oral health.

Keywords: Dental Caries; Fasting; Gingival Index; Plaque Index; Secretory immunoglobulin A (sIgA); Statherin,

Introduction

Ramadan, the ninth month of the lunar calendar, lasts 29–30 days. During this period, Muslims fast from dawn until sunset, refraining from food and drink for 11–18 hours daily. This change in eating habits and alterations in relaxation and exercise routines disrupt daily rhythms (1). Religious fasting has implications for physiology and disease pathogenesis mechanisms (2). Maintaining good oral hygiene can significantly mitigate dental issues, habits such as neglecting nighttime brushing, overeating, or consuming high-fat meals can lead to oral disorders. These include dental caries, gingivitis, and periodontitis (3). Dental caries is a prevalent and complex global oral health concern, resulting from interactions among acid-producing bacteria, fermented carbohydrates, host factors, and saliva (4,5).

Saliva plays a pivotal role in oral health and physiology. It's also a trusted tool for detecting biomarkers indicative of host protective mechanisms. Salivary proteins help clean teeth, prevent abrasion, delay demineralization, facilitate remineralization, neutralize acids, and protect the oral cavity from infections (6).

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Components like Lysozyme, lactoferrins, lactoperoxidase, immunoglobulin, albumin, mucins, histatins, statherin, defensins, cathelicidin LL-37, and other immunoglobulins shield oral tissues (7,8). Secretory IgA (sIgA) is an antibody prevalent in mucous membranes, including those in the oral cavity, respiratory, and digestive tracts. As the primary immunoglobulin in saliva, sIgA is vital for defending the oral cavity against microbial invasions. Bacteria, especially Streptococcus mutans, form biofilms (plaque) on teeth and are the main cause of fermenting dietary sugars. The resultant acids, especially lactic acid, demineralize tooth enamel, leading to cavity formation (8,9). sIgA antibodies in saliva combat dental caries in several ways: by binding to bacterial toxins, enzymes, and adhesins, inhibiting bacterial adherence and colonization, causing bacterial agglutination, and tagging bacteria for immune system destruction (9). Additionally, sIgA forms a barrier on the tooth, impeding bacterial penetration (10). Statherin, a 43-amino acid peptide weighing 5.4 kDa, protects teeth by capturing calcium ions and fostering remineralization. It also manages bacteria by clustering them, preventing them from sticking to hard tissues and epithelium (11). Statherin is increasingly recognized as a potent salivary

indicator of bacterial infection and dental caries (12). Fasting has the potential to alter immunological functions. It augments catabolism and macrophage activity through cellular breakdown (13). While fasting boosts B cell-mediated immunity, its effects on cell-mediated immunity are less clear. It enhances the activity of neutrophils (bactericidal), monocytes, and natural killer cells (14). This study aims to detect the activity and concentrations of secretory IgA and statherin levels in saliva during and after Ramadan fasting and evaluate the association between these biomarkers and dental caries.

Materials and Methods

Subjects

This cross-sectional study included forty participants (20 females and 20 males) who attended the Dental College Teaching Hospital/ Baghdad University and Specialized Dental Center /Al-Sadr City for treatment and follow-up of tooth decay. Their ages ranged from 20 to 25 years. During the patient's follow-up, a second saliva sample was retaken two weeks post-fasting.

Ethical approval

The study protocol was approved by the scientific committee at the Basic Sciences Department/College of Dentistry/University of Baghdad, on 1/4/2023 (Project No. 824823). All patients were given detailed information about the study's objectives and informed consent was signed to represent the patient's acceptance of being involved in the study.

Inclusion criteria:

1. Individuals suffering from tooth decay between the ages of 20-25 years.
2. Individuals who take their last meal two hours before sunrise (at dawn).
3. Those who do not mind following up after fasting.

A follow-up was conducted for this category two weeks after Ramadan fasting.

Exclusion criteria

1. Individuals with systemic, chronic, autoimmune diseases or hypersensitivity reactions, in addition to those having DM, periodontal diseases, or any inflammatory conditions.
2. Non-fasting subjects.
3. Individuals with structural dental defects.
4. Patients undergoing caries treatment.
5. Smoking subjects.

Oral examination:

During the study, each subject underwent a clinical examination conducted by a dentist. This included assessing the periodontal status of all teeth using a periodontal probe. The key periodontal parameters measured were the plaque Index (PI) and the gingival Index (GI).

The presence of plaque is a primary factor in the development of cavities (dental caries) and gingival disease (periodontal disease). When using the Plaque Index, a dental professional typically examines the teeth and assigns a score based on the thickness of plaque and the area it covers. The scoring ranges from 0 (no plaque) to a higher number indicating a thicker and more extensive plaque covering. The Gingival Index system was used to evaluate gingival inflammation, with the presence of inflammation on two surfaces of each tooth being noted. The scoring system for this index ranged from 0, indicating no plaque, to 1, signifying the presence of gingival inflammation (15).

Saliva Collection

One to three milliliters of whole unstimulated saliva were collected for the same participant at two different times; the first, during the fourth week of fasting and the second, two weeks after Ramadan. The samples were gathered between 9 a.m. and 12 p.m. After collection, each sample was placed in a sterile plain tube and centrifuged at 2000 rpm for 15 minutes. The supernatant was then transferred to an Eppendorf tube. All samples were subsequently stored in a deep freezer at -80°C, until analysis (9). Enzyme-linked immunosorbent Assay (ELISA) was employed for the detection of pro-inflammatory biomarkers using the Human ELISA quantitative immunoassay kit (Secretory IgA/Lot No: E23DYH836, Feiyuo company, China) and (Statherin/Lot No: E23DAJ660, Feiyuo company, China) in saliva samples. The readings were obtained using an ELISA reader from BioTek (USA). **Principle of the Procedure:** This kit uses sandwich enzyme immunoassay. This package includes a microtiter plate pre-coated with either Secretory IgA and Statherin antibody. Microtiter plate wells containing standards or samples get a biotin-conjugated Secretory IgA and Statherin antibody. Incubate microplate wells with avidin-HRP. TMB substrate solution only colors wells containing Secretory IgA and Statherin, biotin-conjugated antibodies, and enzyme-conjugated avidin. Sulfuric acid blocks the enzyme-substrate reaction, and the color change is measured at 450 nm±10 nm. The concentration of biomarkers in the samples is then determined by comparing the optical density (OD) of the samples to the standard curve (16).

Statistical analysis

The data processing utilized Statistical Package for Social Sciences (SPSS) version 26 and Microsoft Excel 2010. Statistical analyses included Chi-squared and *t*-test, as well as Wilcoxon Signed Ranks tests. The significance level was set at $P<0.05$ for significant results.

Results

The study includes 40 young individuals between the ages of (20-25) years. The data revealed that there was a non-significant difference between female and male participants regarding their mean age as it was found to be (22.80±1.936) years for the females and (22.85±2.007) years for the males. Statistical analysis

(*P*>0.05) indicated that the observed differences in mean age for both sex was not statistically significant. According to the findings of this study, there is no significant difference (p > 0.05) in both the Plaque Index (PI) and the Gingival Index (GI) between the fasting and post-fasting follow-up periods. The mean values for the PI were (0.18±0.15) for the fasting group and (0.16±0.15) for the post-fasting group. Similarly, the mean values of the GI for both periods were (0.21±0.23) and (0.24±0.11), respectively, as shown in Table 1.

Table 1: Levels of Plaque Index (PI) and Gingival Index (GI) parameters in study groups (fasting, post-fasting)

Variable	Study group		*t*- test	*P*-value
	Fasting	Post-fasting		
	mean±SD	mean±SD		
Plaque index (PI)	0.18±0.15	0.16±0.15	0.455	0.651
Gingival index (GI)	0.21±0.23	0.24±0.11	0.623	0.537

The results for sIgA are presented in Table 2. The level of sIgA (ng/L) decreased in the fasting group (2.14±0.21 ng/L) compared to the subsequent post-fasting measurement (3.34±0.35 ng/L), with a statistically significant difference (p=0.001),

Table 2: The changes in salivary concentration of sIgA (ng/L) in individuals with dental caries (fasting and post-fasting).

Wilcoxon Signed Ranks Test	sIgA concentration (ng/L)	
	Fasting	Post-fasting
mean±SD	2.14±0.21	3.34±0.35
p-value	0.001	

The results in Table 3 indicated a non-significant decrease in statherin levels between the fasting group compared to post-fasting one. The mean±SD values were (2.25±0.18) and (2.85±0.22), respectively, (*P*>0.05).

Table 3: The changes in Statherin (ng/L) levels among individuals with dental caries(fasting and post-fasting).

