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The Editor of **Iraqi Journal of Medical Sciences**

College of Medicine

Baghdad, Iraq

Tel.: + 964 7717516090

P.O.Box 70044, Kadhimiya, Baghdad, Iraq.

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# Iraqi Journal of Medical Sciences

**A Medical Journal Encompassing All Medical Specializations**

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## Probiotic Therapy: Review

Wala'a Sh. Ali<sup>1</sup> PhD, Nibras N. Mahmood<sup>2</sup> PhD

<sup>1</sup>Dept. of Biology, College of Science, University of Baghdad, <sup>2</sup>Dept. of Biology, College of Science, Al-Mustansiriyah University, Baghdad, Iraq

### Abstract

**Background** Although the concept of probiotics was known since the era of Elie Metchnikoff for more than one century ago, the attention of probiotic therapy as effective approach to prevent and treat a wide range of diseases and disorders increased in the recent years. Probiotic therapy is the treatment of diseases by use live micro-organisms known as probiotics and the aim of this therapy is to increase the numbers of microbiota and enhance their activities until such time that microbiota can be rebalanced. In this review; the definition and properties of probiotics, their types and mechanisms of action, as well as their health beneficial effects and safety have been reported.

**Key words** Probiotics, Bacteriotherapy, Safety, LAB, GRAS

### Introduction

The concept of probiotics comes back to the times of the Ukrainian professor of biology, Elie Metchnikoff (1845-1916) <sup>(1)</sup>. He proposed that the consumption of lactic acid bacteria offered health benefits to the human host <sup>(2)</sup>. The word "probiotic" is derived from the Latin and Greek languages (Latin *pro* meaning "for" and Greek *bios* meaning "life") <sup>(3,4)</sup>, it was used for the first time by Kollathin 1953; he coined probiotics as "probiotika" to indicate active substances that are essential for a healthy life <sup>(5)</sup>.

In 1965 the word probiotic introduced for the first time into scientific literature by Lilly and Stillwell <sup>(6)</sup> to describe "substances secreted by one microorganism which stimulates the growth of another", but Parker in 1974 is considered the first one who used this term as we know it today <sup>(3)</sup>. Fuller <sup>(7)</sup> defined probiotics as "A live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance." Salminen *et al* <sup>(8)</sup> redefined the probiotics as "microbial cell preparations or components of

microbial cells that have a beneficial effect on the health and well-being of the host".

However, there are many definitions of probiotics but the most widely used and accepted one is that proposed by the Food and Agriculture Organization of the United Nations (FAO) and World Health Organization (WHO) in 2001, they defined probiotics as "Live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host" <sup>(9,10)</sup>.

Probiotics are considered one of the preventative and therapeutic potential of alternative agents in the pharmaceutical field <sup>(6)</sup>. Probiotics represent one agent of bacteriotherapy, which consists of three agents; Prebiotics, probiotics and synbiotics. Prebiotics are non-digestible compounds that selectively enhance the growth and activity of intestinal microbiota while synbiotics are probiotics and prebiotics together <sup>(11)</sup>. The treatment by use probiotics called probiotic therapy and its goal is to increase the numbers and activities of those microorganisms with health-promoting properties until such time

that the normal flora can be reestablished<sup>(12)</sup>. Because the importance of probiotic therapy, this review focused on properties of probiotics, their types and mechanisms of action, their health beneficial effects as well as their safety.

### Properties of probiotics

The microorganisms that are used as probiotics must have the following properties:

- They should remain viable and stable after culture, during use and storage before consumption<sup>(2)</sup>.
- Able to survive in the intestinal tract under gastric conditions<sup>(13)</sup> by exhibiting acid and bile tolerance<sup>(14)</sup> as well as pancreatic digestion<sup>(2)</sup>.
- Able to adhere to intestinal epithelial surfaces, proliferate, and colonize the gut<sup>(13-15)</sup>.
- They should be nonpathogenic, nontoxic, and generally recognized as safe<sup>(6)</sup>.
- The host should gain direct and indirect beneficial effects from the probiotics after consumption<sup>(16)</sup> such as; anti-carcinogenic activities, reduced intestinal permeability, stimulate immune system<sup>(6)</sup>, and antimicrobial activity<sup>(15)</sup>.
- They should have resistance to antibiotics.
- They should be isolated from the same species as its intended host, and have good sensory characteristics<sup>(6)</sup>.

In addition to these properties, some authors have suggested that probiotic bacteria should be of "human origin"<sup>(2)</sup>.

### Types of probiotics

The microorganisms that are used as probiotics are strains of different bacterial species belong to gram positive bacteria includes lactic acid bacteria (*Lactobacillus*, *Lactococcus*, *Streptococcus*, *Enterococcus*, *Pediococcus* and *Leuconostoc*), *Bifidobacterium*, *Bacillus*, and *Propionibacterium* as well as Gram negative bacteria *Escherichia* and nonpathogenic yeast *Saccharomyces*<sup>(16-20)</sup> (table 1) but the commonly used probiotics are *Lactobacillus* and *Bifidobacterium*<sup>(5,20)</sup> as well as

nonpathogenic yeast<sup>(4,21)</sup>. However, the most studied probiotics are; *Lactobacillus rhamnosus*, *Bifidobacterium lactis*, and *Streptococcus thermophilus*<sup>(22)</sup>. Some of probiotics are of human origin and others are nonhuman strains used in the dairy industry<sup>(23)</sup>. The different strains at the same species may show different effects and thus may exhibit overlapping or specific therapeutic actions to different organs<sup>(3,24)</sup>.

### Mechanisms of action

The beneficial effects of probiotics on host may be direct or indirect<sup>(25)</sup>. The mechanisms of action of probiotics are still a significant question regarding clinical use of them<sup>(26)</sup>, the broad-based definition of probiotics makes the study of their mode of action difficult<sup>(27)</sup>, but the scientists during the last decades have studied the mechanisms of action of probiotics<sup>(28)</sup> and proposed many mechanisms of action for them<sup>(12)</sup>. Guarner *et al*<sup>(19)</sup> recorded immunologic and non-immunologic mode of action, whereas Patel and DuPont<sup>(11)</sup> proposed three general mechanisms of action; antimicrobial activity, immune modulation, and improvement of mucosal barrier integrity (Fig. 1). On the other hand, Binns<sup>(4)</sup> suggested two main modes of action; Impact of microorganisms or their metabolites on the gastrointestinal tract and microbiota, and interaction with the cells and immune system of the host. However, depending on the function involved, each one of the mechanisms could be further subdivided<sup>(27)</sup>.

The proposed mechanisms of the effect of probiotics on host health to prevent or cure the different diseases could be summarized as follows:

#### 1- Production of antimicrobial agents

Probiotics produce many antimicrobial agents such as organic acids, hydrogen peroxide and antimicrobial peptides known as bacteriocins<sup>(12)</sup> that can inhibit the growth both of Gram positive and Gram negative bacteria, as well as the other pathogens<sup>(25)</sup>. Also, probiotics

produce hydrolytic enzymes which contribute increasing amounts of acids and this leads to reduce pH value and thus inhibition of pathogenic bacteria due to the acidic environment<sup>(11)</sup>, the effect of the *Lactobacillus*

spp. on *Helicobacter pylori* infection of the gastric mucosa is one of the important examples on the antimicrobial effect of probiotics<sup>(27)</sup>.

**Table 1. The microorganisms that are used as probiotics**

Microorganism		
Gram +ve bacteria	Lactic acid bacteria	<i>Lactobacillus</i> ; including: <i>L. acidophilus</i> , <i>L. rhamnosus</i> , <i>L. plantarum</i> , <i>L. johnsonii</i> , <i>L. crispatus</i> , <i>L. paracasei</i> , <i>L. casei</i> , <i>L. gasseri</i> , <i>L. fermentum</i> , <i>L. salivarius</i> , <i>L. delbrueckii</i> , <i>L. helveticus</i> , <i>L. gallinarum</i> , <i>L. mylovarus</i> , <i>L. reuteri</i> , <i>L. brevis</i> , <i>L. bulgaricus</i> , <i>L. cellobiosus</i> , <i>L. crispatus</i> , <i>L. curvatus</i> , <i>L. lactis</i> , <i>L. sporogenes</i> and <i>L. sakei</i>
		<i>Lactococcuslactis</i>
		<i>Streptococcus</i> ; including: <i>S. thermophiles</i> , <i>S. salivariussubsp. thermophiles</i> , and <i>S. diaacetylactis</i>
		<i>Enterococcus</i> including: <i>E. faecium</i> and <i>E. durans</i>
		<i>Pediococcuspentosaceus</i>
		<i>Leuconostoccremoris</i>
	<i>Bifidobacterium</i> including: <i>B. infantis</i> , <i>B. adolescentis</i> , <i>B. animalis</i> subsp <i>animalis</i> , <i>B. animalis</i> subsp. <i>lactis</i> , <i>B. longum</i> , <i>B. breve</i> , <i>B. thermophilum</i> and <i>B. bifidum</i>	
	<i>Bacillus</i> including: <i>B. subtilis</i> , <i>B. coagulans</i> , <i>B. licheniformis</i> , <i>B. clausii</i> and <i>B. cereus</i>	
	<i>Propionibacterium freudenreichii</i> subsp. <i>shermanii</i>	
Gram –ve bacteria	<i>Escherichia coli</i> Nissle 1917 (EcN)	
Nonpathogenic yeast	<i>Saccharomyces</i> including: <i>S. cerevisiae</i> , <i>S. bayanus</i> , <i>S. florentinus</i> and <i>S. boulardii</i>	

## 2- Competition with pathogens on adhesion sites and nutrients

Competitive inhibition of pathogen and toxin adherence to the intestinal epithelium is one of the mechanisms of probiotic action<sup>(26)</sup>, probiotics strengthening the barrier effect of the intestinal mucosa and release of gut-protective metabolites and thus prevent the adherence of the pathogenic bacteria to the host cells<sup>(25)</sup>. Walker<sup>(28)</sup> reported that the effect of probiotics on the activation and secretion of mucus in the intestine was directly correlated with the inhibition of pathogenic *Escherichia coli* attachment and of damage to the intestinal tract. However, the mechanism of adherence is still under investigation<sup>(25)</sup>. On

the other hand, probiotics digest food and compete with pathogens and other microbiota for the nutrients, although there is no evidence that this occurs *in vivo* but probiotics may consume nutrients otherwise utilized by pathogens<sup>(12,19)</sup>.

## 3- Immunological effects

The most complex proposed mechanisms of action of probiotics are the interaction with gastrointestinal tract immune cells and lymphoid tissue to modulate the immune and inflammatory responses of the host<sup>(4)</sup>. Probiotics affect directly and indirectly ways the function of lymphoids cells<sup>(27)</sup>, they effect on dendritic cells, monocytes, macrophages,

lymphocytes, T cells, regulatory T cells, immunoglobulin A-producing B cells, and natural killer cells<sup>(11,29)</sup>. According to the animal studies, probiotics can modulate both mucosal and systemic immune system<sup>(25)</sup>. They

can modulate cytokine profiles, induce hypo-responsiveness to food antigens, and activate local macrophages<sup>(19)</sup>. However, it's clear that not all the probiotics have the same immunomodulating characteristics<sup>(25)</sup>.

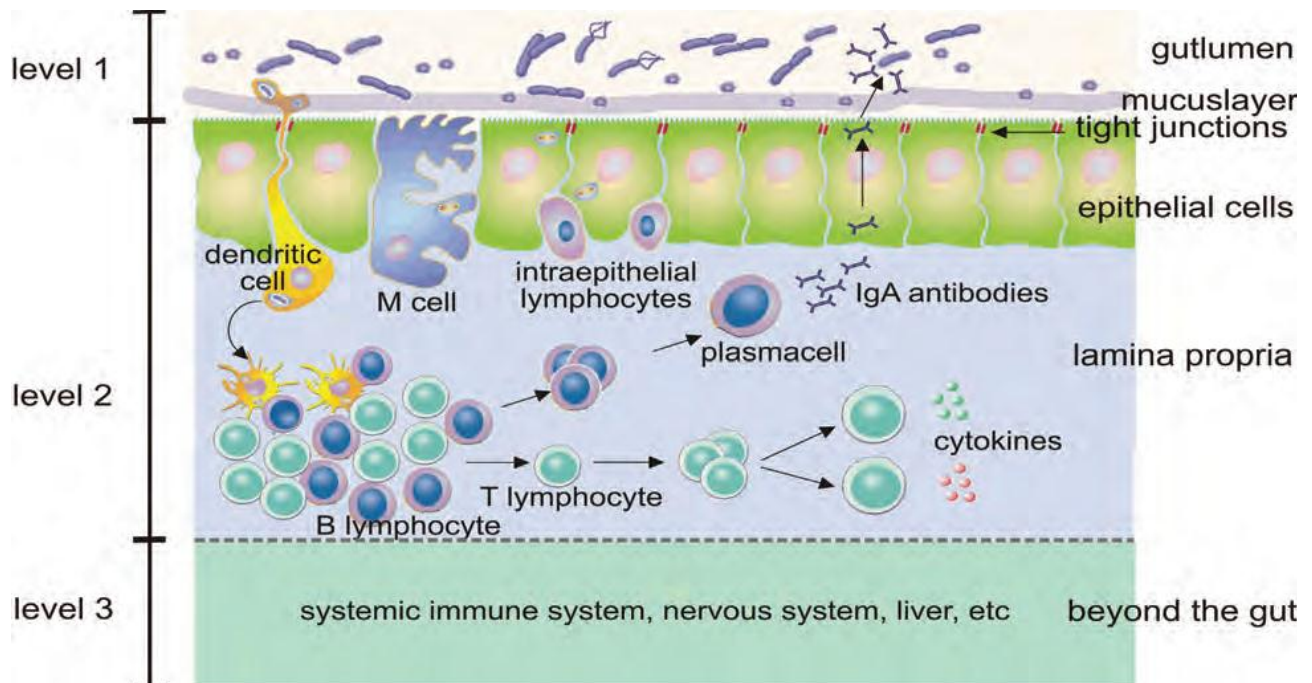


Fig. 1: The three levels or mechanisms of action of probiotics<sup>(4)</sup>

### Health beneficial effects of probiotics

The beneficial effects of probiotics are strain specific<sup>(30)</sup>. However, there are many evidences that numerous probiotics may have similar function with respect to their health effects<sup>(31)</sup>. Recently, there is great interest in probiotic therapy as alternative to antibiotic therapy in many health disorders<sup>(12)</sup>.

Depending on the results of animal models studies, probiotics can be used for treatment or prevention wide range of human diseases and disorders<sup>(6)</sup> such as; cryptosporidiosis<sup>(32)</sup>, urinogenital infections in women<sup>(5)</sup> such as vaginosis and vaginitis<sup>(33)</sup>, *Helicobacter pylori* infections<sup>(34)</sup>, allergic diseases<sup>(2)</sup> such as, food allergy<sup>(35)</sup>, and eczema<sup>(33)</sup>, hypertension<sup>(36)</sup>, inflammatory bowel disease<sup>(33)</sup>, irritable bowel syndrome<sup>(20)</sup> and other diseases. Also probiotics can be used to promote emotional behavior and may influence underlying brain mechanisms<sup>(24)</sup>. Heikkilä *et al*<sup>(37)</sup> reported that

probiotics can be used to remove the microbial toxins from solutions, such as; aflatoxins, ochratoxin A, Shiga toxin, microcystin-LR and cholera toxin.

However, actually the clinical benefits of probiotic treatment based upon many factors, such as the probiotic strain, its dose, age the host, his diet and period of treatment<sup>(9)</sup>.

### Probiotics' safety

The most important requirement for probiotics is safety<sup>(8)</sup>. In fact, the safety of probiotics has not been studied scientifically<sup>(6)</sup>. However, probiotics are divided into two groups depending on their risk to health; Risk group 1 and Risk group 2, the first group has no risk, while the other one has small risk<sup>(5)</sup>. There is no requirement to demonstrate purity, safety, or potency for probiotics before marketing, because they regulated as dietary supplements rather than as biological products or pharmaceuticals<sup>(23)</sup>.

Most LAB strains used in the food supply are nonpathogenic, nonvirulent, and nontoxigenic microorganisms<sup>(2)</sup>. To date, the products that contain probiotics seem to be safe for human health<sup>(22)</sup>. Saavedra<sup>(2)</sup> reported that more than 70 clinical studies involving more than four thousands infants and children consuming products containing probiotics, with no reports of adverse or side effects of probiotics that used in these products.