Wilcoxon Signed Ranks Test	Statherin level (ng/L)	
	Fasting	Post-fasting
mean±SD	2.25±0.18	2.85±0.22
p-value	0.05	

Discussion

The present study consisted of 40 individuals diagnosed with dental caries, equally divided into males and females. This intentional gender parity in the study design was crucial to offset potential biases associated with gender variations. As posited by McGregor *et al*. (2016) upholding a gender-balanced sample is essential due to the potential influence of gender discrepancies on clinical outcomes and biomarker calculations (17).

The main focus of this study revolved around participants aged between 20 and 25 years old, which's a demographic for understanding the development and progression of dental and oral conditions. This age group is particularly important because it signifies a period of transition. As people move from adolescence to adulthood, there are often changes in their preferences, dental care habits, and overall health behaviours that can be observed (18). The mean ages for both genders were very similar; females had an age of 22.80±1.936 years, while males had an age of 22.85±2.007 years. This similarity in ages ensures that any potential variations related to age are equally represented in both genders, thus enhancing the reliability of the findings regarding biomarkers.

Nevertheless, it's pivotal to acknowledge that when applying these findings to populations outside this age bracket, there may be variations in outcomes. Analyzing differences between this young cohort and middle-aged or senior groups could offer deeper insights into the development and presentation of oral ailments (19).

The overall well-being of a person's health is closely connected to the buildup of dental plaque. Within the field of study, the Plaque Index (PI) and Gingival Index (GI) play roles as key measures in evaluating oral hygiene and gingiva health (15).

As elucidated by Jasim *et al*. (2023) dental plaque, predominantly a bacterial biofilm, is identified as the chief instigator of dental caries and periodontal diseases (16). Consequently, fluctuations in the Plaque Index (PI) can shed a light on susceptibility to certain oral maladies. The recorded PI values in this study, for both fasting and post-fasting periods, appear relatively subdued (20).

A heightened GI often signals an escalation in inflammation, potentially pointing to susceptibilities towards gingival and periodontal complications. The minimal discrepancies in GI values between the two fasting phases, while not statistically profound, hint at sustained gingival health throughout (21).

Previous research has documented shifts in PI and GI values explicitly during Ramadan. Peedikayil and Narasimhan, (2019) noted a modest, yet statistically significant surge in periodontal inflammation during fasting, possibly tied to alterations in dietary habits or diminished oral hygiene practices (22). Contrarily, Telgi *et al*. (2013) discerned no remarkable variances in GI values throughout fasting, an observation aligning with the current study's outcomes (23). These disparate findings across studies may stem from diverse factors, including research methodologies, cohort sizes, and divergent dental care habits across populations.

While this study did not delve deeply into the underlying mechanisms, it's plausible that fasting might influence oral hygiene practices, the composition of saliva, and bacterial dynamics, thereby potentially affecting both PI and GI (24). The modest variations observed in this study's outcomes suggest that any potential impacts of fasting were either marginal or effectively counteracted by the prevailing oral conditions.

Secretory immunoglobulin A (sIgA) plays a role in protecting the mucosa from infections. The concentration of sIgA can potentially impact a

person's vulnerability to health issues (9). It plays several primary roles, including neutralizing invasive pathogens, preventing their adhesion to epithelial cells, and aiding in the clearance of antigens and microbes from the mucosal surface. The current study observed a significant drop in Statherin levels during fasting compared to the after fasting groups.

Decreased levels of sIgA could potentially compromise the body's defence mechanisms, making the oral mucosa more susceptible to pathogens (25). When it comes to sIgA production and secretion, both fasting and diet can have an impact. Fasting has been observed to reduce sIgA levels as it puts strain on the system. However, once food is consumed after a period of fasting, there is a boost in sIgA synthesis. This could be attributed to increased metabolic activity and an immune response to antigens and microbes that are introduced through feeding (26).

The findings of this study were in agreement with Nagai *et al*. (2019) who noted diminished sIgA levels during food restriction phases (27). They inferred that metabolic shifts and decreased antigenic stimulation of the oral mucosa's immune system contributed to this decline. However, Pietrzak (2020), postulated that extended fasting consistently elevated sIgA levels, highlighting potential immunoregulatory benefits of fasting (28). The discrepancies observed across these studies might stem from various factors, including the duration of fasting, post-fasting dietary attributes, and individual immune response variations. Identifying variations in sIgA levels due to fasting could offer valuable insights into periods marked by heightened vulnerability to oral infections. Therefore, devising strategies to bolster oral immunity, especially during periods of increased vulnerability like extended fasting, might be beneficial.

Statherin plays a crucial role in maintaining oral health. When present in saliva in large amounts, it performs functions for dental well-being. Its primary responsibility is to regulate the levels of calcium and phosphate ions in saliva to prevent them from forming calcium phosphate salts (29). Statherin helps maintain the balance between tooth demineralization and remineralization, which are responsible for the development and prevention of cavities. Moreover, statherin possesses properties that inhibit the growth of bacteria while also aiding their attachment to tooth enamel. Understanding the composition and growth patterns of biofilm is imperative for maintaining oral health (30).

This study noted a considerable non-significant decrease in Statherin levels during fasting compared to after fasting. The diminished levels might imply a reduced capability of saliva to sustain calcium and phosphate ion supersaturation during fasting. This could heighten tooth enamel's susceptibility to demineralization, especially if fasting extends without proper hydration and nutrient replenishment. Additionally, a decrease in Statherin might alter microbial colonization patterns on tooth surfaces, potentially influencing plaque's composition and properties (31).

Similarly, A study by Chennaoui et al. (2009) who, when examining Ramadan fasting effects on salivary components, identified similar Statherin level reductions, suggesting possible links to diminished salivary flow and glandular secretion changes during fasting (32). In contrast, Besbes et al. documented non-significant shifts in Statherin levels in individuals practicing intermittent fasting. Such differences could stem from variations in, during and after fasting, dietary habits (33).

Conclusion

The study observed salivary levels of sIgA elevated during the fasting period, whereas statherin levels were higher post-fasting. These biomarkers play crucial roles in maintaining oral health by defending invasions and regulating the balance of calcium and phosphate ions in saliva.

Limitation:

Challenges encountered in this study primarily stem from issues related to participant compliance and sample collection. Locating suitable participants was complicated by the fact that some did not adhere to oral hygiene instructions, crucial for ensuring valid sample collection. Additionally, inconsistent adherence to fasting among participants posed further difficulties, leading to considerable effort in collecting and validating samples.

Authors' declaration:

We confirm that all the Figures and Tables in the manuscript are mine/ ours. Besides, the authors have signed an ethical consideration's approval-Ethical Clearance. The project was approved by the local ethical committee in the College of Dentistry/University of Baghdad, according to the guidelines on biomedical research, The license has the code number 824823 dated 1/4/2023

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دور صيام رمضان في مستويات الغلوبولين المناعي A الإفرازي (sIgA) والستاثيرين لدى الأفراد المصابين بتسوس الأسنان

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الخلاصة

خلفية البحث: يحدث تسوس الأسنان عندما تنتج البكتيريا الموجودة في الفم أحماضا من الكربوهيدرات القابلة للتخمير. هذا، بالإضافة إلى العوامل الفردية مثل الجينات أو عادات النظافة الفموية، فضلا عن اللعاب، يؤدي إلى تكوين التجاويف. خلال صيام شهر رمضان، يمكن أن تؤثر التغييرات في عادات الأكل على الصحة، وخاصة المؤشرات الحيوية اللعابية المهمة لصحة الأسنان.

الاهداف: هدفت هذه الدراسة المقطعية إلى الكشف عن نشاط وتركيزات مستويات اللعاب من الغلوبولين المناعي الافرازي A (IgA) والستاثيرين خلال فترة الصيام في رمضان وبعدها، وتقييم العلاقة بين هذه المؤشرات الحيوية وتسوس الأسنان .

طرق العمل :تضمنت الدراسة 40 فردا، تتراوح أعمارهم بين 20 و25 عاما، تم تشخيصهم بتسوس الأسنان. تم تقييم المشاركين لمعلمات اللثة باستخدام مؤشر البلاك (PLI) ومؤشر اللثة. تم جمع عينات اللعاب خلال الأسبوع الرابع من شهر رمضان ومن ثم خلال أسبوعين بعد الصيام. تم قياس تركيزات الغلوبولين المناعي الافرازي A (IgA) والستاثيرين في عينات اللعاب باستخدام تقنية الفحص المناعي الإنزيمي المرتبط(ELISA)

النتائج: لوحظ انخفاض كبير في متوسط تركيز sIgA خلال صيام رمضان (2.14 ± 0.21) مقارنة بعد الصيام (3.34 ± 0.35) (p = 0.001). ومع ذلك، كان هناك فرق غير كبير (ع = 0.05) في مستويات ستاثرين بين مجموعة الصيام (2.25 ± 0.18) ومجموعة ما بعد الصيام (2.85 ± 0.22). لم يتم العثور على فروق ذات دلالة إحصائية فيما يتعلق بكل من مؤشر البلاك (PI) ومؤشر اللثة (GI) داخل مجموعات الدراسة.

الاستنتاجات: قد يشير انخفاض تركيز sIgA و Statherin خلال صيام رمضان إلى تغير نشاط الغدد اللعابية أو استجابة جهازية للصيام، مما قد يؤثر على صحة الفم.

مفتاح الكلمات: صيام رمضان، الغلوبولين المناعي A الإفرازي ، ستاثرين، مؤشر البلاك (PI)، مؤشر اللثة (GI)، تسوس الأسنان..

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Preparation and Characterization of Dutasteride Nanoparticles as Oral Fast-Dissolving Film

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Abstract

Background: Dutasteride, is a drug whose mechanism of action is inhibition of the enzyme 5-alpha reductase. It has been approved for use in the treatment of benign prostatic hyperplasia. Dutasteride has low solubility and high permeability, which classifies it as Biopharmaceutics classification system class II, according to the Biopharmaceutics Classification System. It has a water solubility of only 0.038 ng/mL and a slow dissolving rate, resulting in its exclusive availability in the market as a formulation contained within soft gelatin capsules.

Objective: The aim of this study involves two parts. First, is the enhancement of dutasteride dissolution rate, by the creation of dutasteride nanosuspension, and second is the enhancement of patient compliance by the transformation of this nanosuspension to oral fast-dissolving film, which is characterized by its fast disintegration, stability, and ease of administration.

Methods: The solvent anti/solvent precipitation method was used to formulate dutasteride nanosuspension. In addition, dutasteride nanoparticles oral fast dissolving films were prepared by using the solvent casting method.

To compare the in vitro release patterns of pure dutasteride film and selected dutasteride nanoparticles film, the statistical analysis for the dissolution investigation was conducted using the model-independent technique (employing similarity factor f2) utilizing a DD solver. The selected dutasteride nanoparticle film was supposed to be the test material, while the pure dutasteride film was supposed to serve as the reference.

Results: dutasteride nanosuspension demonstrated a high enhancement of the dissolution rate. In addition, the prepared dutasteride nanoparticles oral fast-dissolving film exhibited a further increase in the rate of dissolution and fast disintegration, and the administration is easy, all of these properties making it a promising dosage form.

Conclusion: Nanosuspension is an excellent approach for enhancing the solubility, dissolution rate, and effectiveness of drugs with limited aqueous solubility such as dutasteride. In addition, the oral fast-dissolving film can be considered a promising dosage form that will increase patient compliance due to its high dissolution rate, fast disintegration, and easy administration.

Keywords: Benign prostatic hyperplasia; Dutasteride; Oral films; Polymers; Solvent casting; Solvent antisolvent precipitation.

Introduction

5-α-Dihydrotestosterone (DHT) plays a major role in the development of benign prostatic hyperplasia (BPH) (1). It is formed by the action of the 5-alpha reductase (5AR) enzyme (2,3). Dutasteride is a drug that functions by inhibiting the enzymatic activity of 5-alpha reductase (5AR). The utilization of this treatment has received official approval for its application in the management of benign prostatic hyperplasia (BPH). Dutasteride is classified as BCS class II according to the Biopharmaceutics Classification System due to its low solubility and high permeability. As a result, it is exclusively available in the market in the form of soft gelatin capsules (4).

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Particle size reduction is one approach that is used for the enhancement of drug solubility and dissolution rate (5,6).

Because solubility plays a pivotal role in drug effectiveness (7), its enhancement by using the nanosuspension approach will provide a solution for the formulation problems that are related to drug solubility (8). Multiple studies have been conducted to document the creation of nanosuspensions, which have been found to result in increased dissolution rates and enhanced bioavailability (9,10). The improved dissolution properties of dutasteride can be attributed to the augmentation of the surface area available for dissolution (11). The procedure for generating nanosuspensions is uncomplicated and applicable to all pharmaceuticals that demonstrate limited solubility in water (12).

The oral route of drug administration is widely regarded as a highly effective strategy for drug delivery. It offers several benefits, including enhanced convenience, cost-effectiveness, and ease of delivery, significantly improving patient adherence. A majority of medication forms are ingested through the oral cavity, however, after being consumed, the drugs can be broken down by enzymes and undergo a substantial reduction in effectiveness due to the first-pass effect, which occurs as they pass through the liver (13). Additionally, many pediatric and geriatric patients display hesitance in consuming solid oral preparations due to their concerns about choking (14). Recently, there has been a significant increase in both popularity and favorability of fast-dissolving drug delivery systems (15). This approach shows great potential for tackling the issue of non-compliance due to their quick disintegration and enabling self-administration without the use of water or the need for chewing (15). Two suggested forms of dosage that rapidly disintegrate in the mouth are the orally disintegrating tablet (ODT) and the orally disintegrating film (ODF). The orally fast disintegrating film, which is a thin film created using hydrophilic polymers, is formulated to disintegrate when it comes into contact with a moist surface, such as the tongue, within a few seconds. The quick disintegration can be attributed to its large surface area (16). The primary drawback of the oral fast-dissolving film is its limited ability to hold a significant amount of the drug and its restricted options for effectively masking the taste (17). The study aim is to prepare dutasteride nanosuspension and transform it into thin film formulations that provide both stability and convenient administration (18). Thin films can additionally enhance drug solubility (15).

Materials and Method

Preparation of dutasteride nanosuspension: Dutasteride nanosuspension was carried out through the solvent anti-solvent precipitation method (19). This approach included the establishment of two discrete stages. Initially, the organic phase was formed by dissolving 0.5 mg of dutasteride in 1 ml of methanol. In contrast, the formation of the aqueous phase involved the dissolution of 0.5% w/v of the stabilizer soluplus in a 10 ml solution of deionized water. The organic part was gradually introduced into the aqueous phase using a syringe, and carefully monitored at a 1 ml/min rate. The resultant mixture was subsequently subjected to mechanical agitation at a speed of 1500 revolutions per minute (rpm) and held at 37°C for duration of 30 minutes to facilitate the volatilization of the solvent.

Dutasteride nanosuspension preparation as oral fast-dissolving film: The method of solvent casting was employed to create fast-dissolving films (20), employing Polyvinyl alcohol (PVA), Hydroxypropyl methylcellulose E5 (HPMC E5), Polyvinyl pyrrolidone k30 (PVP K30), and a combination of both PVA and HPMC E5 as film-forming polymers. Each film has a surface area of 6 cm^2, which contains Dutasteride nanosuspension equivalent to 0.5 mg dutasteride, utilizing Petri dishes with a diameter of 6 cm and a surface area of 28 cm^2.

A petri dish capable of holding four films. The requisite quantity of polymers for one petri dish was dissolved in 10 ml of deionized water, this mixture was heated to 60 °C while being continuously stirred on a magnetic stirrer (1000 rpm) for 1 hour, until the polymer was fully dissolved. The mixture was then allowed to cool, and a plasticizer (glycerin) was introduced. Mannitol, serving as a cooling and sweetening agent, Vanilla serving as a flavoring agent; and cross povidone, acting as an efficient super disintegrant, were dissolved in 3 ml of deionized water. The resulting solution was subsequently combined with the polymeric solution under continuous stirring for a duration of 1 hour, resulting in the formation of a clear solution. Meanwhile, four dutasteride nanosuspension formulas were prepared with a total volume of 10 ml. These formulations were subsequently introduced into the polymer solution and were thoroughly mixed for 3 hours, ensuring uniform distribution of the drug particles inside the polymer matrix and resulting in a more homogeneous and consistent formulation. This ensures that the medicine is evenly distributed, avoiding difficulties such as dosage variability within the final oral film. The mixture was left undisturbed for a minimum of 24 hours to allow the removal of trapped air before being poured into the petri dish. The resulting homogeneous mixture was then spread onto a 6 cm^2 Petri dish, ensuring the absence of air bubbles, and subjected to drying in an oven set at 40 ºC for 24 hours. Once dried, the film was carefully detached from the Petri dish employing a sharp blade and cut into films of suitable shapes and sizes, followed by packaging in aluminum foil. These films were stored in a dry environment (21). This information is presented in the provided Table (1)

Table (1): Composition of various formulations of dutasteride oral films

Ingredient	F1	F2	F3	F4	F5	F6	F7	F8	F9
Dutasteride nanosuspension	50.5	50.5	50.5	50.5	50.5	50.5	50.5	50.5	0.5
PVA (mg)	66		33		50	100	50	100	33
PVP K 30 (mg)				66					
HPMC (mg)		66	33		50		50		33
Glycerin (mg)	20	20	20	20	30	15	30	30	20
Cross povidone (mg)	7.5	7.5	7.5	7.5	10	16	10	10	7.5
Mannitol (mg)	6	6	6	6	5	12	9.5	9.5	6
Vanilla (mg)					4	6			

**Weight of film 150 mg*

**Weight of film 200 mg*

**The oral film contains pure dutasteride*

Dutasteride nanosuspension evaluation: The nanosuspension that was generated was subjected to evaluation in terms of its particle size, drug content, and entrapment efficiency (EE%). In addition, the dissolution characteristics of dutasteride nanosuspension and pure dutasteride powder were

evaluated in an in vitro study using a phosphate buffer (pH 6.8) containing 1% sodium dodecyl sulfate (SDS). A comparative analysis was conducted to evaluate the degree of similarity, as measured by the similarity factor (f2), between the release profiles of a nanosuspension formulation of dutasteride and dutasteride's pure powder, which was employed as a reference.