The potential of health risk of probiotics must keep in mind before pharmaceutical factories adding them into the products<sup>(5)</sup> and assessment of risk must be made before use the probiotic products for hospitalized patients<sup>(6)</sup>. However, any probiotic that used for treatment or prevent the disease need Food and Drug Administration review and approval because it is classified as biological product<sup>(22)</sup>. Probiotics can be prepared as fermented or pelleted feed, powder, capsules, granules, and paste<sup>(16)</sup>. Finally, the suitable description of a probiotic product as reflected on the label should include; genus and species identification, strain designation and viable count of it, recommended dose, precise description of the physiological effect, recommended storage conditions, safety under recommended use conditions, and contact information for post-market surveillance<sup>(19)</sup>.

In conclusion, the well and full understanding of probiotics and their mechanisms of action will support development new methods to prevent and treat a wide range of diseases.

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Correspondence to Dr. Wala'a Sh. Ali

E-mail: microrose.2788@yahoo.com

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## Immunohistochemical Expression of CD44v6 and P53 Status in Borderline and Malignant Ovarian Surface Epithelial Tumors. A Clinico-Pathologic Study.

Mohanad M. Abdul Ghany<sup>1</sup> MSc, Yaarub I. Khattab<sup>2</sup> FICMS, Mohammed A. Al-Kurtas<sup>1</sup> FICMS

<sup>1</sup>Dept. of Pathology & Forensic Medicine, Al-Kindy College of Medicine, Baghdad University, <sup>2</sup>Dept. Pathology & Forensic Medicine, College of Medicine, Al-Nahrain University, Baghdad, Iraq

### Abstract

<b>Background</b>	Ovarian epithelial cancer is a leading cause of death among gynecological malignancies due mainly to advanced stage at presentation.
<b>Objectives</b>	To investigate the expression of CD44v6 and p53 in borderline tumors and malignant ovarian surface epithelial tumors, correlation with clinic-pathological parameters.
<b>Methods</b>	A cross sectional study included a total of (101) formalin-fixed paraffin-embedded ovarian tissue blocks; of which (19) cases were borderline tumors and (82) cases were overt ovarian carcinomas. Sections from each block were immunohistochemically stained for CD44v6 and p53.
<b>Results</b>	The expression of CD44v6 was higher in ovarian carcinomas (57.3%) than borderline tumors (26.3%) and it was significantly correlated with FIGO stage and histological grade of ovarian carcinomas. p53 was overexpressed significantly in invasive carcinoma compared to borderline tumors, and it was significantly associated with higher grade and FIGO stage of invasive carcinomas.
<b>Conclusions</b>	CD44v6 and p53 expressions were correlated with less differentiated, advanced-stage tumor and these markers may be important molecular markers for poor prognosis.
<b>Key words</b>	Borderline tumor, ovarian carcinoma, p53, CD44v6.

**List of abbreviations:** FIGO = International Federation of Gynecology and Obstetrics, IHC = immunohistochemical, SI = staining index.

### Introduction

Ovarian cancer represents one of the most common malignant conditions in adult women <sup>(1)</sup>. In Iraq it rank 6<sup>th</sup> among top 10 cancers affecting female according to the consecutive reports of the Iraqi cancer board in 2008 and 2011 <sup>(2)</sup>. Surface epithelial tumors represent about 90% of primary ovarian cancers <sup>(3)</sup> they may be solid, cystic or mixture of both may be benign, borderline or malignant <sup>(4)</sup>. These tumors represent 15% of all epithelial ovarian cancers, with the serous and mucinous types making up the vast majority of cases <sup>(5)</sup>. Over 75% of

ovarian cancer patients had already developed metastases when they were first diagnosed since it often results in poor prognosis <sup>(6)</sup>.

Adhesion processes are involved in all levels of the metastatic cascade. Most of the cell adhesion molecules including CD44 family have played a great role in various stages of tumor progression and metastasis <sup>(7)</sup>.

CD44v6 is an important isoform of CD44 family. It is a transmembrane glycoprotein widely distributed among different tissues and is a receptor of the extracellular matrix component hyaluronic acid <sup>(8)</sup>.

It has been established that CD44v6 plays role in tumor development and progression in a variety of human cancers including ovarian cancer <sup>(9)</sup>.

The p53 tumor suppressor gene mutation is the most common genetic aberration in human malignancy, including ovarian carcinomas<sup>(10)</sup>. In contrast to borderline tumors where p53 overexpression is a rare event, p53 is the most commonly identified somatic genetic alteration in invasive ovarian carcinomas<sup>(11)</sup>. Cancers with p53 mutation demonstrated a trend toward more aggressive tumor behavior such as poor cellular differentiation and distant metastasis<sup>(12)</sup>.

The aim of this study is to evaluate the immunohistochemical expression of CD44v6 and p53 in borderline and malignant ovarian surface epithelial tumors and to correlate these expressions with clinicopathological parameters (FIGO stage and grade).

## **Methods**

This cross sectional study was approved by Institute Review Board of the College of Medicine, Al-Nahrain University. The collection of the samples last for the period from Mar. 2014 to Feb. 2015. A total of one hundred and one formalin fixed paraffin embedded ovarian tissue of which sixty cases were of serous carcinoma, ten cases were of endometrioid carcinoma, eight cases were of mucinous carcinoma, two were of clear cell carcinoma and two cases were malignant Brenner.

Moreover, nineteen cases of borderline tumor, of which fourteen cases with serous differentiation and five cases of mucinous type were retrieved from the histopathology archive of the Teaching Laboratories in the Medical City, Al-Yarmok Teaching Hospital and Al-Imamain Al-Kadhimiyyin Medical City for the period from Jan. 2011 to Dec. 2014.

All the clinic-pathological parameters such as (age; histopathological type of ovarian carcinoma and borderline tumors as well as grade and FIGO (International Federation of Gynecology and Obstetrics) pathological stage of ovarian carcinomas were obtained from patients' admission case sheets and pathology reports. Any sample lacking the clinic-

pathological information was excluded from this study.

For each case, one representative (4 $\mu$ ) section was stained with Hematoxylin and Eosin and the histopathological diagnosis was revised, while two (4 $\mu$ ) sections were placed on positively charged slides and stained immunohistochemically using three steps- indirect streptavidin method for monoclonal mouse antibodies including anti CD44v6 antibody, clone (VFF-7) and anti-p53 antibody, clone (BP53-12), both manufactured by Abcam.

## **Interpretation of the results of immune-histochemical staining**

**1. CD44v6:** Brown membranous &/or cytoplasmic staining pattern of epithelial cells even if staining was focal in tumor cell is considered positive. Positive control is tonsil. Technical negative control was obtained by omission of primary antibody.

**2. p53 protein:** Brown nuclear staining is considered positive. Positive control is the lymphoid tissue in non Hodgkin lymphoma. Technical negative control was obtained by omission of primary antibody.

The results of immunohistochemical expression of the above molecular markers were analyzed in a semi-quantitative fashion as follow:

**CD 44v6:** was scored semi-quantitative by assessing both staining intensity as absent (= 0); weak/moderate (= 1), intense (= 2) and percentage of stained cells (staining ratio) in relation to the total number cells as follows: 0 = no staining; 1 = staining of 1–20% of cells; 2 = staining of 21-50% of cells; and 3 = staining of 51-100% of cells with final staining index range from 0, 2-5<sup>(13)</sup>.

**p53:** The interpretation of the p53 staining was based on the percentage of tumor cell nuclei staining and the staining intensity. The percentage of stained cells (staining ratio) was used to score a slide semiquantitatively in one of four categories: (a) 1+, 5-25% staining; (b) 2+, 26-50% staining; (c) 3+, 51-75% staining; and (d) 4+, 76-100% staining. Sections with less than 5% tumor nuclei staining were considered



negative. Intensity was graded from weak (1+) to strong (3+). The staining index was calculated for each case as the product of staining intensity and staining ratio (staining index = staining intensity + staining ratio), with final staining index range from 0, 2-7<sup>(14)</sup>.

#### Statistical Analysis

Statistical analysis was performed with SPSS V. 16 (statistical package for social sciences) and also Excel 2007 programs. Continuous variables were expressed as mean±SEM (standard error of the mean), while categorical variables were expressed as numbers and percentages.

Statistical relations between two categorical variables were tested using Chi-square or Fisher exact tests. Relations between categorical and continuous variables were tested using unpaired t-test and ANOVA. Values were considered statistically significant when p-value < 0.05.

#### Results

The clinicopathological parameters of ovarian borderline tumors and carcinoma cases included in the present study (Table 1).

**Table 1. Clinicopathological parameters of borderline tumors and invasive ovarian carcinoma cases**

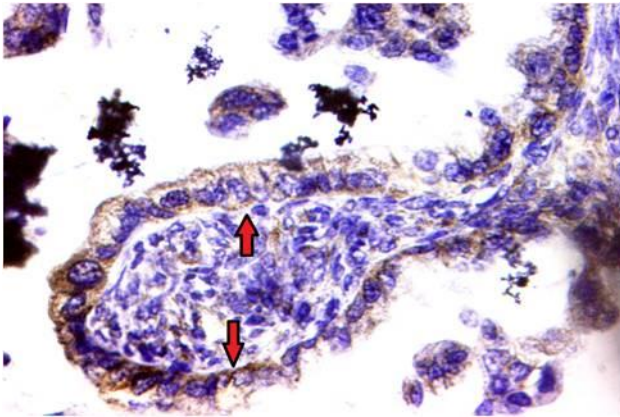
Parameters	No. (%)	
Histopathological diagnosis	borderline tumors	19
	ovarian carcinoma	82
Age (years) Mean range ±SEM	borderline tumors	38.05 ± 2.61 (18-62)
	ovarian carcinoma	57.89 ± 1.13 (30-85)
Histopathological types of ovarian carcinoma	Serous	60 (73.2%)
	Mucinous	8 (9.8%)
	Endometrioid	10 (12.2%)
	Clear cell carcinoma	2 (2.4%)
	Malignant Brenner	2 (2.4%)
Histopathological types of borderline tumors	Serous	14 (74%)
	Mucinous	5 (26%)
Grade of ovarian carcinoma	Well-differentiated	30 (37%)
	Moderately-differentiated	34 (41%)
	Poorly-differentiated	18 (22%)
FIGO stage of ovarian carcinoma	I	27 (29%)
	II	18 (53%)
	III	32 (39%)
	IV	5 (6.1%)

FIGO = International Federation of Gynecology and Obstetrics

#### CD44v6 immunohistochemical expression

Of 82 cases ovarian carcinomas, CD44v6 expression was recognized in 47 (57.3%) cases compared to 5 (26.3%) cases of borderline tumors (Fig. 1) this data failed to achieve statistical significance ( $p > 0.05$ ) (Table 2). Expression of CD44v6 was statistically correlated with histological grade ( $p = 0.004$ )

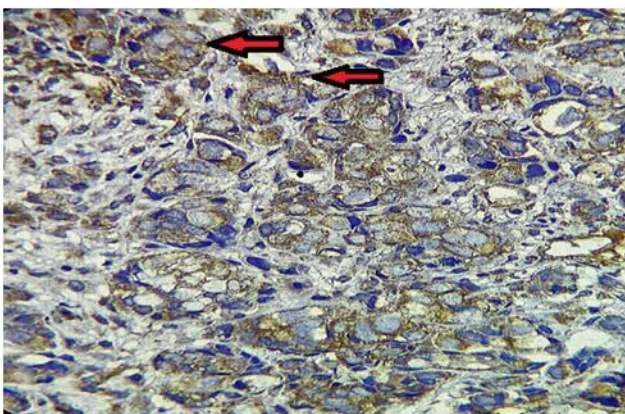
(Fig. 2) and FIGO stage of ovarian carcinoma ( $p < 0.001$ ), but not correlated with age and histological type in both carcinoma and borderline. The correlation found between CD44v6 expression in ovarian carcinoma and clinicopathological parameters are shown in (Table 2).



**Fig. 1. Borderline serous tumor stained with anti CD44v6 showing positive brown cell membrane (arrows) and cytoplasmic immunostaining with scoring index 3, (40X).**

#### **p53 immunohistochemical expression**

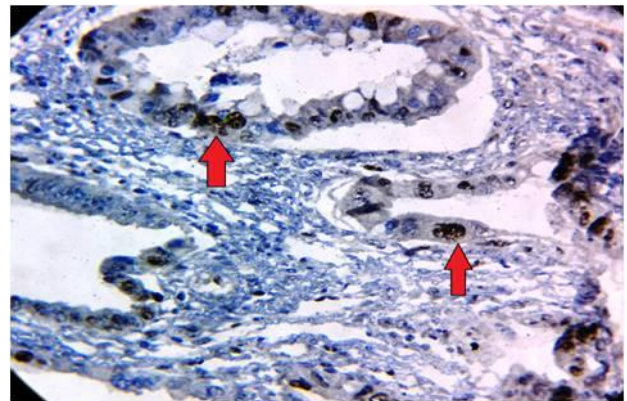
p53 was expressed immunohistochemically in 47 (57.3%) cases of ovarian carcinomas compared to only 1 (5.3%) case of borderline tumors (figure 4) this difference in expression was significant statistically ( $p = 0.004$ ), (Table 3). The table also show significant association between IHC expression of p53 with FIGO stage and grade of ovarian carcinomas, ( $p = 0.01$ ) and ( $p < 0.001$ ), respectively. The current study failed to express association between IHC expression of p53 with different types of ovarian carcinoma (Fig. 3) and age in borderline tumors or ovarian carcinoma, (Table 3).



**Fig. 2. Poorly differentiated serous carcinoma stained with anti CD44v6 showing positive brown cell membrane (arrows) immunostaining with scoring index 5, (40x).**

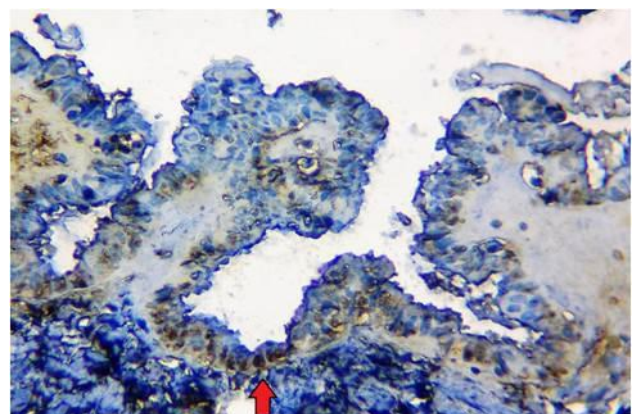
#### **Discussion**

CD44v6 is involved in the production of experimental metastasis. Previous reports have indicated that the overexpression of CD44v6 was correlated with poor prognosis of human cancers<sup>(8,15)</sup>. FIGO stage and grade are the two most important prognostic factors in ovarian cancers.



**Fig. 3. Mucinous carcinoma stained with anti-p53 showing positive brown nuclear staining with scoring index of 4, (arrows), (40X).**

The current study showed significant association between FIGO stages of ovarian carcinoma and CD44v6 immune expression, the expression were increased with higher stage of ovarian carcinoma. These findings were in agreements with Shi *et al*<sup>(13)</sup>, Zhou *et al*<sup>(16)</sup> and Bian<sup>(17)</sup>.



**Fig. 4. Borderline serous tumor stained with anti-p53 showing positive brown nuclear staining with scoring index of 3, (arrow), (40X).**

The current study also demonstrates increase in CD44v6 expression with loss of differentiation of malignant cells of in ovarian carcinoma. This tendency for increase CD44v6 expression along with increase grade of ovarian tumor had been demonstrated by several previous studies like Bar *et al*<sup>(18)</sup>, Shi *et al*<sup>(13)</sup>, Zhou *et al*<sup>(16)</sup> and Ryabtseva *et al*<sup>(19)</sup>. The positive correlation between histological grade, FIGO stage and expression of CD44v6 may indicate that this

marker play an important role predicting the prognosis of patient with ovarian carcinoma. Regarding the expression of CD44v6 in relation to histological types of ovarian tumors, this study showed no statistically meaningful correlation neither among different types of invasive ovarian carcinoma, nor between the 2 subtypes of borderline tumors. This is in agreement with Shi *et al*<sup>(13)</sup> and Zhou *et al*<sup>(16)</sup>.