Measurement of drug content in dutasteride nanosuspension formula: A volumetric flask was utilized to hold 1 ml of nanosuspension formula, which was then diluted with 9 ml of methanol. The resulting mixture underwent sonication for a duration of 1 hour. The collected sample was subjected to analysis via a UV-visible spectrometer, specifically at the wavelength (λ max) where the drug in methanol displayed its highest absorbance, which was measured at 240 nm. The percentage of drug content was determined by applying a designated equation (1).

Drug content % = (detected drug content /Theoretical drug content) * 100 %…. Eq. (1) [22].

Determination of Entrapment Efficiency: The entrapment efficiency refers to the proportion of a drug or substance that is successfully incorporated within the nanoparticles. It's usually expressed as a percentage and indicates how effectively the nanoparticles entrap and hold the active ingredient. Entrapment efficiency (EE%) of the prepared dutasteride nanosuspension formula was evaluated using an Amicon ultra-4 centrifugal filter with Mwt 10 KD. A total of 4 ml of dutasteride nanosuspension was placed in the Amicon tube and centrifuged at 4000 rpm for 30 minutes. Subsequently, the concentration of concentrated dutasteride particles was assessed using UV spectrophotometry at a wavelength of 240 nm. EE% was then measured using the following equation: EE% = obtained dutasteride amount / theoretical dutasteride amount * 100 %…. Eq. (2) [23].

Evaluation of dutasteride oral fast-dissolving film

Visual appearance: The visual features of the film, including its level of transparency or semi-transparency, were evaluated using a straightforward visual inspection (24).

In vitro disintegration study: The film was placed in a small petri dish, containing 10 milliliters of deionized water. The petri dish underwent constant shaking until the film underwent complete disintegration. The period starting from the initiation of the disintegration process until the disintegration of the film is completed, is documented as the disintegration time (25, 26).

Film's thickness: The film's thickness was measured at different positions using an electronic vernier caliper. The assessment in this study aims to evaluate the uniformity of thickness across different films, as it directly impacts the accuracy of dosage administration within the film (27).

Drug content of the film: The films were solubilized in 100 ml of phosphate buffer solution with a pH value of 6.8, supplemented with 1% sodium dodecyl sulfate (SDS). Subsequently, the mixture was agitated for a duration of 30 minutes utilizing a magnetic stirrer. Subsequently, samples were obtained from the resulting solution and subjected to filtration using a syringe filter with a pore size of 0.1 µm. The absorbance of each sample was measured using a UV spectrophotometer at a wavelength of 244 nm. The quantity was determined using an equation that was derived from the calibration curve of the drug in a buffer solution with a pH of 6.8, which also contained 1% SDS (28, 29).

Weight of films: The weight variation investigations consisted of individually weighing eight films for each formula, followed by the calculation of the average weight (30,31).

Surface pH measurement: The measurement of the pH of the surface was performed by dissolving the film in 2 ml of deionized water at ambient temperature. The surface pH value was determined by placing the pH meter electrode touching the dissolved film and allowing it to remain stable for a duration of 1 minute (32).

Folding Endurance measurement: The measurement of Folding Endurance involved the manual folding of the film at a consistent location until it underwent rupture. The folding endurance value is ascertained by quantifying the number of times the film can be folded until it reaches the point of fracture (33).

In vitro dissolution study of the oral fast-dissolving films: The evaluation of the film's release was conducted utilizing the USP dissolution test apparatus type II. The film was immersed in a dissolution medium comprising 200 ml of a 6.8 buffer solution containing 1% SDS. The paddle was subjected to a rotational speed of 50 revolutions per minute at a temperature of 37°C (34). Samples were extracted at time intervals (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, and 15 minutes) in 5 ml volumes. To maintain sink condition, the withdrawn sample was immediately replaced with 5 ml of fresh dissolution medium. Subsequently, the withdrawn sample was filtered using a 0.1 syringe filter. The measurement of absorbance for each sample was conducted utilizing a UV-visible spectrophotometer at a wavelength of 244 nm (35).

Compatibility study: The assessment of the compatibility between the dutasteride nanoparticles (NPs) and the excipients included in the formulation of the film was conducted using Fourier Transform Infrared (FTIR) analysis. To validate the absence of any interactions and ascertain the presence of characteristic peaks of the drug, a comparative analysis was conducted between the spectra of dutasteride nanoparticles (NPs) and the chosen film formulation (36).

Results

Assessment of dutasteride nanosuspensions:

The Malvern Zeta Sizer was employed to examine a sample of dutasteride nanosuspension. The analysis yielded a particle size measurement of 73.24 nm and a polydispersity index (PDI) value of 0.184. The examination of the drug content and entrapment efficiency (EE%) of the formulation of dutasteride

nanosuspension yielded a drug content of 99.58% ± 0.0121, accompanied by an EE% of 99% ± 1.41. Additionally, it was observed that the dutasteride nanosuspension exhibited complete release within a 15-minute timeframe, whereas the release of pure dutasteride powder was only 30% after duration of 1 hour.

The calculated similarity factor value is 12.37, indicating that it falls below 50. This observation indicates a notable discrepancy in the dissolution properties between the dutasteride nanosuspension that was prepared and the pure dutasteride powder (37). , as depicted in Figure (1)

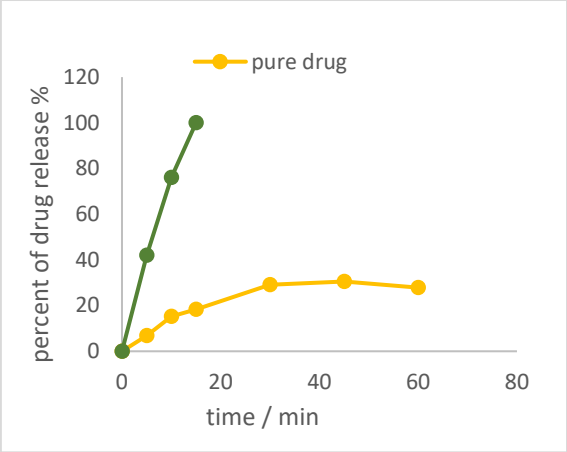

Figure (1): In vitro release of the pure dutasteride and dutasteride nanosuspension formula in 6.8 buffer with 1% SDS

General evaluation parameter of dutasteride films:

Visual appearance: The dutasteride nanoparticle films were subjected to a range of evaluations as a means of drug administration. the F1, F2, F3, and *F9 films exhibited a uniform and transparent appearance, with a smooth and consistent texture on their surfaces. However, the F5, F6, F7, and F8 films showed inconsistencies in homogeneity and clarity. Additionally, the F4 film displayed strong adhesiveness, making it impossible to separate from the Petri dish.

As depicted in Figure (2).

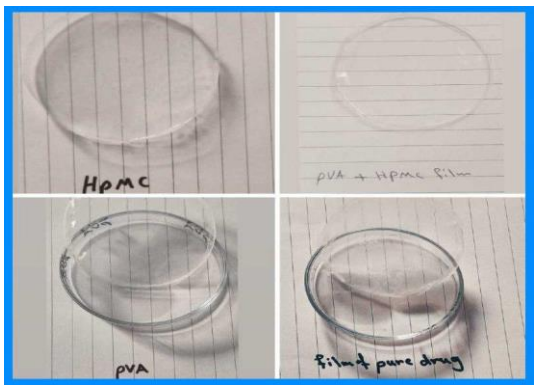

Figure (2): Film formulas containing HPMC E5 (F2), PVA(F1), a combination of HPMC E5 and PVA (F3), and film of pure dutasteride (*F9)

In vitro disintegration study: Analysis of in vitro disintegration revealed the following film disintegration times: 29 ± 1 second for (F1), 30 ±1.2 seconds for (F2), 28 ± 1.7 seconds for (F3), and 53 ± 1.4 seconds for a pure drug film (*F9), whereas the disintegration time of other films exceeded 30 seconds. They were consequently eliminated from the other examination.