**Table 2. Association of CD44v6 immunohistochemical expression with clinicopathological parameters of borderline tumors, and ovarian carcinoma**

Clinicopathological parameter		CD44v6		p value
		Positive	Negative	
Age (years) mean± SEM	Borderline tumors	42.80 ± 5.17	36.36 ± 3.01	0.245
	Ovarian carcinoma	59.30 ± 1.56	56.00 ± 1.62	0.336
Histopathological diagnosis	Borderline tumors	5 (26.3)	14 (73.7)	0.228
	Ovarian carcinoma	47 (57.3)	35 (42.7)	
Histopathological type of Borderline tumors No. (%)	Serous	4 (28.5)	10 (71.5)	0.554
	Mucinous	1 (20.0)	4 (80.0)	
Histopathological type of invasive carcinomas No. (%)	Serous	38 (63.3)	22 (36.7)	0.312
	Mucinous	4 (50.0)	4 (50.0)	
	Endometrioid	2 (20.0)	8 (80.0)	
	Clear cell Carcinoma	2 (100.0)	0 (0)	
	Malignant Brenner	1 (50)	1 (50.0)	
Grade of invasive carcinoma No. (%)	Well-differentiated	12 (40.0)	18 (60.0)	0.004
	Moderately-differentiated	19 (55.9)	15 (44.1)	
	Poorly-differentiated	16 (88.9)	2 (11.1)	
Pathological stage of invasive carcinoma No. (%)	I	4 (14.9)	23 (85.1)	<0.001
	II	14 (77.7)	4 (22.3)	
	III	24 (75.0)	8 (25.0)	
	IV	5 (100)	0	

Although there is a similarity in expression pattern between different types ovarian carcinoma, there are some differences; a possible explanation for the difference may be that ovarian carcinomas are heterogeneous entities, some derived from borderline tumors and others arising de novo.

Comparing CD44v6 expression in carcinoma versus borderline groups, the expression was higher among invasive carcinoma group (57.3%) than borderline tumor (26.3%), this results were in concordance with Zagorianakou

*et al* (50 %vs. 42.9 %) <sup>(20)</sup> and Hong *et al*. (40% vs. 27%) <sup>(21)</sup>. However, this difference in expression of CD44v6 didn't achieve statistical significance either in the current study, or in the two other studies mentioned, while Bian <sup>(17)</sup> prove a significant difference among the two groups. This may attributed to larger sample of borderline tumors (32 cases) compared to (19 cases) in the current study, (15 and 14 cases) in Hong *et al* <sup>(21)</sup> and Zagorianakou *et al* <sup>(20)</sup>, respectively.

**Table 3. Association of p53 immunohistochemical expression with clinicopathological parameters of borderline tumors, and ovarian carcinoma**

Clinicopathological parameter		p53		P value
		Positive	Negative	
Age (years) (mean± SEM)	Borderline tumors	40.0 ± 0.00	37.94 ± 2.76	0.867
	Ovarian carcinoma	57.60 ± 1.31	58.29 ± 2.02	0.767
Histopathological diagnosis	Borderline tumors	1 (5.3)	18 (94.7)	0.004
	Ovarian carcinoma	47 (57.3)	35 (42.7)	
Histopathological type of invasive carcinomas No. (%)	Serous	38 (63.3)	22 (36.7)	0.948
	Mucinous	5 (62.5)	3 (37.5)	
	Endometrioid	3 (30.0)	7 (70.0)	
	Clear cell Carcinoma	1 (50.0)	1 (50.0)	
	Malignant Brenner	0	2 (100.0)	
Grade of invasive carcinoma No. (%)	Well-differentiated	10 (33.3)	20 (66.7)	<0.001
	Moderately-differentiated	24 (70.6)	10 (29.4)	
	Poorly-differentiated	13 (72.2)	5 (27.8)	
Pathological stage of invasive carcinoma No. (%)	I	6 (22.3)	21 (77.7)	0.01
	II	10 (55.5)	8 (44.5)	
	III	26 (81.25)	6 (18.75)	
	IV	5 (100)	0	

The p53 tumor suppressor gene mutation is the most common genetic aberration in human malignancy, including ovarian carcinomas <sup>(10)</sup>. p53 was detected in only one case of borderline tumor (5.3%), compared to 47(57.3%) out of 82 case of carcinoma. These data were similar to those of Anreder *et al* <sup>(22)</sup> where only 2 (10.5%) of 19 borderline tumors showed reactivity to p53 compared to 30(62.5%) of 48 carcinomas. Kupryjanczyk *et al* <sup>(23)</sup> demonstrated immune-reactivity with anti-p53 in 14% of the borderline tumors while Zagorianakou *et al* <sup>(20)</sup> found very low expression (less than 10%) or even absent in all 14 cases of ovarian borderline tumors compared to 23 (47.9%) of 48 carcinoma cases show greater than 10% expression of p53. These statistically significant results can be interpreted to justify use of the p53 status as an immunohistochemical marker to help differentiate invasive from borderline ovarian neoplasms. Although p53 mutations have been detected in all histological types of ovarian carcinoma, the relationship between p53

protein expression and the histopathological subtype in ovarian carcinomas is still controversial.

In studies of Milner *et al* <sup>(24)</sup> and Gottlieb and Berek <sup>(25)</sup> both show that the highest incidence of p53 positivity were reported in serous carcinoma and The lowest rate of p53 expression was found in endometrioid carcinoma. These observations were in agreement with the results of the current study which also showed that serous carcinoma expressed p53 more frequently than other types of invasive carcinoma, furthermore the only positive single case of borderline tumor that express p53 was of serous differentiation, however statistical comparison did not reach significance in this field. This statistical non-significant correlation between histological types of invasive ovarian carcinoma and p53 IHC expression was found by Hamdi and Saleem <sup>(26)</sup>, Skirnisdottir *et al* <sup>(27)</sup> and Levesque *et al* <sup>(28)</sup> as well. This controversy of findings in this field could be due to random case selection and different types of antibodies used in different studies.

Focusing on association of p53 IHC expression with grade of ovarian carcinoma many authors found a significant association between grade and p53 expression<sup>(29-31)</sup>.

The current study also demonstrates this association, i.e., increase in IHC expression of p53 was associated with increase grade of ovarian carcinoma which was highly significant from statistical point of view. The study show that 13 (72.2%) out of 18 cases with poorly differentiated tumors are positively expressed the marker compare to 10 (33.3%) out of 30 cases of well differentiated tumors. Apparently the most important determinant of clinical outcome of ovarian carcinoma is the clinicopathologic stage at the initial time of diagnosis.

Shelling *et al*<sup>(32)</sup> found that the prevalence of TP53 gene alterations appears to raise with increasing stage and it occur more often in stage III and IV ovarian cancers when compared to stage I and II, i.e., in 58% versus 37% in stage III/IV and in stage I/II, respectively.

In the current study we are in agreement with these finding as there were a significant association between p53 immune expression and stage of invasive ovarian carcinoma. The data of the current study show that while expression of p53 did not exceed 25% in stage I we found the expression hit 100% in stage IV, this raise in immune expression of P53 with increase in FIGO stage was supported by findings of many previous studies<sup>(23,33,34)</sup>. The current study show no significant correlation between p53 IHC expression and age of the patients and this is consistent with many previous studies<sup>(26,31,35)</sup>.

We concluded that CD44v6 expression was higher with higher grade and FIGO stage of ovarian carcinoma, ovarian carcinoma with p53 mutation demonstrated a trend toward more aggressive tumor behavior such as poor cellular differentiation and distant metastasis and CD44v6 and p53 may be important molecular markers for poor prognosis.

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### Author contributions

All authors coordinated study subject recruitment, implementation and progress of this study, and helped with data interpretation and manuscript organization and editing.

### Conflict of Interest

The authors have no conflicts of interest

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**Correspondence to Dr Mohanad M. Abdul Ghany**

**E-mail: hamammy@yahoo.com**

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## Serum Soluble fms-like Tyrosine Kinase-1 (sflt-1) Level at Third Trimester of Pregnancy and One Month Following Delivery in Women with Preeclampsia

Radhwan M. Hussein<sup>1</sup> MSc, Najat A. Hasan<sup>2</sup> PhD, Bushra J. Al-Rubayae CIBOG

<sup>1</sup>Ministry of Health, <sup>2</sup>Dept. of Chemistry & Biochemistry, College of Medicine, Al-Nahrain University, <sup>3</sup>Dept. of Obstetrics & Gynecology, Babylon Medical College, Iraq

### Abstract

<b>Background</b>	Preeclampsia is characterized by abnormal vascular response to placentation that is associated with increased systemic vascular resistance, enhanced platelet aggregation, activation of the coagulation system, and endothelial cell dysfunction.
<b>Objective</b>	To investigate the serum level of anti angiogenic factor soluble fms-like tyrosine kinase-1 (sFlt-1) in the third trimester of preeclamptic pregnant in relation to the Doppler ultrasonography indices.
<b>Methods</b>	One hundred and five pregnant women at the Pediatric and Maternity Teaching Hospital in Hilla City were studied. All of them were in their third trimester and with singleton pregnancy. All participants were reexamined one month following delivery.
<b>Results</b>	The body mass index, spot urine protein to creatinine ratios and mean pulsatility index, resistance index), systolic and diastolic blood pressures were increased in preeclamptic groups. Post-delivery systolic and diastolic blood pressures increased in the control pregnant group and significant reduction in the both preeclamptic groups compared with the systolic and diastolic blood pressures at third trimester of pregnancy. sFlt-1 levels were increased in severe than mild preeclampsia and control groups. In post-delivery period, the sflt-1 decreases in the control pregnant, mild and severe preeclamptic groups in comparison to the third trimester. In preeclampsia, the sflt-1 is positively correlated with blood pressure, protein: creatinine ratio, body mass index, resistance index, pulsatility index, total serum protein, and negatively correlated with neonatal weight and gestational age at delivery.
<b>Conclusion</b>	The changes in anti angiogenic factors sFlt-1 during pregnancy and in post-delivery period stress its role in the pathogenesis of preeclampsia and could be used to predict the disease progression and impact on the outcome of pregnancy.
<b>Keywords</b>	Preeclampsia, sFlt-1, Doppler indices.

**List of abbreviation:** PE = preeclampsia, BMI = body mass index, P/C ratios = spot urine protein to creatinine ratios, PI = pulsatility index, RI = resistance index, sBP = systolic blood pressure, dBP = diastolic blood pressure, TP = total serum protein, sFlt-1 = soluble fms like tyrosin kinase-1, VEGF = vascular endothelial growth factor, ALT = alanine aminotransferase, AST = aspartate aminotransferase, ELISA = enzyme linked immunosorbent assay, ROC = receiver operating characteristic, PlGF = placental growth factor.

### Introduction

**P**reeclampsia (PE) is a pregnancy associated multisystem disorder that complicates 2%-10 % of pregnancies of

western world. It is accompanied with a neonatal and perinatal mortality rate of 10 % worldwide <sup>(1)</sup>. The clinical features of preeclampsia are proteinuria and hypertension that occur after 20 weeks of gestation in women who were not previously diagnosed to be hypertensive. Other signs and symptoms of PE are edema and headache <sup>(2)</sup>. PE can be divided into mild and severe forms, according to the amount of proteinuria, the severity of

the hypertension, and the degree of effect on other organ systems<sup>(3)</sup>. The etiology of PE is unknown and, actually the entity is remarkably poorly understood. Delivery of the placenta usually halts the disease progression but it does not reverse all of the associated pathophysiological changes of preeclampsia<sup>(4)</sup>. Soluble fms (feline sarcoma virus)-like tyrosine kinase-1 (sFlt-1) is an anti-angiogenic protein. It is a shortened splice variant of the vascular endothelial growth factor (VEGF) receptor-1 that signals angiogenesis. The sFlt-1 captures vascular endothelial growth factor thus preventing its interaction with ligands and down regulating the biological effects of VEGF<sup>(5)</sup>. sFlt-1 is upregulated in PE, leading to increased circulating levels. The increased sFlt-1 levels are associated with decreased circulating levels of VEGF and placental growth factor (PlGF), resulting in endothelial dysfunction. Infusing sFlt-1 to pregnant rats provides a convincing animal model of PE<sup>(6)</sup>. The lowered oxygen tension in primary cytotrophoblast culture and villous explants was found to increase the sFlt-1 expression suggesting the role of hypoxia in stimulating sFlt-1 gene expression<sup>(7,8)</sup>. It has been reported that uteroplacental ischemia, results in a PE-like phenotype accompanied by elevated circulating sFlt-1<sup>(9)</sup>. Placental ischemia/ hypoxia may alone be sufficient to induce PE through sFlt-1 up-regulation. When the trophoblastic invasion is defective, an enhanced placental vascular resistance is likely to occur as evidenced in abnormal Doppler ultrasound spectrum of the uterine vessels, indicating that the women are at risk for serious pregnancy disorders like PE<sup>(10)</sup>. So, this study was conducted to test the relationship of the antiangiogenic factor sFlt-1 and the Doppler ultrasound finding and to monitor their impact on the neonatal health and the post-delivery hemodynamics.

## **Methods**

This research was conducted on 105 pregnant women at their third trimester of pregnancy

over a period of fourteen months from May 2014 till Aug. 2015 at the Pediatric and Maternity Teaching Hospital in Hilla city. The practical part was conducted at Research Laboratories in the Department of Chemistry and Biochemistry, College of Medicine, Al-Nahrain University, and at the Department of Radiology and at laboratories of Pediatric and Maternity Hospital in Hilla city.

Out of total 105 pregnant, 70 women suffer from PE with a maternal age of 19-41 year and the other 35 were normotensive pregnant (Maternal age range = 18-36 year) who were served as control. All of studied women were carrying single fetus and at their third trimester of pregnancy. The PE group was further subdivided according to the severity of the disease into mild (n=35) and severe PE (n=35) according to the standard criteria<sup>(3)</sup>.

The study was approved by the Institute Review Board of the College of Medicine, Al-Nahrain University and a written consent was provided from each participant to be enrolled in this study. All pregnant women were subjected to clinical examination, blood pressure (BP) measurement, and routine antenatal ultrasonography. Doppler ultrasonography has been used for each pregnant to determine the pulsatility index (PI) and resistance index (RI).

Five milliliters of venous blood samples were withdrawn for the spectrophotometric determination of serum creatinine, total serum protein (TP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), and glucose, and the serum sflt-1 concentration by Enzyme linked immunosorbent assay (ELISA). Spot urine samples were used immediately for the measurement of creatinine, and protein.

**Statistical Analyses:**

Group comparisons and data correlation were carried out using mean, standard error of mean, analysis of variance and Pearson correlation. Paired t-test was used to compare the pre delivery and post-delivery levels of aforementioned parameters and a P value of less than 0.05 was considered to be statistically



significant. The receiver operating characteristic (ROC) curve was used to demonstrate the cut-off values, sensitivity, specificity and area under the curve of the studied research variables<sup>(11)</sup>.

## Results

The results of maternal age, gestational age at delivery, BMI, neonatal weight, spot urinary protein / creatinine ratio (P/C) and Doppler findings (PI and RI) for both control pregnant group and patients groups (mild and severe preeclampsia) at third trimester are listed in table 1. There were very high significant decreases ( $p < 0.001$ ) in mean gestational age

at delivery and of neonatal weight in mild and severe preeclampsia group when compared with those of the control pregnant group. There were high significant increase in means of body mass index (BMI), spot urine protein, to creatinine ratios and mean RI in severe PE ( $p < 0.01$ ) and in mild PE ( $p < 0.01$ ) in comparison to their respective values of the control group. The elevation in the PI indices in mild PE was less significant as compared to control group ( $p < 0.05$ ). The means of all of aforementioned parameters were significantly higher in pregnant women with severe PE as compared with pregnant with mild form of PE.

**Table 1. Illustrates the demographic and biochemical data the preeclamptic patients and controls at third trimester of pregnancy**

Parameter	Control group	preeclampsia	
		Mild	Severe
Number	35	35	35
maternal age (year)	25.6 ±0.84	27.43 ±0.99	26.8 ±0.84
gestational age at delivery (weeks)	38.00 ±0.2	36.92±0.26 <sup>a▼***</sup>	36.17±0.19 <sup>a▼***</sup>
BMI (Kg/m <sup>2</sup> )	27.92±0.55	31.23±0.5 <sup>a▲**</sup>	34.3±0.86 <sup>a▲***, ▲b**</sup>
neonatal weight (Kg)	2.66 ±0.05	2.45±0.05 <sup>a▼**</sup>	2.34±0.4 <sup>a▼***</sup>
urinary spot protein /creatinine ratio	0.1 ±0.003	1.04±0.05 <sup>a▲***</sup>	3.55±0.23 <sup>a▲***, b▲***</sup>
pulsatility index	0.71 ±0.02	0.84±0.153 <sup>a▲*</sup>	1.02±0.06 <sup>a▲***, b▲**</sup>
resistance index	0.56± 0.01	0.65±0.01 <sup>a▲**</sup>	0.72±0.02 <sup>a▲***, b▲*</sup>

a = ANOVA test between mild, severe preeclampsia group versus control pregnant group: ▼\*\*\* = very high significant decrease ( $p < 0.001$ ); ▲\*\* = high significant increase ( $p < 0.01$ ); ▲\*\*\* = very high significant increase ( $p < 0.001$ ); ▼\*\* = high significant decrease ( $p < 0.01$ ). b = ANOVA test between mild and severe preeclampsia groups: ▲\*\* = high significant increase ( $p < 0.01$ ); ▲\*\*\* = very high significant increase ( $p < 0.001$ ).