Thickness of films: Within each formulation, the thickness of the films was consistent between 0.13 and 0.18 mm. All of the films fall within the acceptable thickness limit (less than 0.3 mm) for oral films (38). Extremely low standard deviation (SD) values illustrate the reproducibility of the method and the uniformity of film thickness (39).

Drug content: The drug content of the film formulation was determined to be 97.2% ± 0.0007, 95% ± 0.12, 98.6% ± 0.001195, and 96.5% ± 0.011 for PVA-based film (F1), HPMC E5-based film (F2), the combination of PVA and HPMC E5-based film (F3), and ordinary film (*F9), respectively. According to the results, all formulations adhered to the British Pharmacopoeia criteria for drug content (85-115%) (40). These results indicate that the drug nanoparticles have a uniform distribution throughout the film and that the film production method is effective, resulting in a homogeneous film with a high drug content (41,42).

Weight of films: The recorded weights of the prepared films were found to be 148.3 ± 5.7, 147.3 ± 15.5, and 149 ± 4.2 for films F1, F2, and F3, respectively. The findings indicate that the mean weight of the films aligns with the weight of the initial formulation.

Surface PH measurement: The pH values of the films ranged from 6.5 to 6.8. The pH range of these films aligns with that of the oral mucosa, and none of the films cause any mouth irritation, making them appropriate for utilization (43).

Folding Endurance measurement: As indicated in Table 2, all of the films exhibit a folding capacity that exceeds 300.

Table (2): Physicochemical characteristics of the selected dutasteride oral films after preparation

F. Code	Weight of film (mg)	Film thickness (mm)	Folding endurance	Drug content	surface pH	In vitro DT (sec)
F1	148.3 ± 5.7	0.147 ± 0.0194	> 300	97.2% ± 0.0007	6.5± 0.07	29 ± 1
F2	147.3 ± 15.5	0.150 ± 0.014	> 300	95% ± 0.12	6.8 ± 0.05	30 ± 1.2
F3	149 ± 4.2	0.143 ± 0.0171	> 300	98.6%± 0.0011	6.6 ± 0.02	28 ± 1.7

In vitro dissolution study of the films: The assessment of the dissolution of dutasteride nanoparticle film formulations and the pure dutasteride film was conducted using the USP dissolution test apparatus type II. The dissolution medium employed in the experiment consisted of 200 milliliters of phosphate buffer solution with a pH value of 6.8, supplemented

with 1% sodium dodecyl sulfate (SDS). According to the data presented in Figure (3), it can be observed that the film composed of PVA and HPMC E5 (referred to as F3) exhibited a complete release of its contents in an in vitro setting within 2 minutes. On the other hand, the film composed solely of PVA (F1) achieved complete release after duration of 5 minutes. The film containing HPMC E5 (F2) exhibited a release rate of 73% after 15 minutes, whereas the pure dutasteride film (*F9) demonstrated a release rate of only 28% during the same time frame. The study involved a comparison of the release patterns of films containing F1, F2, and F3, as well as a pure dutasteride film (*F9) that served as a reference.

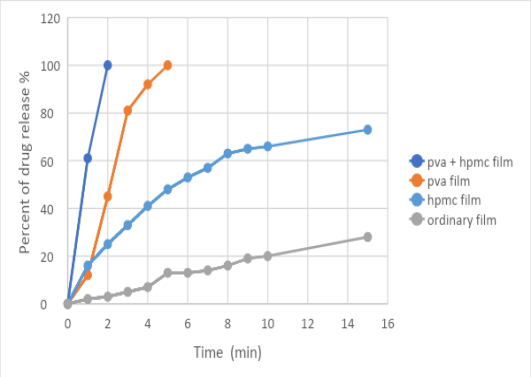

Figure (3): In vitro dissolution of the pure dutasteride oral film and dutasteride NPs films in phosphate buffer pH 6.8 containing 1% SDS

The similarity factor f2 was utilized for this purpose. According to the data provided in Table 3, the obtained similarity factor value was determined to be below 50.

Table (3): Similarity factor $f_2$values for the dissolution profiles of the oral films containing dutasteride nanoparticles as compared to the oral film containing dutasteride in pure form

Formula name	f_2
F1	8.79
F2	22.03
F3	6.53

Based on the aforementioned findings, encompassing disintegration time, drug content, and release profile, the formulation denoted as F3, which incorporates both PVA and HPMC E5 polymers, was chosen as the favored option, as depicted in Table (4).

Table (4): The characteristics of optimized dutasteride nanoparticle oral film.

Parameter	F3
Weight	149± 4.2
Drug content	98.6%± 0.0011
drug release %	100%
In vitro disintegration time	28 ± 1.7
pH of surface	6.6 ± 0.02
Thickness	0.143 ± 0.017
Folding endurance	>300

The comparative analysis of the in vitro dissolution performance of the optimal dutasteride nanoparticles oral film (F3) was conducted to evaluate the impact of the film component on drug release, as depicted in Figure (4). This assessment involved comparing the results with those obtained from the prepared dutasteride nanosuspension formula.

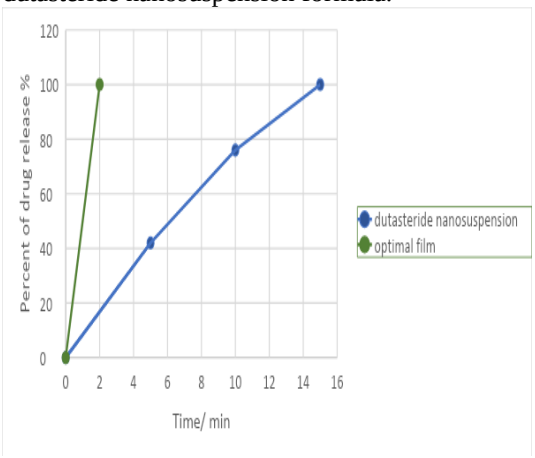

Figure (4): In vitro dissolution of dutasteride nanosuspension formula and optimal film (F3) in phosphate buffer pH 6.8 with 1% SDS

Compatibility study: There was no observed interaction between dutasteride nanoparticles (NPs) and the selected excipients for the film formulation, as evidenced by Figures (5) and (6). The Fourier Transform Infrared (FTIR) spectrum of the optimized film formulation (F3), depicted in Figure (7), exhibits prominent peaks corresponding to the drug (44).

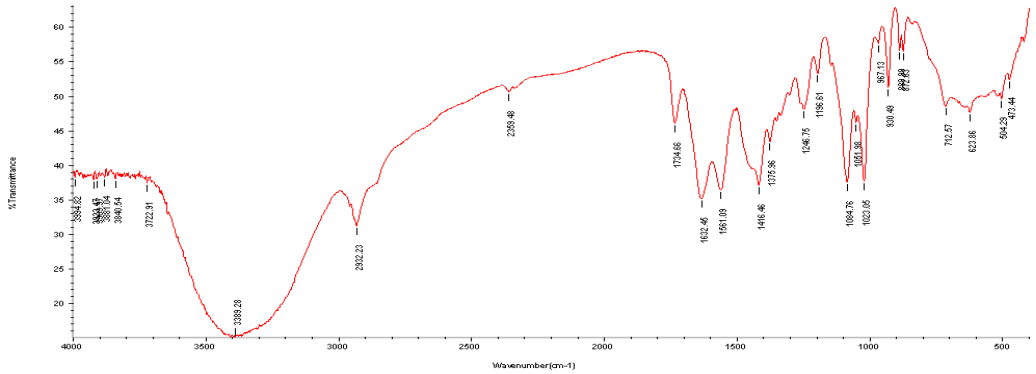

Figure (5): FTIR spectrum of dutasteride nanoparticles

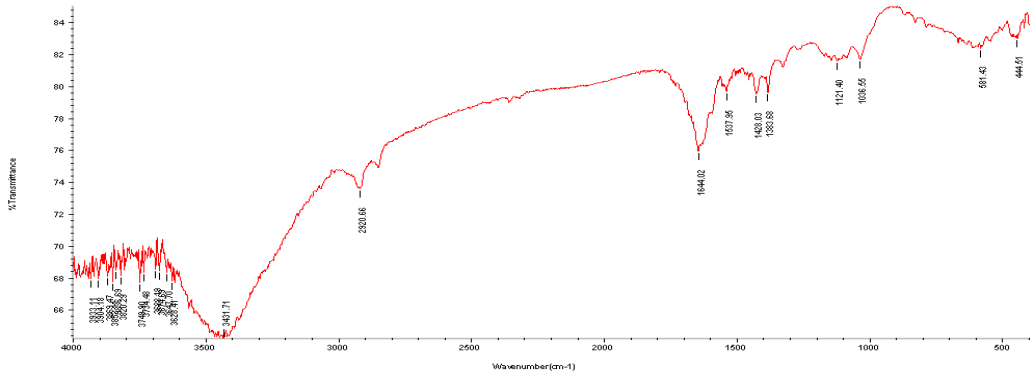

Figure (6): FTIR spectrum of the selected film formulation (F3)

Discussion

The impact of the concentrations of polymers on the films' appearance: The experimental findings indicate that the F5, F6, F7, and F8 films exhibited a lack of homogeneity and clarity. Moreover, it was observed that as the polymer concentration increased, the thickness of the films also increased, leading to a decrease in transparency (45).