The results of systolic blood pressure (sBP), diastolic blood pressure (dBP), total serum protein (TP) and sflt-1 for both control pregnant group and patients groups (mild and severe preeclampsia) at third trimester and one month following delivery are listed in table 2.

There were very high significant increases ( $p < 0.001$ ) in mean of sBP and dBP in both mild and severe preeclamptic groups when compared with control pregnant group at third trimester.

Comparison of the results of the blood pressure before and after delivery using paired t-test showed very high significant increases ( $p < 0.001$ ) in the mean of sBP and dBP in the control pregnant group after one month following delivery compared with the sBP and dBP mean value of the same group at third trimester of pregnancy. On the contrary, the mean values of sBP and dBP were significantly lowered in both mild and severe preeclamptic groups after one month following delivery

compared with the sBP and dBP mean value of the same groups at third trimester of pregnancy (p < 0.001). The mean post-delivery

sBP and dBP levels were continued to be significantly higher in severe PE above those with mild PE (p < 0.01).

**Table 2. Illustrates the differences in systolic and diastolic blood pressure, total serum protein and soluble fms like tyrosin kinase-1 between preeclamptic patients and controls at third trimester of pregnancy and one month following delivery**

Parameter		Control No = 35	Preeclampsia	
			Mild No = 35	Severe No = 35
sBP (mmHg/hr)	Before delivery	10.92±0.1	13.52±0.18 <sup>a▲***</sup>	14.78±0.23 <sup>a▲***,b▲***</sup>
	After delivery	11.31±0.11 <sup>c▲***</sup>	11.48±0.12 <sup>c▼***</sup>	12.07±0.19 <sup>a▲***,b▲**c▼***</sup>
dBP (mmHg/hr)	Before delivery	7.3±0.11	9.4 ±0.15 <sup>a▲***</sup>	10.45±0.22 <sup>a▲***,b▲***</sup>
	After delivery	7.51±0.09 <sup>▲***</sup>	7.64 ±0.13 <sup>c▼***</sup>	8.15±0.14 <sup>a▲***,b▲**c▼***</sup>
(TSP) (g/dl)	Before delivery	6.02±0.11	5.61 ±.09 <sup>a▼**</sup>	5.23±0.08 <sup>a▼***,b▼**</sup>
	After delivery	6.72±0.09 <sup>c▲***</sup>	6.43±0.07 <sup>a▼*c▲***</sup>	6.19 ±0.07 <sup>a▼***,b▼*c▲***</sup>
Sflt-1 (ng/ml)	Before delivery	2.15±0.11	2.78±0.14 <sup>a▲**</sup>	3.55±0.15 <sup>a▲***,b▲***</sup>
	After delivery	1.96±0.1 <sup>c▼***</sup>	2.15±0.1 <sup>c▼***</sup>	2.50±0.1 <sup>a▲***,b▲*,c▼***</sup>

a = ANOVA test between mild, severe preeclampsia group versus control pregnant group: ▼\*\*\* = very high significant decrease (p < 0.001); ▲\*\* = high significant increase (p < 0.01); ▲\*\*\* = very high significant increase (p < 0.001); ▼\*\* = high significant decrease (p < 0.01). b = ANOVA test between mild and severe preeclampsia groups: ▲\*\* = high significant increase (p < 0.01); ▲\*\*\* = very high significant increase (p < 0.001). c = paired t-test for control, mild, severe, mean concentration at third trimester versus one month following delivery mean values: ▲\*\*\* = very high significant increase (p < 0.001); ▼\*\*\* = very high significant decrease (p < 0.001)

There were high significant decreases in the third trimester mean TSP concentration in mild (p < 0.01) and severe preeclamptic (p < 0.001) groups when compared with control pregnant group in the third trimester, with significant decreases (p < 0.01) in severe preeclamptic group when compared with mild PE. The post-delivery mean TSP levels showed significant elevation (p < 0.001) in control, mild and severe PE groups compared with their concentration at late pregnancy.

There was significant increase (p < 0.01) in mean serum sflt-1 in mild preeclamptic group and very high significant increase (p < 0.001) in severe preeclamptic group in comparison with those of the control pregnant group at third trimester. In severe PE, the sflt-1 levels were significantly above those in mild PE group (p < 0.05). There were very high significant decreases (p < 0.001) in means of serum sflt-1 levels in the control pregnant, mild and severe preeclamptic groups after one month following

delivery compared with the respective groups mean values at third trimester of pregnancy.

Receiver operating characteristic curve (ROC) analyses of serum sflt-1 revealed the ability of this marker to differentiate preeclamptic from normal pregnancies (Fig. 1). In this figure the cut-off value of sflt-1 was 2.74ng / ml with sensitivity and specificity of 67%, and 69%, respectively.

In severe PE, the sflt-1 values showed very high positive significant correlation (p < 0.001) with systolic BP (r = 0.69, Fig. 3), diastolic BP (r = 0.68, Fig. 3), P/C ratio (r = 0.82, Fig. 4), BMI (r = 0.8, Fig. 4), PI (r = 0.6, Fig. 2), statistically less but significant positive correlation (p < 0.01) with total serum protein (r = 0.56) and RI (r = 0.36, Fig.2).

On the other hand, in mild PE the sflt-1 concentration exhibited very high significant positive correlation (p < 0.001) with systolic BP (r = 0.62, Fig. 6), P/C ratio (r = 0.68, Fig. 7), BMI, and RI (r = 0.65, r = 0.64, respectively; Fig. 5),

and positive correlation ( $p < 0.01$ ) with total serum protein and dBp ( $r = 0.55$ ,  $r = 0.47$ , in an order; Fig. 6) and less strong positive correlation ( $p < 0.05$ ) with PI ( $r = 0.39$ , Fig. 5), significant negative correlation with gestational age at delivery and neonatal weight ( $r = -0.48$ ,  $r = -0.4$ , respectively; Fig. 8).

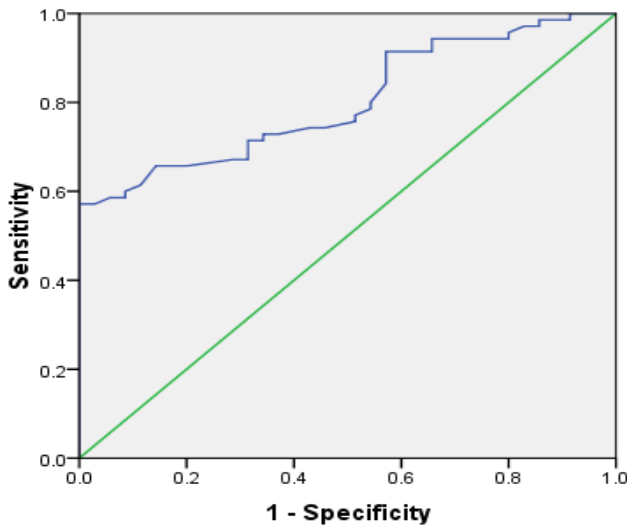


Fig. 1. Receiver operating characteristic curve showing sensitivity and specificity for prediction of preeclampsia by serum soluble fms like tyrosine kinase-1 levels.

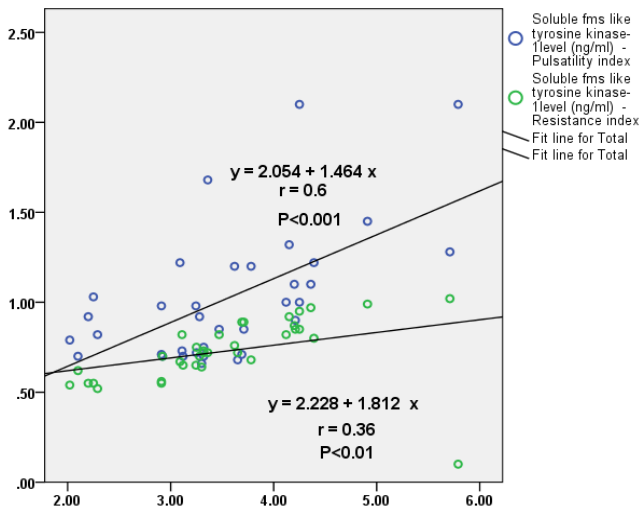


Fig. 2. The correlation of serum levels of soluble fms like tyrosine kinase-1 (ng/ml) with pulsatility index and resistance index in the severe preeclampsia group.

## Discussion

In this study, the significant decrease in the means of gestational age at delivery in mild and severe PE were in line of those reported by Aggarwal *et al* (2011), Stepan *et al* (2013), Akihide *et al* (2014), Hanita *et al* (2014) and

Sophia *et al* (2014) who stated that gestational age at delivery was significantly lower in the group of PE women compared to control normotensive group. The decrease in gestational age at delivery occur due to the clinician's decision to start delivery based on fetal condition, presence of signs of labor, cervical Bishop score, and fetal gestational age (18).

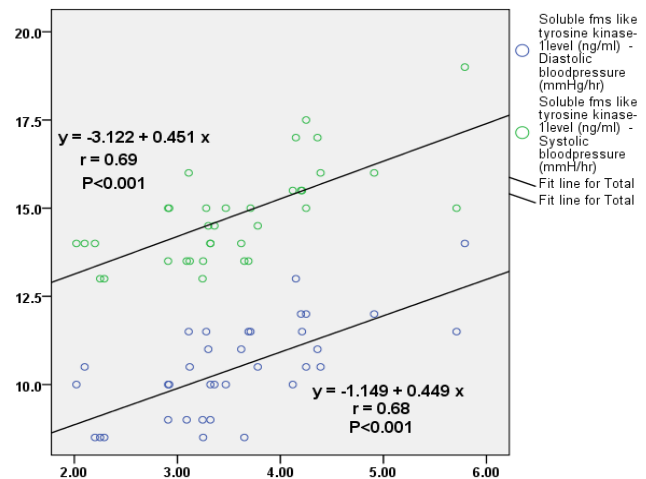
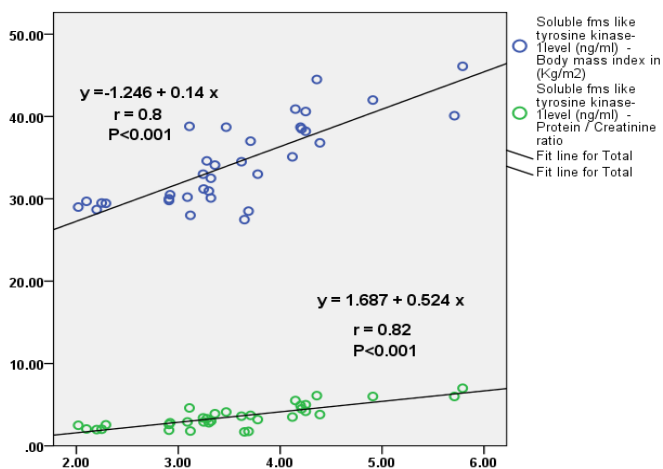


Fig. 3. The correlation of serum levels of soluble fms like tyrosine kinase-1 (ng/ml) with systolic and diastolic blood pressure (mmHg/hr.) in the severe preeclampsia group.

The significant elevation reported herein of maternal BMI in PE groups were comparable to those of Bibi *et al* (2011); Bian *et al* (2015) and disagreed with Bahia *et al* (2014) findings who stated that the means of body mass indices were not significantly different between the PE and normal groups.

Sharami *et al* (2012) concluded that women with PE had higher BMI than controls. A secondary analysis was performed by Athukorala and colleagues, (2010) on data collected from nulliparous women with a singleton pregnancy. Women were categorized into three groups according to their BMI: normal; overweight and; obese. Obstetric and perinatal outcomes were compared by univariate and multivariate analyses. They suggested that the rate of overweight and obesity is increasing amongst the obstetric population. Women who are overweight and obese have an increased risk of adverse

pregnancy outcomes. In particular, obese women are at increased risk of gestational diabetes, pregnancy induced hypertension and preeclampsia.



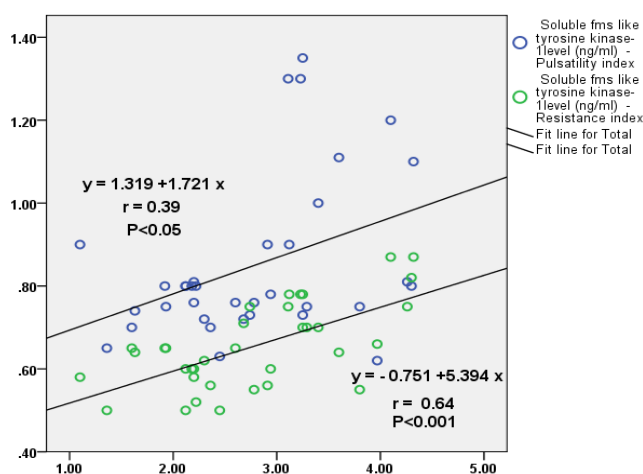
**Fig. 4. The correlation of serum levels of soluble fms like tyrosine kinase-1 (ng/ml) with protein/creatinine ratio and body mass index (Kg/m<sup>2</sup>) in the severe preeclampsia group.**

There were very high significant decreases in mean of baby weight of mothers suffering from severe PE when compared with neonates of healthy mothers. Also there was high significant reduction in mean of neonatal weight in mild PE mother compared with those of healthy mothers. These results are in line with those of Stefan *et al* (2010), Megha and Sunita (2013), Hanita *et al* (2014), Akihide *et al* (2014), Bian *et al* (2015), Spracklen *et al* (2015) who revealed significant decrease in the neonatal weight in PE group compared to those of normal pregnant groups. On the contrary, Bahia *et al* (2014) revealed that the means of neonatal birth weights were not significantly different between the PE and normal groups.

There are two explanations for the decrease in the weight of newborn for PE mothers. First: faulty placentation manifests in the mother as PE with vascular damage, enhanced systemic inflammation, and insulin resistance; in the placenta as oxygen and nutrient transfer restriction and oxidative stress; and in the fetus as growth restriction and progressive hypoxemia <sup>(27)</sup>. Second: The observed overall

effect on birth weight may depend on the relative proportions of full-term and preterm deliveries among patients with preeclampsia (17).

In this study there were very high significant increases in mean of sBP and dBP in both mild and severe PE groups when compared with control pregnant group at the third trimester and with increase in the disease severity. These findings were in agreement with those of Stefan *et al* (2010); Bibi *et al* (2011); Aggarwal *et al* (2011); Stepan *et al* (2013); Magna and Sitikantha, (2013).

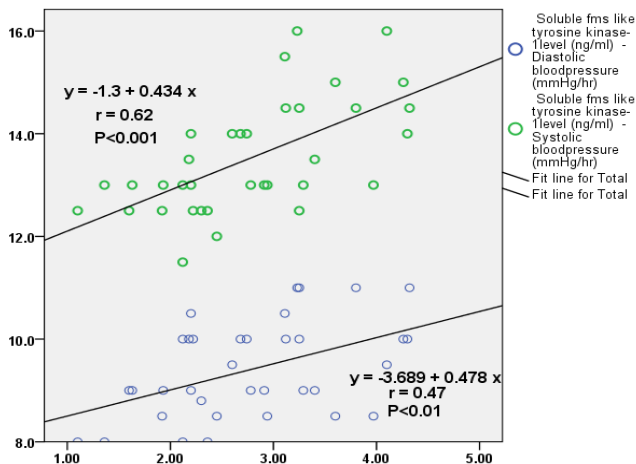


**Fig. 5. The correlation of serum levels of soluble fms like tyrosine kinase-1 (ng/ml) with pulsatility index and resistance index in the mild preeclampsia group.**

One month following delivery, the sBP and dBP in control pregnant group were found to increase significantly compared with the same group mean values at third trimester of pregnancy. The causes that lead to decrease BP levels at third trimester of pregnancy (although, these BP values are within the normal ranges) include the circulatory expansion, increased flow of blood in pregnant body to maintain the supply of oxygen and food nutrients to the fetus, and hormonal changes such as progesterone which relaxes the walls of blood vessels thus reduce the blood pressure.

There were very high significant decreases in means of sBP and dBP in mild and severe PE group at post-delivery period compared with their respective mean values at third trimester

of pregnancy. This occur due to the resumption of the normal physiology of the body and removal of factors that cause the increase in the BP (factors that lead to development of PE) such as presence of the placenta which is central to the pathogenesis of preeclampsia, stress, antiangiogenic agents, immunological factors and decrease in the angiogenic factors.

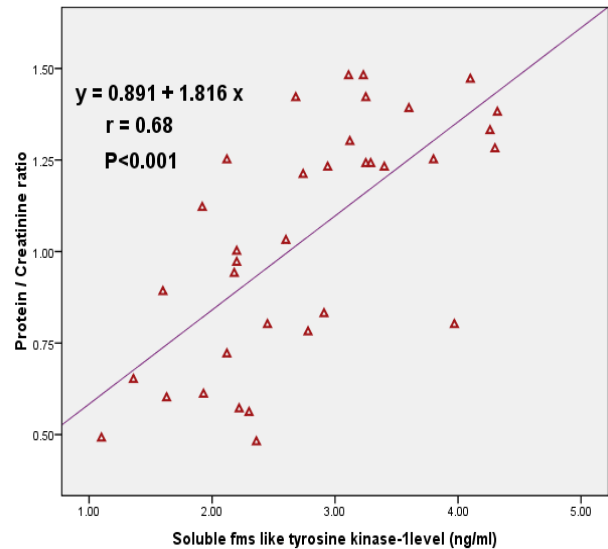


**Fig. 6.** The correlation of serum levels of soluble fms like tyrosine kinase-1 (ng/ml) with systolic and diastolic blood pressure (mmHg/hr.) in the mild preeclampsia group.

There were high significant decreases in mean TP in mild and severe PE groups when compared with those of the control pregnant group and the reduction in serum protein was more significant with the disease severity. Adedeji *et al* (2012) had observed that the concentration of TP was not significantly decreased in normotensive pregnant women compared to that of non-pregnant women. Olooto *et al* (2013) showed that TP levels were significantly lowered while urinary protein was significantly elevated in pregnancy related hypertension and PE, respectively as compared to those of the normal pregnant women.

The main cause of the relative decrease in the TP in control pregnant group and PE groups (mild and severe PE) is the hemodynamic changes accompanying the expansion of the plasma volume and the increases in cardiac output up to 50% which creates dilution of plasma proteins. As a result, the concentrations of albumin and  $\alpha$ 1-acid glycoprotein decrease

up to 20-40% at term<sup>(30)</sup>. In PE groups (mild and severe) the loss of glomerular barrier charge and size selectivity and glomerular endotheliosis result in poor filtration and increased urine protein<sup>(31)</sup>.



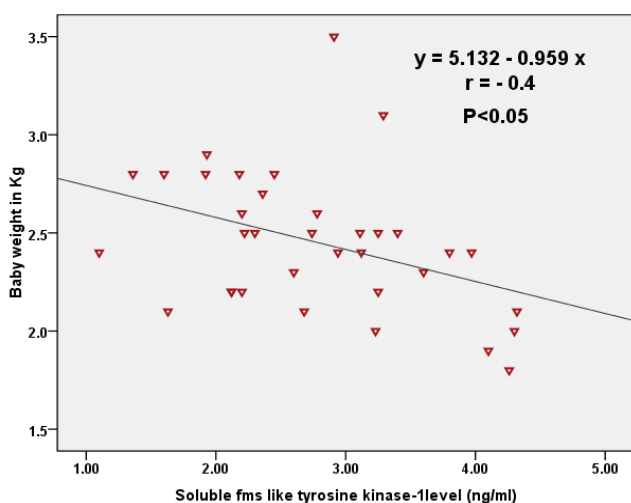
**Fig. 7.** The correlation between serum levels of soluble fms like tyrosine kinase-1 (ng/ml) and protein/creatinine ratio in the mild preeclampsia group.

The post-delivery mean serum protein revealed very high significant elevation in the control pregnant group and PE (mild and severe) as compared with their respective mean values at third trimester period. This can be explained by the hemodynamic changes in which there is a decrease in the expansion of plasma volume post-delivery with a decline in the renal loss of protein especially in PE groups (mild and severe).

In this study, there were significant increases in the PI and RI in mild PE with very high significant increases in means of PI in severe PE group compared with those of the control pregnant group. The mean PI and RI were found to be higher with the increase in the severity of the PE. Similar findings were recorded by Dahiana *et al* (2014), Guedes-Martins *et al* (2014), Martínez-Ruiz *et al* (2014), and Bian *et al* (2015).

The present study showed significant increases in the means of sflt-1 in severe PE compared with mild PE and the control pregnant group

mean values. Similar observations were reported by many other researchers<sup>(20,24,35-38)</sup>. In 2014, Sophia and co researchers analyzed the serum levels of antiangiogenic factor sflt-1 in addition to the neonatal adverse outcomes in 40 women with established PE and 40 normotensive women. They found that the sFlt-1 levels were significantly higher in the PE women compared to normotensive women. The increased serum values of the anti-angiogenic sFlt-1 were associated with increased rates of late preterm, early term births and very low birth weight.



**Fig. 8. The correlation between serum levels of soluble fms like tyrosine kinase-1 (ng/ml) and neonatal weights (Kg) in the mild preeclampsia group.**

Placental overexpression of sFlt-1 specifically in the fetal derived trophoblast cells was implicated as the underlying cause of PE. Fan and colleagues (2014) suggested that the fetal-derived trophoblastic cells over express sflt-1 as a self-defense against excessive VEGFA produced by maternal decidual cells.

The results of the significant positive correlation reported herein between the anti-angiogenic factor sFlt-1 with PI and RI and sBP and dBP in pregnant patients with mild or severe PE supported the finding of Sophia *et al* (2014) who demonstrated a significant contribution of sFlt-1 to the pathogenesis of endothelial dysfunction and the consequent systematic vascular disorder.

According to the results of present study, the higher concentrations of sFlt-1 were negatively correlated with the adverse neonatal outcomes in the form of reduction in the newborn births weight. This can be attributed to the decreased uteroplacental blood flow, and hypoxic stress to the fetus and placenta, also to the poor vascular remodeling and the induced placental and endothelial damage<sup>(40)</sup>.

The finding of significant positive correlation between anti-angiogenic factor sFlt-1 concentration and protein/creatinine ratio occur due to direct effects of sflt-1 on renal tissue that might lead to glomerular endotheliosis<sup>(41)</sup>. Aggarwal *et al* (2011) showed an abnormally increased production of sFlt-1 and sEng, two powerful antiangiogenic molecules, by preeclamptic placentae. The sFlt-1 and sEng are believed to exert their pathogenic effects by limiting the availability of their pro-angiogenic ligands (PlGF, VEGF and transforming growth factor- $\beta$ ) to their native cell surface binding partners on the endothelium. This angiogenic imbalance is believed to induce endothelial dysfunction, systemic vasoconstriction, hypertension and proteinuria.

There were very high significant decreases in the means of sflt-1 in preeclamptic groups (mild and severe PE) one month post-delivery compared with the mean sFlt-1 concentration of those groups at late gestational period. This observation can be explained by the postpartum removal of the placenta which represent the major source of sFlt-1 in which placental syncytiotrophoblasts and in particular syncytial knots were identified as a major source of sFlt-1<sup>(42)</sup>.

In conclusion, the association between anti angiogenic factor soluble fms-like tyrosine kinase-1 (sFlt-1) during pregnancy and one month post-delivery period stress its role in the pathogenesis of PE and could be used to predict the disease progression and impact on the outcome of pregnancy, also there were significant association of preeclampsia and sflt-1 with low neonatal weight.

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## Author contributions

Dr Hasan put the study concept and design; Dr. Al-Rubayae did the physical examination, diagnosis, and the clinical follow up of cases; and Hussein collect blood samples, did the laboratory analyses and preparation of the manuscript.

## Conflict of interest

None

## Funding

None

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Corresponding to Dr. Radwan M. Hussein

E-mail: radwan.asal@yahoo.com

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## Adsorption of Folic Acid on Iraqi Bentonite and Kaolin from Aqueous Solution

Raid J.M. Al-Timimi<sup>1</sup> PhD, Hiba S. Jassim<sup>2</sup> MSc, Ghassan M. Suleiman<sup>2</sup> PhD

<sup>1</sup>Dept. of Chemistry and Biochemistry, College of Medicine, Al-Nahrain University, <sup>2</sup>Section of Biochemical Technology, Dept. of Applications Sciences, University of Technology, Baghdad, Iraq.

### Abstract

**Background** Using specific antidotes for the treatment of acute poisoning that is due to drug overdose by drugs adsorption is of significant importance in physical pharmacy for the preparation of physical antidotes.

**Objective** The adsorption of folic acid from aqueous solution was investigated on two adsorbents (bentonite and Iraqi kaolin).

**Methods** The present study involves studying adsorption of folic acid on Iraqi clays (bentonite and kaolin) from aqueous solution. Adsorption isotherm of folic acid on both surfaces used obeyed Freundlich isotherm. UV- Spectrophotometric technique was used to obtain the quantities of adsorption data at different conditions of temperature.

**Results** The quantities adsorbed of the folic acid for bentonite and kaolin was increased at 37°C. Adsorption characteristics were described using Freundlich isotherm. The value of  $\Delta G^\circ$  showed the spontaneous nature of the folic acid adsorption on both adsorbents.

**Conclusion** This result indicated the surface heterogeneity leading to different adsorption forces from site to site and different affinities toward drug molecules.

Thermodynamic parameters have been calculated at different temperatures. The adsorption of folic acid increases with increase in temperature and positive value of  $\Delta H^\circ$  indicates endothermic nature of the adsorption process. Negative values of free energy at all studied temperatures indicate that the adsorption process is spontaneous and favorable for folic acid. Positive value of entropy suggests increased randomness at the solid/solution interface.

**Keyword** Adsorption, folic acid, bentonite, kaolin, thermodynamic parameters.

### Introduction

Folic acid or folate is a vitamin B<sub>9</sub> soluble in water, identified as an anti-anemia and growth factor. It is produced by plants (green leaves, algae) and micro-organisms (bacteria, yeast). In mammals, folic acid and its derivatives, serve as acceptors and donors of carbon units and are involved in amino acid and nucleotide biosynthesis, also prevents neural tube defects such as spina bifida<sup>(1)</sup>, in addition the action of mechanisms of folic acid acts role as a methyl donor in a range of metabolic and nervous system

biochemical processes, as well as being necessary for DNA synthesis.

Vitamin B<sub>9</sub> is essential for numerous bodily functions. Humans cannot synthesize folates *de novo*; therefore, folic acid has to be supplied through the diet to meet their daily requirements. The human body needs folate to synthesize DNA, repair DNA, and methylate DNA as well as to act as a cofactor in certain biological reactions.

There is a complex interaction between folic acid, vitamin B<sub>12</sub> and iron. A deficiency of one may be "masked" by excess of another so the

three must always be in balance <sup>(2)</sup>. The risk of toxicity from folic acid is low, because folate is a water-soluble vitamin and is regularly removed from the body through urine. One potential issue associated with high dosages of folic acid is that it has a masking effect on the diagnosis of pernicious anemia (vitamin B<sub>12</sub> deficiency), and a variety of concerns of potential negative impacts on health. Drug poisoning has been defined as a condition produced by any substance which when swallowed, inhaled, injected, or absorbed precutaneously is capable of causing death, injury, toxic reactions. One of perspective methods for emergency treatment of accidental poisoning by drug is adsorption.

In cases where no specific antidotes exist, prevention of further adsorption of a drug from the oral route is by use of oral adsorbents. Using adsorption method, the adsorbents have to answer to number of requirements, to be active, stable, and accessible cheap, and the most important is that the exchange ions should be harmless. The use of natural adsorbents clays in the prevention of further adsorption of drug, are recognized in clinical practice.

The thermodynamic parameters associated with the adsorption processes can be calculated from the variation of thermodynamic equilibrium constant (K) with change in temperature, during the adsorption reaction, at pH=8.

Adsorption is usually a decrease in free energy change and entropy of the system. This process is affected by a number of factors such as concentration of adsorbate, surface area of adsorbent, temperature, ionic strength, pH <sup>(3)</sup>, solubility of adsorbate in water and hydrophilic characteristics i.e. the effect of hydrogen bond. The increase in the solubility of solute in water and hydrophilic properties causes a decrease in the adsorbed amount <sup>(4)</sup>.

The adsorption at different types of adsorbents such as kaolin, activated charcoal, attapulgite, talc, magnesium trisilicate and bentonite has been studied <sup>(3,5)</sup>. The Kaolin (high adsorption

capacity) and bentonite can reduce both undesirable and desirable components, such as aroma and flavor compounds <sup>(5,6)</sup>.

Therefore, the Bentonite is considered as a good adsorbent material for heavy metal ions such as Pb<sup>2+</sup> in aqueous solution due to its typically elevated surface area, high availability and low cost <sup>(7)</sup>. Bentonite consists of one octahedral alumina sheet lying between two tetrahedral layers of silica. The negative charge of bentonite is attributed to the isomorphs replacement of Al<sup>3+</sup> for Si<sup>4+</sup> in the tetrahedral layer and Mg<sup>2+</sup> for Al<sup>3+</sup> in the octahedral layer <sup>(8)</sup>.

The adsorption data are tested for a number of isotherm equations (Langmuir equation and Freundlich equation). Moreover, the current study has been to visualize the pattern of adsorption of this drug on the two different adsorbents to various situations such as, pH, temperature, and contact time.

Langmuir behavior assumes a rapid reversible adsorption, and interaction only between adsorbate molecules and a surface site of adsorbent.

The Langmuir equation could be expressed as:

$$C_e/Q_e = 1/K + a/k \cdot C_e \dots\dots\dots(1)$$

Where Q<sub>e</sub> is the amount of adsorbate (mg/g), C<sub>e</sub> is the equilibrium concentration (mg/L), and a, k are constants related to adsorption capacity and energy of adsorption respectively or sometimes called Langmuir constants.

Freundlich Isotherm is one of the most important isotherms that deal with adsorption at solid-liquid interface. Most of surfaces are heterogeneous, so the change in potential energy is regular, and the adsorption sites are not equivalent in energy hence the multilayer formation is highly expected.

Freundlich equation could be written as follows:-

$$Q = k_F C_e^{1/n} \dots\dots\dots(2)$$

$$\log Q = \log k_F + 1/n \log C_e \dots\dots\dots(3)$$

Where Q is the adsorbate quantity (mg/g), C<sub>e</sub> is the concentration of adsorbate at equilibrium (mg/L), k<sub>F</sub>, and n is the adsorption capacity and

an empirical parameter, respectively and also called Freundlich constants<sup>(9)</sup>.

## Methods

The instruments used were UV - VIS Spectrophotometer (UV-1800) Shimadzu, thermo stated Shaker bath/GFL (D-3006), Germany, pH Meter/HM -73, TDA Electronics Ltd., Centrifuge / eppendorf 5804 R, electronic Balance/Sartorius Lab. BP 3015. The material used is NaOH (Emscope laboratories Ltd). The drug used was folic acid that is obtained (Hopkin & Williams, Ltd.) England. The molecular formula of folic acid is  $C_{19}H_{19}N_7O_6$ , and its molecular weight is 441.4. It has a melting point of  $250^{\circ}\text{C}$ , a density of  $1.68\text{ g/cm}^3$ . Folic acid is practically insoluble in water. It dissolves in diluted acids and in alkaline solutions<sup>(10)</sup> (Fig. 1).

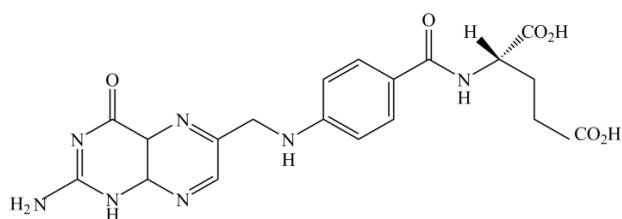


Fig. 1. Structural formula of folic acid<sup>(10)</sup>.

## Adsorbents

Iraqi kaolin (Fig. 2) was obtained from (Dwaikhla) opened mine (north of Rutba) in the Iraqi Western desert supplied by the "General Company for Geological Survey and Mining", Baghdad, Iraq.