The impact of polymer concentration on the disintegration time of the films: The longer than 30-second disintegration time of F5, F6, F7, and F8 films can be explained by the fact that a greater polymer concentration results in the formation of a thicker gel upon contact with the medium (46).

The impact of a plasticizer on the folding endurance of the films: All of the manufactured films have folding endurance values greater than 300, which is indicative of success (46). By decreasing the glass transition temperature, glycerin acts as a plasticizer. The film's flexibility is increased by the decrease in its glass transition temperature, which in turn increases its folding endurance (47).

The impact of polymer type on the release of dutasteride np from the resulting films: Films made with hydroxypropyl methylcellulose (HPMC) and polyvinyl alcohol (PVA)(F3), showed the desired degree of flexibility and ease of peeling, as found by Bhikshapathi et al. A large amount of water can be absorbed by these polymer systems, causing them to gel. Drug molecules are released from the film via diffusion after the film expands in response to the penetration of a dissolution medium or biological fluid (48,49). As shown in F1, a higher concentration of the hydrophilic polymer PVA causes a more rapid and extensive swelling process, which contributes to the film's quicker release (50). HPMC's retardant properties cause HPMC-based film (F2) to have a slow-release profile. Its high viscosity causes a thicker, swollen gel layer to form, which in turn increases the time it takes for drug molecules to diffuse out of the gel (51,52).

The effectiveness of the dutasteride nanosuspension formulation in terms of in vitro dissolution was compared to that of the chosen dutasteride np oral fast-dissolving film (F3). Figure 4 shows that the dissolution properties are significantly different. Thin films have the potential to further enhance drug solubility, as has been demonstrated (53).

Conclusion

The present study has successfully showcased the ability to produce a dependable, swiftly disintegrating film composed of dutasteride nanoparticles through the utilization of a solvent-casting technique and the incorporation of diverse polymers. This approach aims to improve patient compliance, drug dissolution, and the extent to which the drug is absorbed and available for use in the body. Shorter disintegration and faster dissolution times were observed in formulations utilizing the combination of polyvinyl alcohol (PVA) and hydroxypropyl methylcellulose E5 (HPMC E5) as the film-forming polymer (F3). The drug delivery system described herein exhibits substantial potential for various patient populations, with a particular emphasis on individuals encountering challenges related to swallowing, such as geriatric patients. Therefore, it can be deduced that dutasteride oral fast-dissolving films (OFDFs), with their exceptional patient compliance and numerous advantages, present innovative and promising opportunities for the future.

Abbreviations	Meaning
BPH	Benign Prostatic Hyperplasia
BCS	Biopharmaceutical Classification System
ODF	Oral disintegrating film
OFDF	Oral fast-dissolving film
EE%	Entrapment Efficiency
PDI	Polydispersity index
FTIR	Fourier transform infrared spectroscopy
SD	Standard deviation
DT	Disintegration time
NPS	Nanoparticles
UV	Ultraviolet
USP	United states pharmacopeia
pH	Is a numeric scale that is used to determine the acidity or basicity of an aqueous solution.
f_2	Similarity factor
cm	Centimeter
cm^2	Square centimeter
ml	Milliliter
nm	Nanometer
mg	Milligram
Min.	Minute
rpm	Revolution per minute
PVA	Polyvinyl alcohol
PVP k30	Polyvinyl pyrrolidone k30
HPMC E5	Hydroxypropyl methylcellulose E5
SDS	Sodium dodecyl sulfate

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Ethics Statements
In vitro study, no ethical statements are required.

Conflicts of Interest: None
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Author Contribution
The authors confirm their contribution to the paper as follows: data collection, analysis and interpretation of results, and draft manuscript preparation: Rusul Wahhab Kadhum. Shaimaa N Abd-Alhammid reviewed the results and approved the final version of the manuscript.

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تحضير وتوصيف جسيمات الدوتاستيرايد النانوية كأفلام ذات ذوبان سريع عن طريق الفم

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الخلاصة
الخلفية :دوتاستيرايد هو مثبط لانزيم 5-الفا ريداكتيز. تمت الموافقة عليه لعلاج تضخم البروستات الحميد. تم وذلك لقلة ذوبانتيته في الماء. يتوفر الدوتاستيرايد في السوق فقط على شكلBCSتصنيفه كفئة ثانية من نظام
كبسولات جيلاتينية ناعمة .
الهدف: هدفنا من هذه الدراسة هو لغرضين الأول زيادة معدل الذوبان للدوتاستيرايد عن طريق تحضير معلق نانوي من الدوتاستيرايد، الثاني هو زيادة التزام المريض بالعلاج وذلك من خلال تحويل المعلق النانوي الى أفلام فموية تتميز بانها سريعة الذوبان، سريعة التفتت، وسهلة الاستخدام.
الطرق :استخدمت تقنية ترسيب المذيب والمضاد للمذيب في تخضير المعلق النانوي للدوتاستيرايد. بالنسبة لإنتاج الأفلام الفموية ذات الذوبان السريع، فقد تم استخدام طريقة صب المذيب .
النتائج :المعلق النانوي للدوتاستيرايد الذي تم الحصول عليه أدى إلى زيادة معدل الذوبان. إضافة الى ذلك،
الفلم الفموي سريع الذوبان الذي يحتوي على جزيئات الدوتاستيرايد النانوية أظهر زيادة اكثرفي سرعة الذوبان والتفتت وأيضا سهولة الاستخدام، كل هذه الصفات جعلته شكل دوائي واعد.
الاستنتاج: المعلق النانوي هو وسيلة ممتازة لتحسين ذوبانية، سرعة الذوبان وفعالية الدواء الذي يمتلك ذوبانيه محدودة في الماء مثل الدوتاستيرايد، بالإضافة الى ذلك فان الأفلام الفموية سريعة الذوبان يمكن اعتبارها كشكل دوائي واعد الذي سيحسن من التزام المريض بالعلاج بسبب تحسينه للذوبانية، لسرعة الذوبان وسهولة استخدامه .
الكلمات المفتاحية: تضخم البروستات الحميد، دوتاستيرايد، أفلام سريعة الذوبان، بوليمرات، صب المذيب، ترسيب المذيب والمضاد للمذيب.

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Research Article

Molecular Detection of the *mec*A and some Virulence Determinants in Methicillin-Resistant *Staphylococcus aureus*

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Abstract:

Background: Methicillin Resistant *Staphylococcus aureus* (MRSA) is globally acknowledged as a prominent contributor to both hospital-acquired and community infections. Understanding key virulence factors including coagulase production, hemolysis ability and biofilm formation, is crucial.
Objective: The study aimed to establish a molecular characterization of *mec*A gene and virulence factors genes (*hla, icaA, and coa)* in clinical isolates of MRSA obtained from two hospitals in Baghdad.
Methods: A hundred and five isolates were obtained from clinical sources from November 2022 to March 2023 and their antibiotic sensitivity was assessed using the agar diffusion test against seven different antibiotics (Azithromycin, Ciprofloxacin, Nitrofurantoin, Rifampin, Trimethoprim, Ofloxacin and Oxacillin), through Conventional Polymerase Chain Reaction, the presence of virulence factor genes including *mec*A, *hla*, *ica*A, and *coa*, was determined in MRSA isolates.
Results: All MRSA isolates (100%) harbored the *mec*A, *hla*, and *ica*A genes while the *coa* gene was recognized in 50% of the isolates. Regarding antibiotic susceptibility, all MRSA isolates (100%) demonstrated sensitivity to Nitrofurantoin. Additionally, 96.8% of the isolates were sensitive to Oxacillin.
Conclusion: Molecular detection of methicillin resistance genes and virulence genes can be used to diagnose MRSA isolates in hospitals. The presence of these genes may affect their pattern of sensitivity to antibiotics.
Keywords: Methicillin-resistant *Staphylococcus aureus*, *mecA* gene, *hla* gene. *icaA* gene, *coa* gene.

Introduction

Staphylococcus aureus is one of the most developing worldwide public health issues resulting from the emerging resistance to antimicrobial agents and leading to ineffective treatment (1). *S. aureus is* considered one of the most widely spreading bacterial infection in community and hospital settings as it possesses multiple mechanisms of antibiotic resistance, therefore, leading to severe infections (2). The problem is MRSA strains which previously spread in hospital settings but is currently occurring progressively in community settings. MRSA exhibits high infection and virulence (3).
Understanding the prevalence of MRSA is of utmost significance for infection control, the prevention of severe infections and gaining insights into the mechanisms of resistance (4). These bacteria are responsible for widespread diseases spanning from acute skin abscesses to chronic endocarditis and osteomyelitis, affecting both hospital and community settings (5). In recent years, MRSA has become a global concern, with variations in its distribution from one region to another (6). For instance, in Zakho city MRSA frequency is 85% as reported by Hami & Ibrahim (7), while its reported 90% by Hameed *et al.* in Karbala city (8). MRSA strains exhibit resistance to β-lactam antibiotics through two mechanisms: first, they hydrolyze penicillin β lactams by production of penicillinase, and reduce binding affinity for β lactams through altered Penicillin-Binding Protein (PBP). This alteration results in fighting almost all available β-lactam antibacterials except for the latest cephalosporins such as ceftaroline (9). The resistance mediated by penicillinase is determined by the *bla*Z gene while the resistance associated with PBP is determined by the *mec*A gene sited in the SCCmec gene (10, 11).
Locally, a study conducted at the Maternity and Children teaching hospital and Al Diwaniya teaching hospital in Iraq, *S. aureus* isolates from different cases were found to be highly resistant to the usually used antibiotics (12). Another Iraqi study, revealed thar MRSA strains were resistant to various antibiotics such as azithromycin, methicillin, and ciprofloxacin, but not to ceftaroline (13).
The pathogenicity of MRSA strains relies on several virulence factors; for example, hemolysins which lead to development of diseases and are categorized into three types: alpha(α), beta(β) and gamma(γ). The alpha hemolysin is a toxin produced by the *Hla* gene of *S. aureus* acting as a virulence factor that forms pores in cell membranes, disrupting epithelial barriers and leading to cell lysis and death (14).

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Biofilms which are multi-cellular bacterial communities embedded in an extracellular matrix pose a major challenge in the setting of non-communicable diseases. These biofilms provide protection to bacteria and increasing their resistance to antibiotics. Antimicrobial resistance is a prominent feature of biofilm-associated bacteria and facilitates their adhesion to infected areas (15). MRSA strains are known to produce biofilms by expressing polysaccharide intracellular adhesion (PIA), which is generated by transcription of the *ica*A gene operon products on chromosomes (16).

Furthermore, a main virulence factor of *S. aureus* is coagulase, which contributes to pathogenic infections such as endocarditis, abscess formation and staphylococcal bacteremia (17). Coagulase, an enzyme-like protein, converts fibrinogen to fibrin and leads to the formation of a plasma clot. Consequently, it enhances the pathogenesis of *S. aureus*, promotes persistent infection, plays a role in immune evasion and facilitates the spread of the bacterium through host tissues (18). Therefore, the current study aims to find the molecular characterization of *mec*A gene and some virulence factors genes (*hla*, *ica*A, and *coa*) in clinical isolates of MRSA obtained from patients.

Materials and Methods

Samples collection: Between November 2022 and March 2023, 105 specimens (nostril, throat, urine, sputum and wound samples) were collected in sterilized transport tube media from Abu Ghraib General Hospital and Al Yarmouk Teaching Hospital laboratories.

Isolation and Identification of *S. aureus* and detection of MRSA isolates: All specimens were inoculated on Mannitol salt agar (MSA) (Accumix, England). The cell culture plates were incubated overnight at 37°C. Biochemical tests for isolated bacteria were identified by oxidase, coagulase, urease and catalase tests (10).

Phenotypic detection of MRSA isolates was done by utilizing a Methicillin disc (10 µg) and measurement of inhibition bacterial zone of growth after cultured on Muller Hinton medium according to the CLSI guidelines (19). Next, the confirmation of identified isolates was carried out via the VITEK 2 identification device.

Antibiotics Susceptibility Test (AST): Antibiotic susceptibility test using seven different antibiotics [Azithromycin (15µg), Rifampin (5µg), Trimethoprim (10µg), Nitrofurantoin (100µg), Ciprofloxacin (10µg), Ofloxacin (5µg) and Oxacillin (5µg)] was performed for MRSA isolates by KB test according to Bauer *et al*. in 1966 (20) and CLSI (19).

Molecular Detection of *mec*A, *ica*A, *coa, and hla* genes

DNA Extraction

Norgen's Blood DNA Isolation Mini Kit (Norgen, Canada), OneTaq DNA Polymerase Kit (NEB, England) and the Qubit Double-stranded DNA high sensitivity Kit (ThermoFisher, USA) were used for extracting pure DNA from Methicillin-Resistant *S. aureus* isolates depending on their manufacturer's guidelines. After that, NanoDrop spectrophotometer was used to accurately determine sample concentration between 10 pg./µl and 100 ng/µl (21).

Polymerase Chain Reaction assay: All Methicillin-Resistant *S. aureus* isolates were checked molecularly for *hla, icaA, coa* and *mecA* genes using conventional PCR. The amplification procedure for these genes included the initial denaturation phase heated to 94°C for 5 minutes then, at 94°C, 38 cycles of denaturation for 30 seconds, annealing for 45 seconds at 57°C, extension for 45 seconds at 72°C, and final extension for 7 minutes at 72°C. Electrophoresis of the conventional PCR product in a 2% Tris-acetate-EDTA (TAE) agarose gel electrophoresis with 1x TAE buffer for 80 minutes at 80 volts and dyed with RedSafe dye (22). The primer sequences used in conventional PCR for the detection of *mec*A, *ica*A, *coa*, and *hla* genes in MRSA isolates, and the primer sequences are shown in Table 1.

Table 1: The Primers sequences used for detection of *ica*A, *mec*A, *coa,* and *hla* genes

Genes name	Sequence	Size (bp)
*mec*A	F: GTTGTAGAAGGTCCATTATGG R: TAGAACCTTGAGCCTCTTTT	226
hla	F: TTTTCTTTTCAGGAAGCGAG R: CTTCGATTAATACTGTCCGTC	400
coa	F: CTGGGAGTAAAAATGGGAAAC R: CAGGTATTGGTCTTCCTCTAA	179
*ica*A	F: GTATTAAGCGAAGTCAGACAC R: CCAGCTTACAAATATGAGTCC	554

Statistical analysis: The R software package was used to analyze the data to determine the sensitivity of the bacteria under study to different antibiotics. The percentages and numbers of resistant and sensitive ones for each type of antibiotic used were determined at a significance level of ($p<0.05$) (23).

Results

Isolation of *Staphylococcus aureus* and MRSA

Only 84 (80%) samples grew on MSA, and based on the primary diagnosis, 47 isolates of these were *S. aureus* (Table 2) and were examined for antibiotic sensitivity tests by the Kirby-Bauer method to identify methicillin sensitivity of *S. aureus*. Out of these 47 isolates, 31 (66.0%) were resistant, 8 (17.0%) were intermediate, and 8 (17.0%)

Table 2: The biochemical tests of *S. aureus* isolates

Test	Result
Gram stain	(+)
Catalase	(+)
Coagulase	(+)
Indole	(-)
Capsule	(-)
Oxidase	(-)

Were sensitive (Table 3). The confirmation of identification was performed by the VITEK2 system. All 31 bacterial isolates were recognized as MRSA based on the primary diagnosis and the VITEK2 system results.

Table 3: Susceptibility of 47 MRSA isolates to Methicillin

Sensitivity	Resistant	Intermediate	Sensitive	P- value
S. aureus	31 (66%)	8 (17%)	8 (17%)	0.0006***
Chi Square test (X^2)				

Antibiotics susceptibility test

A sensitivity test was conducted using seven different antibiotics (Azithromycin, Ciprofloxacin, Nitrofurantoin, Rifampin, Trimethoprim, Ofloxacin and Oxacillin) to determine the sensitivity of MRSA isolates by agar diffusion test.

The results showed that all 31 MRSA isolates (Table 4) were sensitive to Nitrofurantoin at a rate of 100%. However, they exhibited no sensitivity to Ciprofloxacin. Azithromycin, on the other hand, showed a sensitivity rate of 35.5% for MRSA and a resistance rate of 54.8%. Additionally, 29.0% of MRSA isolates were resistant to Trimethoprim, while 9.7% showed resistance to Rifampin and Ofloxacin. Moreover, 96.8% of the isolates were sensitive to Oxacillin, 83.9% were sensitive to Rifampin and 71% were sensitive to Ofloxacin.