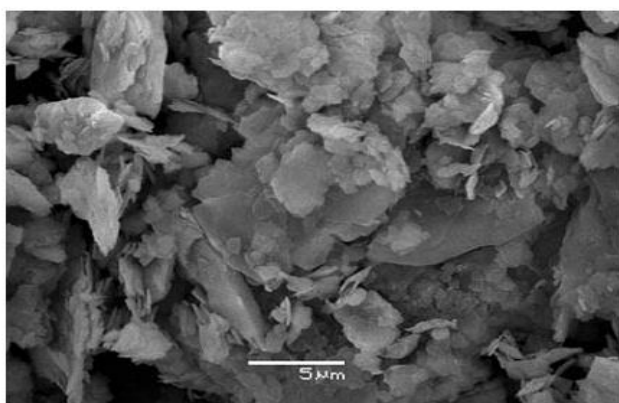


Fig. 2. The shape characterization of kaolin<sup>(3)</sup>

The weight percentages of the Iraqi kaolin clay were:  $\text{SiO}_2$  (54.68%),  $\text{Al}_2\text{O}_3$  (30.19%),  $\text{Fe}_2\text{O}_3$  (1.02%),  $\text{TiO}_2$  (1.00%) and loss on ignition (10.94%).

## Bentonite

The bentonite clay (Fig. 3) size  $75\ \mu\text{m}$  used in this study was supplied by Geological scanning company and has the following composition (by percentage weight):  $\text{SiO}_2$  (56.77%),  $\text{Al}_2\text{O}_3$  (15.67%),  $\text{Fe}_2\text{O}_3$  (5%),  $\text{CaO}$  (4.48%)  $\text{MgO}$  (3.42%),  $\text{K}_2\text{O}$  (0.60%),  $\text{Na}_2\text{O}$  (1.11%), L.O.I (12.49%).

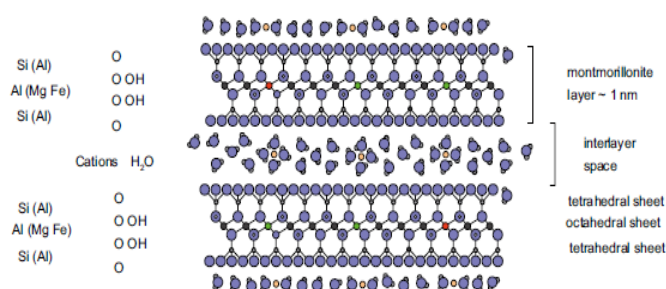


Fig. 3. The Structural formula of bentonite<sup>(12)</sup>

Kaolin and Bentonite adsorbents were in powder form. Each of them was washed several times with excessive amounts of distilled water then dried at ( $170^{\circ}\text{C}$ ) in the oven for three hours and kept in airtight containers. Each adsorbent was ground and sieved using Retch test sieve  $150\ \mu\text{m}$ . A stock of (500ml) aqueous solution of folic acid drug ( $0.0005\ \text{mg L}^{-1}$ ) was prepared and its ( $\lambda\ \text{max}$ ) was determined. The maximum absorbance ( $\lambda\ \text{max}$ ) was (210) nm (Fig. 4).

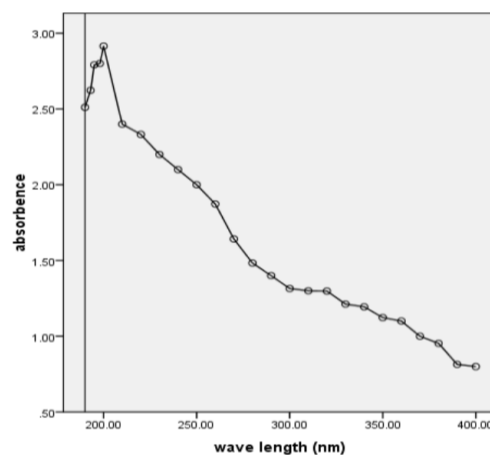
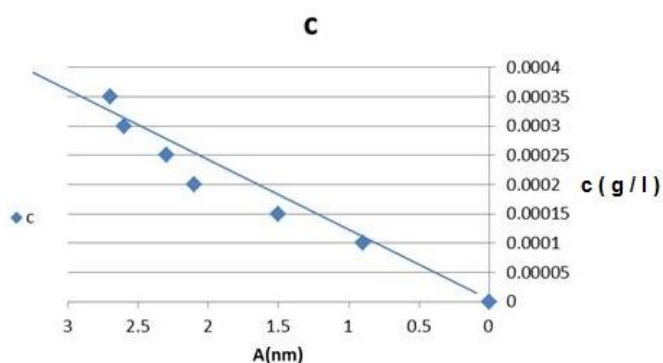


Fig. 4. UV Spectra of aqueous solution of folic acid at pH=8 and temperature  $37^{\circ}\text{C}$

Various drug solutions with different concentrations were prepared by diluting the stock solution with distilled water (0.0001, 0.00015, 0.0002, 0.00025, 0.0003 and 0.00035 mgL<sup>-1</sup>).

In order to obtain the calibration curve for aqueous solutions of Folic acid at pH = 8 the absorbance values of these drug solutions were measured at the specific ( $\lambda_{max}$ ) using UV-Vis double beam Spectrophotometer and plotted versus the concentrations of these drug solutions (Fig. 5).



**Fig. 5. Calibration curve for aqueous solutions of Folic acid at pH=8 and temperature 37°C.**

The time to reach equilibrium state, that is required for full saturation of adsorbent surface at 37°C by the adsorbate has been determined by the following procedure: 500 ml initial concentration (0.0005 mg/L) of adsorbate solution was shaken with (0.5 g) of each adsorbent. The absorbance of adsorbate solutions were measured by UV/Visible spectrophotometer at different intervals 10, 20, 30, 60 ...minutes until reaching equilibrium (no further uptake of adsorbate by adsorbent as the time proceeds).

A systematic procedure was followed to determine the adsorption isotherms for each pair of adsorbent -adsorbate systems. A volume of (50ml) of six different concentrations of drug solution (0.0001, 0.00015, 0.0002, 0.00025, 0.0003 and 0.00035 mg/L) was shaken with (0.5 g) of adsorbent at a certain temperature in a thermostatic shaker. The speed of shaking was 60 cycles per minute. After the equilibrium time (30 min) elapsed,

the mixtures were allowed to settle and the clear liquids were centrifuged at 3500 round per minute (rpm) for 20 minutes. The absorbencies of the filtrate solutions were measured at ( $\lambda_{max}$ ). The equilibrium concentrations of the prepared solutions can be determined from the calibration curve using their absorbencies. Adsorbed amount of the drug was calculated at certain conditions from the concentration of solution before and after adsorption according to equation (1):

$X_m = (C_o - C_e) V / m$ .....(1), where  $C_o$  and  $C_e$  are the initial and equilibrium concentrations of drug solution (mg/L) respectively,  $V$  is the volume of solution in liter,  $X_m$ = the maximum quantity of adsorbate (in mg) that is adsorbed on the adsorbent at certain value of  $C_e$  that was fixed for all temperatures used in the study, ( $m$ ) is the weight of adsorbent in grams.  $X_m$  can be determined from equation (2):

$Q_e = X_m / m$ .....(2), where  $Q_e$ = is the quantity of adsorbate (in mg) held by (0.5 g) of adsorbent.

The equilibrium constant ( $k$ ) for the adsorption process at each temperature is calculated from equation (3):

$K = (Q_e) (0.5 \text{ g}) / (C_e) (0.05 \text{ L})$ .....(3), where (0.5 g) represents the weight of the clay that has been used, (0.05 liter) represents the volume of the drug solution used in the adsorption process.

The change in free energy ( $\Delta G^\circ$ ) could be determined from equation (4):

$\Delta G^\circ = - RT \ln K_c$  .....(4), where  $R$  is the universal gas constant (8.314 kJ/mol K),  $T$  is the temperature (K) and  $K_c$  is the distribution coefficient. Gibbs free energy change of adsorption ( $\Delta G^\circ$ ) was calculated using  $\ln K_c$  values for different temperatures.

The heat of adsorption ( $\Delta H^\circ$ ) may be obtained from equation (5):

$$\ln X_m = -\Delta H^\circ / RT + \text{constant} \text{ .....(5)}$$

The change in entropy ( $\Delta S^\circ$ ) can be determined from equation (6):

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ \text{ .....(6)}$$

## Results

### Temperature effects and thermodynamic parameters

The general shapes of the adsorption of folic acid on kaolin and bentonite at three different temperatures (30, 37 and 40°C) are given in fig. 8 and 9. Figures show that the adsorption of folic acid increases at 37°C temperature.

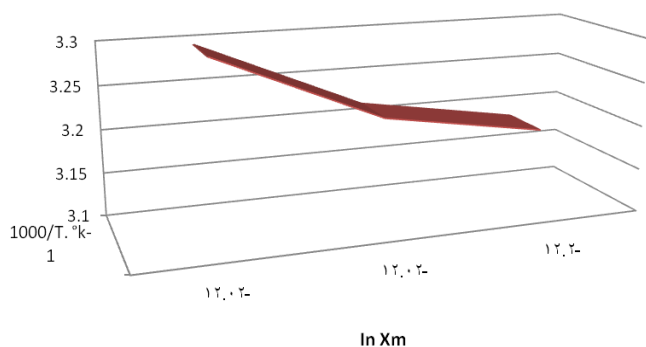
The study of the temperature effects on adsorption helps in finding the basic thermodynamic functions ( $\Delta H^\circ$ ,  $\Delta G^\circ$ ,  $\Delta S^\circ$ ) of the

adsorption processes. Table (1) gives  $X_m$  values at different temperatures at pH=8. Most of the absorption process after digestion process ends at the small intestine pH = 8. Table 1 shows the effect of temperature on the maximum adsorbed quantities of folic acid on kaolin and bentonite; where  $X_m$  is the maximum uptake of adsorbate at certain value of ( $C_e$ ) for all temperatures.

**Table 1. Effect of temperature on the maximum adsorbed quantities of folic acid on kaolin and bentonite**

Adsorbent	T.°C	T.k	1000/T. k <sup>-1</sup>	X <sub>m</sub> (mg)	ln X <sub>m</sub>
Kaolin	30	303	3.30	0.000006	-12.02
	37	310	3.22	0.000006	-12.02
	40	313	3.19	0.000005	-12.20
Bentonite	30	303	3.30	0.000005	-12.20
	37	310	3.22	0.0000065	-11.94
	40	313	3.19	0.000006	-12.02

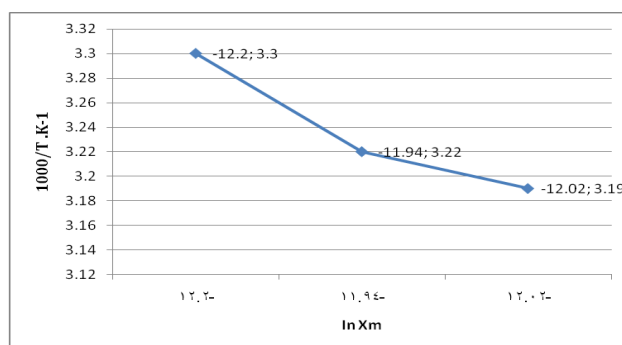
Plotting ( $\ln X_m$ ) versus  $1000/T$  produced a straight line with a slope =  $-\Delta H/R$  as shown in fig. 6 and 7.



**Fig. 6.  $\ln X_m$  plotted against reciprocal absolute temperature for the adsorption of folic acid on kaolin at pH=8 and different temperatures (30, 37 and 40°C).**

Table 2 shows the basic thermodynamical values of adsorption of folic acid on kaolin and bentonite. The negative values of the  $\Delta G$  for the adsorption of folic acid on bentonite and kaolin indicated that the adsorption process of folic acid is spontaneous. The positive values of

$\Delta H$  at different temperature indicated an endothermic reaction. The positive values of  $\Delta S$  for the adsorption of folic acid on bentonite indicated increase in the degree of freedom of the adsorbed species.



**Fig. 7.  $\ln X_m$  plotted against reciprocal absolute temperature for the adsorption of folic acid on bentonite at pH=8 and different temperatures (30, 37 and 40°C).**

### Adsorption isotherms

Adsorption isotherm is the equilibrium relationship between the concentration in the liquid phase and the concentration in the

adsorbent phase in the adsorbent particles at given temperature. The experimental data obtained are analyzed by the adsorption isotherms of folic acid on bentonite and kaolin at pH=8 and different temperatures. In the present study, Freundlich models were used to analyze the data. The adsorption isotherm in fig. 8 and 9 indicated a frundlich isotherm according on Giles classification.

Figures 10 and 11 show a linear relationship between  $(\log Q_e)$  and  $(\log C_e)$ , which indicates that the surface of adsorbent bentonite and kaolin are heterogeneous in character and involve the formation of multilayers.

**Discussion**

The adsorption system can be defined as an equilibrium one including the adsorbent being in contact with the bulk phase and also called interfacial layer<sup>(13)</sup>.

The most important in the investigation of adsorption mechanism is the equilibrium adsorption isotherm. The properties such as surface property, the adsorption affinity of adsorbent and the maximum adsorption capacity can be determined from the adsorption isotherm and correlative constants<sup>(4)</sup>.

Bentonite and kaolin is the clay mineral that was chosen as adsorbent for this study with folic acid as show in figure 1 have( C=O and NH) groups can be adsorbed on different site of each adsorbents.

From adsorption isotherms it can be concluded from Giles classification is S type depend on Freundlich Isotherm used to describe the adsorption characteristics for the heterogeneous surface and the formation of multilayers<sup>(14,15)</sup>, this also indicates that bentonite surface is heterogeneous<sup>(15)</sup>.

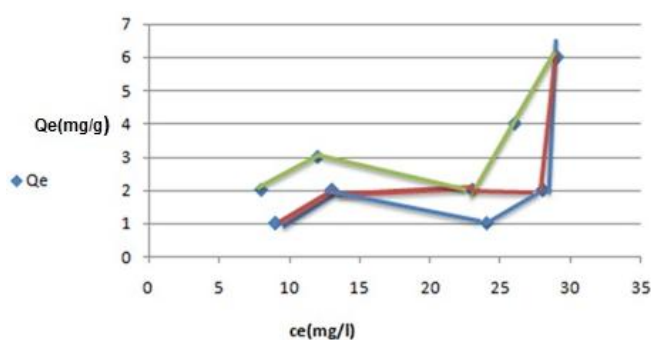
**Table 2. Values of thermodynamic functions for the adsorption of folic acid on Bentonite and kaolin at temperatures.**

Adsorbent	$\Delta H^\circ$ kJ.mol-1	$\Delta G^\circ$ kJ.mol-1	$\Delta S^\circ$ J.mol-1	pH	Temperature °C
Kaolin	+30281	-1007.65	+103.26	8	30
	+30981	-1030.93	+103.26	8	37
	+31748	-234.20	+102.17	8	40
Bentonite	+30734	-226.72	+102.18	8	30
	+30774	-180.41	+99.85	8	37
	+31280	-182.15	+100.51	8	40

It was found that the adsorption processes between the folic acid and negative site of hydroxyle group in the bentonite site surface<sup>(16)</sup>, is attributed to the isomorphous replacement of  $Al^{3+}$  for  $Si^{4+}$  in the tetrahedral layer and  $Mg^{2+}$  for  $Al^{3+}$  in the octahedral layer. This negative charge is balanced by the presence of replaceable cations ( $Ca^{2+}$ ,  $Na^+$ , etc.) in the lattice structure, which enhance adsorbing cationic pollutants<sup>(8)</sup>.

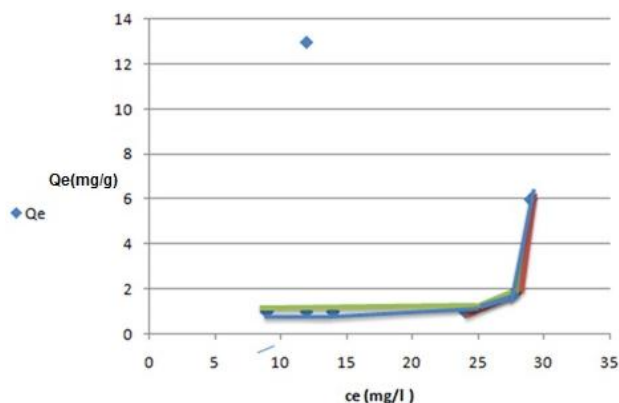
The surface of the adsorbent becomes negatively charged which enhances the adsorption of positively charged functional groups of the adsorbate through the

electrostatic force of attraction on the surface<sup>(17)</sup>.



**Fig. 8. Adsorption isotherms of folic acid on kaolin at pH=8 and different temperatures (30, 37 and 40°C).**

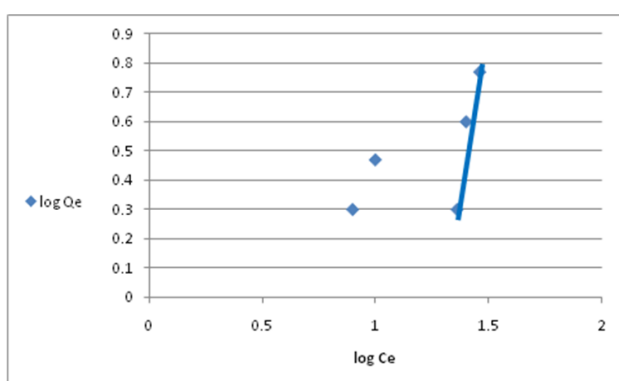
Under alkaline solution at pH=8 the chemical structure of bentonite varies with the variation in pH. The natural bentonite is a net negative charge on the surface as pH reduces<sup>(15)</sup>, while the kaolin clay carries the negative and positive charges<sup>(18)</sup>.



**Fig. 9. Adsorption isotherms of folic acid on bentonite at pH=8 and different temperatures (30, 37 and 40°C).**

Spontaneity of a adsorption process can be determined by thermodynamic parameters such as free energy change ( $\Delta G^\circ$ ), enthalpy change ( $\Delta H^\circ$ ) and entropy change ( $\Delta S^\circ$ )<sup>(19)</sup>.

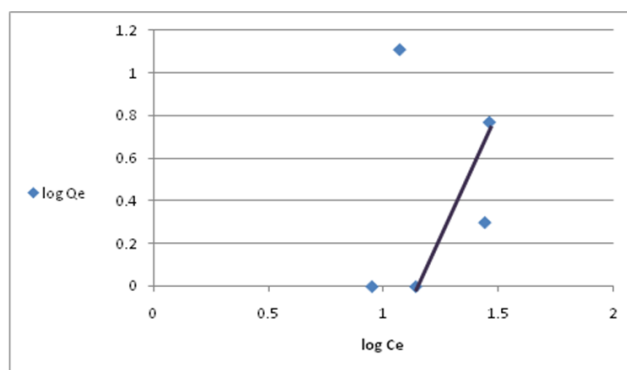
The negative values of  $\Delta G^\circ$  indicate the feasibility and spontaneity of adsorption process. Further, the decrease in the values of  $\Delta G^\circ$  with the increasing temperature indicates the spontaneity of the process at higher temperatures<sup>(20)</sup> which does not require an external energy source for the system<sup>(19)</sup>.



**Fig. 10. Linear form of Freundlich isotherm of folic acid adsorbed on kaolin at 37°C and pH=8.**

The positive values of enthalpy change ( $\Delta H^\circ$ ) which indicate endothermic nature<sup>(20)</sup> was also confirmed from the increase of adsorbate adsorption efficiency on adsorbents as the temperature increased<sup>(19)</sup>.

the positive value of  $\Delta S^\circ$  indicate a good affinity of adsorbate towards the adsorbent materials<sup>(20)</sup>, that there is an increased at the solid/liquid interface during adsorbate adsorption onto the adsorbents and drug molecules remain randomly on the each surface<sup>(19,17)</sup>.



**Fig. 11. Linear form of Freundlich isotherm of folic acid adsorbed on bentonite at 37°C and pH=8.**

In conclusion, Iraqi kaolin and bentonite showed ability to adsorb the folic acid drug from its aqueous media; therefore, both adsorbents can be used as antidotes for dealing with a case of drug overdose. Moreover, adsorption isotherms of the drug folic acid on Iraqi kaolin and bentonite depend on Freundlich isotherm model. These results indicated that the surface heterogeneity of the adsorbents leading to different adsorption strengths from site to site and different affinities towards drug molecules. Thermodynamic parameters such as enthalpy change ( $\Delta H^\circ$ ), free energy change ( $\Delta G^\circ$ ) and entropy change ( $\Delta S^\circ$ ) showed that the adsorption process of folic acid on each adsorbents was endothermic and spontaneous.

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### Author contribution

Both authors share the responsibility in preparing and completing this work.

### Conflict of Interest

The authors declare no conflicts of interest.

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Correspondence to Dr. Raid J.M. Al-Timimi

E-mail: rjtimimi68@yahoo.com

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## Evaluation of Pediatric Head Injuries

Ihssan S. Nema *MBChB FIBMS*

Section of Neurosurgery, Dept. of Surgery, College of Medicine, Al-Nahrain University, Baghdad, Iraq.

### Abstract

<b>Background</b>	Trauma is a leading cause of death in children older than one year in the world; with head trauma representing 80% or more of the injuries. Most head injuries in children occurs secondary to motor vehicle accidents, falls, assaults, recreational activities, and child abuse.
<b>Objectives</b>	To evaluate head trauma in pediatrics age group regarding age, sex, presenting symptoms and signs and the line of management; to know the role of CT scan in assessment of pediatrics head trauma, and to determine the extent of pediatric head trauma in Baghdad city.
<b>Methods</b>	Fifty neurosurgical pediatrics cases with head trauma between the age of one year to fourteen years whom presented to the neurosurgical ward in Al-Imamain Al-kadhimain Medical City. Questioner for gender, age, mechanism of injury, presented signs and symptoms, consciousness, CT scan findings, and the line of management.
<b>Results</b>	The study showed that males have the higher incidence with 72%. Head injury due to road traffic accident accounting for 44%. The loss of consciousness was documented in 60% of the cases. All patients subjected to CT scan imaging modality for assessing head injury as the ideal way. The presence of hematoma was seen in 68% of the cases. Associated injuries were seen in 60% of cases. Vomiting as an important symptom in pediatrics head trauma encountered in 52% of the patients. The developments of fits are very important sequel encountered in 16%. The close monitoring and follow up being the most common line of management accounting for 88%.
<b>Conclusion</b>	Pediatrics head trauma is a common problem with males have higher incidence than females. The most common mechanism of injury was road traffic accident. CT scan is a very vital and important diagnostic tool in evaluating patient with head trauma.
<b>Key words</b>	Pediatrics, head trauma.

**List of abbreviation:** HT = head trauma, ICH = intracranial hemorrhage, PGCS = Pediatric Glasgow Coma Scale, CT scan = computerized tomography scan, CSF = cerebrospinal fluid, RTA = road traffic accident.

### Introduction

Trauma is a leading cause of death in children older than one year in the world, with head trauma (HT) representing 80% or more of the injuries. Patients with HT may experience one or a combination of primary injuries, including scalp injury, skull fracture, basilar skull fracture, concussion, contusion, intracranial hemorrhage (ICH), subarachnoid hemorrhage, epidural

hematoma, subdural hematoma, intraventricular hemorrhage, penetrating injuries, and diffuse axonal injury<sup>(1)</sup>. The secondary injury is represented by systemic and intracranial events that occur in response to the primary injury and further contribute to neuronal damage and cell death<sup>(2)</sup>.

The distribution of HT is relatively stable throughout childhood. An increase in the incidence of HT was identified in two age groups. At approximately age 15 years, a dramatic increase occurs, mainly in males, related to their involvement in sports and driving activities. Infants younger than 1 year

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also have an elevated incidence of HT, which is attributed to falls and child abuse <sup>(3)</sup>. The overall outcome for children with head injuries is better than that for adults with the same injury scores <sup>(4-6)</sup>.

Outcome assessment based on the Pediatric Glasgow Coma Scale (PGCS) can be used as an early predictor, but this scale has limitations regarding long-term outcome. Mechanism of injury appears to be a significant predictor of clinical and functional outcomes at discharge for equivalently injured patients <sup>(7)</sup>.

Seizures are more commonly observed with contusions (more so with subdural hematoma than with epidural hematoma), depressed skull fracture, and severe head injury (PGCS score, 3-5).

Other complications include leptomenigeal cyst, meningitis, cranial nerve injury, post traumatic syndrome and transient cortical blindness <sup>(8)</sup>. Computed tomography (CT) of the head remains the most useful imaging study for patients with severe HT or unstable multiple organ injury <sup>(9,10)</sup>.

Indications for CT scanning in a patient with a head injury include posttraumatic seizures, progressive headache, an unreliable history or examination because of possible alcohol or drug ingestion, loss of consciousness for longer than 5 minutes, physical signs of basilar skull fracture, repeated vomiting or vomiting for more than 8 hours after injury, and instability after multiple trauma <sup>(11)</sup>.

### **Methods**

A cross-sectional study of 50 cases of closed pediatric HT between the ages of 1 year to 14 years whom present to the neurosurgical ward, Al-Imamain Al-Kadhmain Medical City from 1<sup>st</sup> of December 2012 to the 1<sup>st</sup> of May 2013. Questioner about the sex, age, mechanism of the injury, presented signs and symptoms, consciousness level, CT scan findings, and the line of the management.

Vomiting consider seriously in such patient, because it can be an important sign of raised intracranial pressure. Observation for any

watery nasal or ear discharge can give clue to dural laceration with resultant cerebrospinal fluid (CSF) leak. Periorbital swelling and discoloration or post auricular bruises can indicate basilar skull fracture. Focal neurological can indicate hemispheric or brain stem compromised.

All patient managed with first aid measures (airway, respiration and circulation), those whom need surgical intervention managed urgently in the emergency theater, and others with no surgical findings admitted to the neurosurgical ward and close observation ensured. Antiepileptic and antibiotics were given for those with high risk for seizures and infection respectively.

By using the SPSS (Statistical Package for the Social Sciences) the categorical data formulated as frequency and percentage. Chi-square test describes the association of these data. The lower level of accepted statistical significant difference is bellow or equal to 0.05.

### **Results**

A total of 50 pediatric head trauma were enrolled in this study. They were 72% male and 28% female. The age range was between 1-14 years, mean age 4.4 years.

The mechanism of the of most head injury in this study were due to road traffic accident accounting for 44% while fall from height was 42% and trauma or fall of a heavy object were 14%. The loss of consciousness in this study was documented from the history in 60% of the cases while those with no history loss of consciousness were 40% (Table1).

The presence of hematoma was seen in 68% of the cases as seen in table (2). The presence or the development of associated signs like Raccoon eyes which is found in 32% of the cases and battle sign which is found in 16% of the cases which may point to the presence of basilar skull fracture.

Basilar skull fracture may cause CSF to leak from the nose (CSF rhinorrhea) alone or with blood, which is seen in 12% (6 cases). Basilar skull fracture may cause CSF to leak from the

ear with hemotympanum or bleeding from the ear which is encountered in 8% (4 cases). In this study 52% of the patients had vomiting all were projectile in type (Table 3).

**Table 1. Descriptive statistics of the presenting state of consciousness**

Consciousness	Frequency	%	Valid %	Cumulative %
Loss	30	60.0	60.0	60.0
No	20	40.0	40.0	100.0
Total	50	100.0	100.0	

**Table 2. Descriptive statistics of the C-T scan findings**

CT scan finding	CT fracture		Total
	Yes	No	
Hematoma	20	14	34
No hematoma	12	4	16
Total	32	18	50

**Table 3. Associated symptoms and signs**

Symptoms or sign	Frequency / out of 50	%	Valid %	Cumulative %
Vomiting	26	52.0	52.0	52.0
Raccoon eye	16	32.0	32.0	84.0
Battle sign	8	16.0	16.0	100.0
Rhinorrhea	6	12.0	12.0	100.0
Otorrhea	4	8.0	8.0	100.0
Extracranial injuries	8	16.0	16.0	100.0
Focal neurological sign	2	4.0	4.0	100.0
fit	8	16.0	16.0	100

In this study CT scan showed that 64% (32 cases) had skull fracture while 36% (18) had no skull fracture. Forty eight percent of the cases in this study had linear fracture while 16% had depressed fracture (Table 4).

**Table 4. Descriptive statistics of the type of the skull fracture**

Skull fracture	Frequency	%	Valid %	Cumulative %
Linear	24	48.0	48.0	48.0
Normal	18	36.0	36.0	84.0
Depressed	8	16.0	16.0	100.0
Total	50	100.0	100.0	

The presence of hematoma was seen in 68% of the cases. The most common site of hematoma encountered is the subgaleal (extra cranial) which was seen in 40% of the cases either alone mostly or in association with other intracranial hematoma the second most

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common hematoma was the subdural type which is seen in 16% of the cases either alone or with extradural hematoma and then the intracerebral hematoma in 12% either alone or

with subgaleal hematoma and then is the extradural hematoma either alone or in with subdural hematoma accounting for 8% of the cases (Table 5).

**Table 5. Descriptive statistics of the site of hematoma**

Site	Frequency	%	Valid %	Cumulative %
Normal	16	32.0	32.0	32.0
Subgaleal	16	32.0	32.0	64.0
Subdural	6	12.0	12.0	76.0
ICH	4	8.0	8.0	84.0
Extradural and Subdural	2	4.0	4.0	88.0
Extradural	2	4.0	4.0	92.0
Subgaleal and Extradural	2	4.0	4.0	96.0
Total	50	100.0	100.0	100.0

The close monitoring and follow up being the most common accounting for 88% ,while surgical option was done in only 8% of the

cases (4cases) three of them had depressed fracture and the other had a big extradural hematoma. Two patients died (Table 6).

**Table 6. Descriptive statistics of the line of management and outcome**

line of management and outcome	Frequency	%	Valid %	Cumulative %
Follow up	44	88.0	88.0	88.0
Surgery	4	8.0	8.0	96.0
Dead	2	4.0	4.0	100.0
Total	50	100.0	100.0	

### Discussion

Pediatric head trauma is a common problem and is commonly seen in the neurosurgical ward of hospitals.

In this study the age of the patients presented with closed head trauma was between 1 to 14 year with the mean age is 4.42, while in a same study conducted in the USA by the American pediatric association the mean age was 8.9 years an older mean age than our study<sup>(12)</sup>.

The sex distribution in our study shows that the males have the higher incidence with 72% (36 cases out of 50) while in females it was 28% (14 out of 50) as the boys are more playful than girls and have more outside activities that make them more prone to RTA. The same study in the US showed also a higher male

incidence with 64% and the females were 36%<sup>(12)</sup>.

The mechanism of the of most head injury in this study were due to road traffic accident accounting for 44% while fall from height was 42% and trauma or fall of a heavy object were 14% as these percentages are relatively equal to study published in pediatrics in review 2012<sup>(13)</sup>.

The loss of consciousness in this study was documented from the history in 60% of the cases while those with no history loss of consciousness were 40% (Table1), for a period lasted from 5 minutes to about 2 days, which shows a higher incidence of loss of consciousness than a study in the USA was made by the Traumatic Brain Injury Study

Group of the Pediatric Emergency Care Applied Research Network<sup>(14)</sup>.

All patients in this study had undergone C-T scan, the ideal imaging modality for assessing head injury which can detect both intracranial injury and skull fracture reliably. In this study C-T scan showed that 64% (32 cases) had skull fracture while 36 % (18) had no skull fracture. Forty eight percent of the cases in this study had linear fracture while 16% had depressed fracture (Table 4).

In comparison to study made in the USA, fractures were encountered in CT scan in 52% of the cases 39% were had linear fracture and 13% had depressed fracture<sup>(15)</sup>.

The presence of hematoma was seen in 68% of the cases while in another study in the USA the percent was 65%<sup>(15)</sup> which is close to it.

The most common site of hematoma encountered is the subgaleal (extra cranial) which was seen in 40% of the cases either alone mostly or in association with other intracranial hematoma the second most common hematoma was the subdural type which is seen in 16% of the cases either alone or with extradural hematoma and then the intracerebral hematoma in 12% either alone or with subgaleal hematoma and then is the extradural hematoma either alone or in with subdural hematoma accounting for 8% of the cases (Table 5).

In this study patients who had another injury elsewhere in their body were about 16% which varied from single fracture to multiple fractures to abdominal visceral injury most of them are due to RTA (Table 3), and this agreed with a study done in Nationwide Children's Hospital in Columbus, Ohio and Rainbow Babies and Children's Hospital in Cleveland, Ohio<sup>(2)</sup>.