Table 4: Antibiotics susceptibility test of MRSA isolates against seven different antibiotics

Antibiotics	% of isolates			
	sensitive	intermediate	resistant	P - Value
Azithromycin	11 (35.5)	2 (6.5)	18 (58.1)	0.01**
Ciprofloxacin	0	14 (45.2)	17 (54.8)	0.354
Nitrofurantoin	31 (100)	0	0	0.00***
Rifampin	26 (83.9)	2 (6.5)	3 (9.7)	0.0216*
Trimethoprim	1 (3.2)	21 (67.7)	9 (29.0)	0.865
Ofloxacin	22 (71.0)	6 (19.4)	3 (9.7)	0.0453*
Oxacillin	30 (96.8)	0	1 (3.2)	0.004**
Chi square test (x^2)				

Identification of *mecA* gene and virulence factor genes

The conventional PCR test was used to identify the main virulence factor genes including *mec*A, *ica*A, *coa*, and *hla* genes that are responsible for methicillin resistance, polysaccharide production, coagulase production, and blood lysis, respectively. Ten MRSA isolates were used for determining these genes. The results detected that all samples contained *mec*A, *hla*, and *icaA* genes (100%), the *coa* gene was identified in 50% of the isolates. as shown in Figure 1.

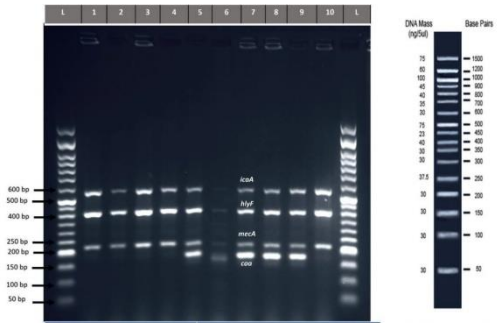

Figure 1: Multiplex Conventional PCR of amplified PCR products for *mecA* (226bp), *hla* (400bp), *ica*A (554bp), and *coa* (179bp) from MRSA isolates

Discussion

S. aureus is a main contributor to the hospital-acquired and community contagions globally. The transformation of *S. aureus* from methicillin-sensitive to methicillin-resistant is primarily due to the acquirement of the *mecA* gene (24, 25).

The prevalence for MRSA in the present study differs from other studies which may be due to various factors, including poor hygiene, antibiotic excessive use, regional conflicts and close contact, such as through religious visits.

The majority of MRSA isolates showed Azithromycin and Ciprofloxacin resistance which agrees with other studies (26), such as Bastidas *et al*. at 61.8% (27) and Ponvelil *et al*. at 16% (28). additionally, studies in Jordan showed a 15% resistance for Azithromycin and a 28.6% resistance to Ciprofloxacin in Pakistan (29). In this study, Nitrofurantoin demonstrated sensitivity among MRSA isolates aligning with previous findings (30). Consequently, Nitrofurantoin is considered an effective antibiotic for treating MRSA infections, as no resistance was observed among the tested isolates. Resistance rates for Rifampin (9.7%) and Trimethoprim (29.0%) were seen in this study, showing some agreement with findings by Bai *et al*. for Rifampin (5.9%) (31), but showing a higher rate (11.8%) for Trimethoprim than in the study by Ibrahiem *et al*. (32).

The frequency of MRSA isolates resistance to Oxacillin was 3.2%, in contradiction to the results of Khasawneh *et al*. (33), who found a resistance rate of 42.1% for MRSA isolates in Jordan. The 9.7% resistance to Ofloxacin disagrees with Sohail *et al*. (34), who found a resistance rate of 98%.

In the current study, Ofloxacin exhibited a sensitivity rate of 71% for MRSA isolates, which is close to the results of Ndedy *et al*. (35), who reported a sensitivity of 50% for Ofloxacin. Hense, the most effective antibiotics for treating MRSA as found in the current study were Ofloxacin, Rifampin and Oxacillin since only a small number of MRSA isolates exhibited resistance to these drugs.

The molecular detection method of the *ica*A, *mecA*, *coa*, and *hla* genes was achieved using multiplex PCR. Vieira *et al*. found that 100% of MRSA isolates from clinical sources were *mecA* producers (36). MRSA is a primary human pathogen causing

significant morbidity worldwide and is closely monitored by the World Health Organization. This pathogen contributes to biofilm formation, acute human infections and the development of antibiotic resistance (37).

A study by Bayirli *et al*. reported that 92.6% of isolates exhibited alpha-hemolysis, while 1.6% displayed beta-hemolysis (38). Additionally, the current study reveals that MRSA isolates which possess the *mecA* gene were phenotypically susceptible to Nitrofurantoin. The expression of the *icaA* gene through the study revealed all isolates had positive results and expressing the gene, which is similar to the findings of Azmi *et al*. (39), who reported a prevalence of 100% for the *icaA* gene in MRSA. Regarding the *coa* gene, the current study reported that it was found in 50% of MRSA isolates, which was lower than that reported by Hezam (86.6%) (40), which may be due to differences in sample treatment, geographical locations, and the dynamic nature of bacterial evolution.

The study revealed that Oxacillin and Nitrofurantoin as effective treatments for MRSA infections but with controlled use to prevent antibiotic resistance.

Limitations: The study was based on only two hospitals (Abu Ghraib General Hospital and Al Yarmouk Teaching Hospital) hence, the findings don't represent the whole population.

Conclusion

Molecular detection of methicillin resistance genes and virulence genes can be used to diagnose MRSA isolates in hospitals. The presence of these genes may affect their pattern of sensitivity to antibiotics, especially with the increasing antibiotic resistance due to antibiotic misuse, and person-to-person transmission.

Authors' declaration:

We confirm that all the Figures and Tables in the manuscript belong to the current study. Besides, the Figures and images, which do not belong to the current study, have been given permission for re-publication attached to the manuscript. Authors sign on ethical consideration's approval-Ethical Clearance: The project was approved by the local ethical committee in the University of Baghdad according to code number (5296/22) on (18/ 10/ 2022).

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الكشف الجزيئي عن جين mecA وبعض محددات الفوعة في المكورات العنقودية الذهبية المقاومة للميثيسيلين

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الخلاصة
خلفية البحث: تُعد المكورات العنقودية الذهبية المقاومة للميثيسيلين (MRSA) بأنها أحد الممرضات المساهمة في العدوى المكتسبة من المستشفيات والمجتمعات عالمياً. إن فهم عوامل الفوعة الرئيسية، مثل تكوين الأغشية الحيوية، والقدرة على إنحلال الدم، وإنتاج إنزيم التخثر، أمرٌ بالغ الأهمية.
الهدف: هدفت الدراسة الحالية إلى تحديد التوصيف الجزيئي لجين *mecA* وبعض جينات عوامل الضراوة (*hla*، *icaA*، و*coa*) بين العزلات السريرية للـ MRSA التي تم الحصول عليها من مستشفيين في بغداد.
المنهجية: تم الحصول على 105 عزلة من مصادر سريرية ما بين نوفمبر 2022 الى مارس 2023، وتم فحص حساسيتها للمضادات الحيوية باستخدام طريقة كيربي باور ضد سبعة مضادات حيوية مختلفة (أزيثروميسين، سيبروفلوكساسين، نيتروفورانتوين، ريفامبين، تريميثوبريم، أوفلوكساسين، وأوكساسيلين). بالإضافة إلى ذلك، تم تحديد وجود جينات عامل الفوعة، بما في ذلك *mecA* و*hla* و*icaA* و*coa*، في عزلات الـ MRSA بواسطة تقنية تفاعل انزيم البلمرة المتسلسل.
النتائج: أظهرت نتائج الدراسة الحالية أن جميع عزلات MRSA (100%) تحتوي على جين الـ *mecA* وجين الـ *hla* وجين الـ *icaA*، في حين تم اكتشاف جين الـ *coa* في 50% من العزلات. فيما يتعلق بالحساسية للمضادات الحيوية، أظهرت جميع عزلات الـ MRSA (100%) حساسة للنيتروفورانتوين. بالإضافة إلى ذلك، وجد أن 96.8% من العزلات حساسة للأوكساسيللين.
الاستنتاج: لخصت نتائج الدراسة الحالية الى ان الكشف الجزيئي لجينات مقاومة الميثيسيلين وجينات الفوعة يمكن استخدامه لتشخيص الـ MRSA في المستشفيات وان وجود هذه الجينات قد يؤثر على نمط حساسيتها للمضادات الحيوية.
الكلمات المفتاحية: المكورات العنقودية الذهبية المقاومة للميثيسيلين، جين *mecA*، جين *hla*. جين *icaA*، جين *coa*.

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