The presence or the development of associated signs like Raccoon eyes which is found in 32% of the cases and battle sign which is found in 16% of the cases which may pointed to the presence of basilar skull fracture (Table 3), these result agreed with a study published by Ringl et al<sup>(10)</sup>.

In addition basilar skull fracture may cause CSF to leak from the nose (CSF rhinorrhea) alone or with blood, which is seen in 12% (6 cases).

Basilar skull fracture may cause CSF to leak from the ear with hemotympanum or bleeding from the ear which is encountered in 8% (4 cases), this is comparable to the result of Ringl et al<sup>(10)</sup>.

Vomiting is an important symptom in pediatric head trauma as it may reflect an increase in the ICP. In this study 52% of the patients had vomiting all were projectile in type varied in frequency from once time only to more than ten time over a period of three days, and this more or less equal to the study done by Traumatic Brain Injury Study Group of the Pediatric Emergency Care Applied Research Network<sup>(14)</sup>.

The development of the fit is a very important encountered in patients with head trauma and the onset of the attack is very important too as the more early the fit is the less likely that the patient will develop epilepsy in the future. In our study 84% of the patients (42 cases), did not develop fit while only 16% (8 cases) developed fit , two of them were immediately after the trauma and two of them within the first 24 hours and the remaining four were after the first 24 hours. Six of the patient who developed fit had depressed fracture as this may cause direct damage to the brain tissue. Most of the fit were of the tonic clonic type, this comparable to Atabaki et al<sup>(15)</sup>.

The subsequent development of focal neurological deficit was only seen in two cases (4%), one of them was having cerebellar ataxia and the second had right upper arm weakness, and these results are more or less equal to the result of Schunk and Schutzman<sup>(12)</sup>.

The line of management decided for each case was directed toward treating the possible brain injury and the extent of the head injury. The close monitoring and follow up being the most common accounting for 88%, while surgical option was done in only 8% of the cases (4 cases) three of them had depressed fracture and the other had a big extradural hematoma.

Two patients died, one after about 12 hours of the presentation, and the second after about two days for unknown reasons and they were referred to the forensic medicine to know the cause, this agrees with study of Haider et al<sup>(7)</sup>.

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### Conflict of Interest

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E-mail: [ihssansubhe2006@yahoo.com](mailto:ihssansubhe2006@yahoo.com)

Phone: + 964 7717515988

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## Immunohistochemical Localization of HNF4 $\alpha$ in the Choroid Plexus of the Rabbit Ventricles with Clinical Implication

Nawras N. Al-Kafagi<sup>2</sup> *MBChB*, Thaer M. Farhan<sup>1</sup> *FIBMS*, Muthanna A. Al-Kaabi *PhD*

<sup>1</sup>Dept. of Anatomy, College of Medicine, Al-Nahrain University, Baghdad, <sup>2</sup>Dept. of Anatomy, College of Medicine, Babil University, Babil, Iraq

### Abstract

- Background** The choroid plexuses are composed of highly differentiated epithelial cells in with connective tissue and permeable capillaries among them. These cells connected by tight junction to form the blood – CSF-barrier that plays important role in protection of CNS. HNF4 $\alpha$  is transcription factor of many drugs transporter proteins and known as nuclear subfamily 2 group alpha encoded by HNF4 alpha gene can be found in the liver, pancreas, intestine, brain and recently in epithelial cell of choroid plexuses.
- Objectives** To evaluation the HNF4 $\alpha$  intensity and localization of the choroid plexus in the lateral and forth ventricles of the rabbit.
- Methods** Choroid plexuses of lateral and forth ventricles of 30 adult male rabbits were studied by morphological and immunohistochemical evaluation of HNF4 $\alpha$  in theses ventricles that play role in regulation of drugs transporters and drugs metabolism in B-CSF-B.
- Results** Histological method showed little different features by subjective examination between various localizations of the choroid plexuses. The immunohistochemical activity of HNF4 $\alpha$  was different between lateral and forth ventricle where the IHC positivity is more in the lateral ventricle (0.067 $\pm$ 0.029) than forth ventricle (0.032  $\pm$ 0.018) and this results is statistically significant.
- Conclusion** The choroidal cells of lateral ventricle showed more IHC activity of HNF4 $\alpha$  and might indicate targeting of many drug transporters proteins, metabolites and eliminate of toxic compounds from brain tissues.
- Key word** Choroid plexuses, HNF4 $\alpha$  in lateral ventricle, drug transporter across the blood brain barrier.

**List of abbreviation:** CP = choroid plexus, CSF = cerebrospinal fluid, HNF4 $\alpha$  = hepatic nuclear factor 4  $\alpha$ , DME = drug metabolism enzyme, H&E = hematoxyline and eosin, IHC = immunohistochemical, ABCB = ATP binding cassette transporter proteins, B-CSF-B = blood CSF barrier, HRP = horse radish peroxidase.

### Introduction

The choroid plexus (CP) consists of highly differentiated vascularized epithelial tissue. It is found in four sites: in the roof of third, fourth ventricle and medial wall of each of lateral ventricle, combined is about 40 cm<sup>2</sup> (1).

Histological evidence of CPs differentiation in several species showed that metencephalic plexuses (forth ventricle) appear first then

telencephalic plexuses (lateral ventricle) and then third ventricle in rabbit and human (2,3).

The CP is multifunctional organ responsible for production of cerebrospinal fluid (CSF) in lateral, third and fourth ventricles by diffusion through the ependymal and pia vessels. In human, it is estimated that 95% of the fluid is formed in the lateral ventricles. Most of the remainder is formed in the third and fourth ventricles (4). Then circulation to subarachnoid space and recycle 4 times per day in order to clean out metabolites and toxins like beta amyloid.

The CP interface between the blood and CSF; it serves as a gateway for immune cell trafficking into the CSF and is in an excellent position to provide continuous immune surveillance by CD4+T cell<sup>(5)</sup>.

The choroidal epithelial cells are closely connected to each other by tight junction and constitute the structural basis of the blood-CSF barrier<sup>(6)</sup>.

Also they produce many substances like transthyretine<sup>(7)</sup>, transferrin<sup>(8)</sup>, in studies on human and rat and growth hormone factor especially during development of CPs cell and in response to brain injury<sup>(9)</sup> in rodent species. HNF4  $\alpha$  (hepatic nuclear factor 4  $\alpha$ ) is zinc-finger protein and known as nuclear subfamily 2 group alpha encoded by HNF4 alpha gene can be found in the liver, pancreas, intestine, brain and recently in epithelial cell of CP, that binds DNA as homodimer plays role in regulation of drug metabolism enzyme (DME) and drug transporters<sup>(10)</sup>, it is required for development of liver and control of many enzymes in liver<sup>(11)</sup>.

The immunohistochemistry study of HNF4 alpha in CP will determine HNF4 alpha –DNA binding activity search transcript expression of various ATP binding cassette (ABC) transporter like (ABCB1, ABCB4, ABCC1) in the CP; this provides evidence of HNF4 alpha to be an important regulator of ABCB drug transport in CPs<sup>(10)</sup>.

HNF4 alpha plays a role in transcriptional control of drug transport<sup>(12)</sup>. Also HNF4 alpha act as a transcription factor of proteins released from CPs in cytoplasm and intracellular like transthyretin<sup>(13, 14)</sup>.

## **Methods**

A total of 30 adult male New Zealand rabbits (*Oryctolagus cuniculus*), animals were sacrificed by deep anesthesia and the skull open dorsally by strong pair of scissors starting from foramen magnum to the nasal bones. After the brain delivered fixated in 10% formalin for 24 hours then take the brain and divided by coronal section into two parts to obtain the CPs of

lateral and forth ventricles, it is difficult to obtain the CPs of third ventricle.

Samples were processed for paraffin blocks, sectioned and then stained with hematoxyline and eosin (H&E) stain for general histological examination according to<sup>(15)</sup>. Paraffin sections on positively charged slides were further stained by anti-HNF4 $\alpha$  antibody.

### **The anti –HNF4 alpha antibody ab 94748 (rabbit polyclonal to HNF 4 alpha)**

- deparaffinize and rehydrate tissue section , then embedding in dry milk 0.5 in pbs solution.
- add enough drops of hydrogen peroxide block to cover the section. Incubate for 10 minutes. wash 2 times in phosphate buffer ph of it 7, then apply protein block and incubate 10 min. at room temperature, then wash 2 times.
- apply primary antibody (anti –HNF4 alpha antibody) diluted as 1/200 by phosphate puffer incubate for 90 min., then wash 2 times.
- apply complement and incubate 10 min. wash 2 times .
- apply HRP conjugate, incubate for 15 min., rinse 4 times in buffer add 1 drope of DAB chromogen to 50 dropes of DAB substrate mixing by swing and apply to tissue incubate 10 min. rinse 4 times .
- apply counter staining (hematoxyllin ) for 7 min., then wash in tabe water for 2 min.
- dehydration and cover slipe with use mounting.

The slides of CPs of lateral and forth ventricle were examined under light microscope and photographic picture was taken and use aperio scope image analysis softt ware positive pixel count (version 9) to measure the intensity of immunehistochemical (IHC) reaction and obtain data.

## **Result**

### **Choroid plexus morphology by H&E stain**

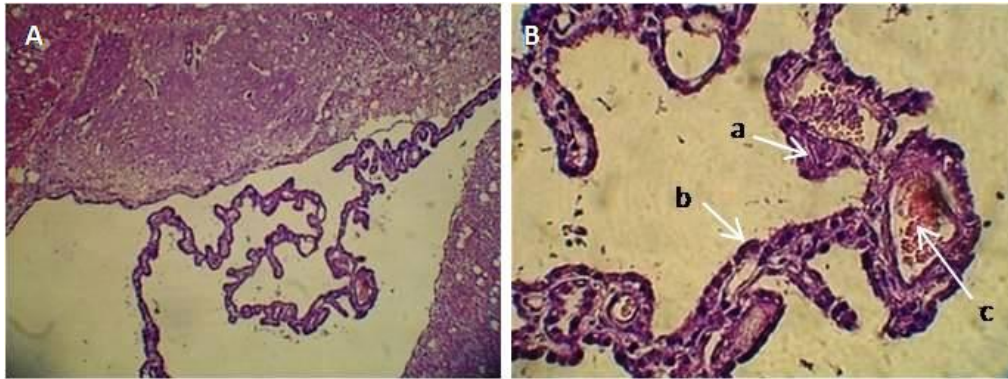
#### **Choroid plexuses of lateral ventricle:**

The CP was seen as cluster of solitary layer of cuboidal to low cylindrical cells with rounded

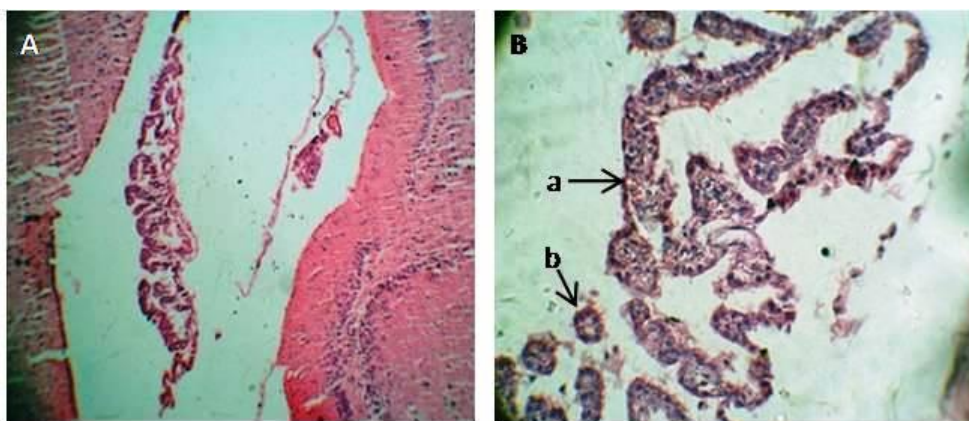


nuclei surrounding vascularised cores rested on a basement membrane, nuclei of endothelial cells of choroidal vessels are flattened also found connective tissue in between vascularized core of CPs. In current study, these clusters of cells, observed with abundant

cytoplasm, the nucleus is larger with obvious nucleolus in the lateral ventricle if compared with forth ventricle and more vessels were observed in the lateral ventricle than forth ventricle and the cells suspended by ependyma in the lateral ventricle as shown in fig. 1 and 2.



**Fig. 1. Coronal sections of cerebral hemisphere with choroid plexuses inside of lateral ventricle by H.&E. show (a) large nucleus, (b) abundant cytoplasm, (c) larger blood vessels if compared with forth ventricle (A -10 X, B-40 X).**



**Fig. 2. Coronal section of brain stem with choroid plexuses inside of forth ventricle by H.&E. stain show (a) round nucleus, (b) less cytoplasm and smaller blood vessels if compared with lateral ventricle (A- 10 X, B- 40X).**

#### **Immunohistochemical identification of HNF 4 alpha in the lateral and forth ventricles:**

The mean intensity of HNF4 alpha was highest in CPs of lateral ventricle and lowest in forth ventricle ( $0.032 \pm 0.018$  Vs  $0.067 \pm 0.029$  ( $p < 0.0001$ )). The IHC staining occurs in the basolateral side of choroidal cell near the lumen of blood vessels with a granular stain occur in this area due to founding of aggregation of transporters proteins across

through the blood CSF barrier (B-CSF-B) as shown in fig. 3.

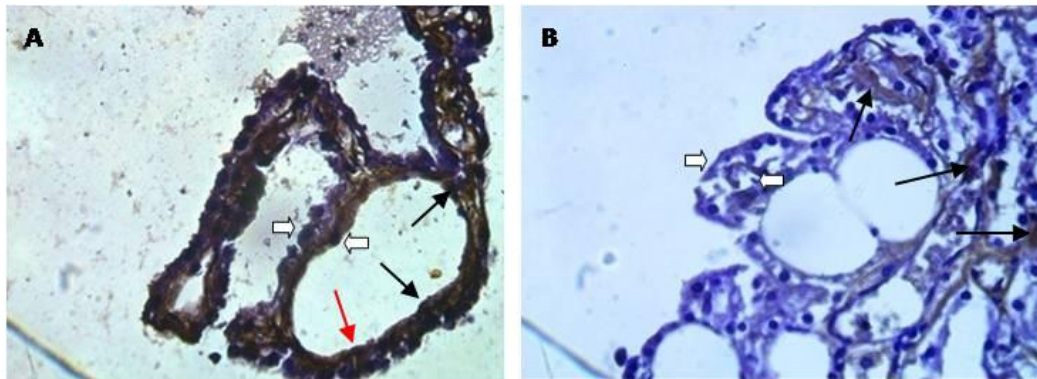
#### **Discussion**

##### **Significance of Immunohistochemical reaction study:**

Previous study on choroid plexuses of lateral, third and forth ventricle were done as a one entity but some authors reported a difference in activity of some enzymes of the lateral ventricle differ from that of forth ventricle <sup>(16)</sup>.

This current study worked to establish the idea that CP of cerebral ventricle is not single entity

by IHC reaction of proliferation process and activity in drug transporter in CSF-B-B.



**Fig. 3. Coronal section of cerebral hemisphere with choroid plexuses inside the (A) lateral ventricle (B) fourth ventricle show expression of anti-HNF4  $\alpha$  in the basolateral site of epithelial cell of choroid plexuses (black arrow), Note that the basal site stained more than apical (white arrow) also the cytoplasm is granular and dark brown (strong positive) as (red arrow) (A & B 40 X).**

The IHC reactivity of CPs of both lateral and fourth ventricles were estimated by using the Aperio software that can detect the cells stained by the marker stain and categorize them into three areas: negative (not show activity), positive, and strong positive. The above is applicable for HNF4  $\alpha$  in the current study; the ependymal cells of CP, endothelial cells are stained by the anti-HNF4  $\alpha$  marker, in addition; other cells stained like RBC and some stromal cells which might give false positive reading and change the real estimation of what the study plane. So to overcome it, the weak positive reading by Aperio should be omitted and excluded and only positive and strong positive reactions are counted in the field of tissue.

In the following manner regarding HNF4 $\alpha$  describe activity as:

The positive stained cells shown as dark and light brown colored which indicate strong and moderate positive reaction respectively and occur more in the basolateral side of choroidal cells near the lumen of blood vessels with a granular stain occur in this area due to the presence of aggregation of transporter proteins across through the B-CSF-B like ABCC, ABCB1, ABCB4, transthyretin, as shown in fig.

3, also HNF 4 $\alpha$  was observed in apical endothelium that means the drug back to blood and this agrees with Monika who said that ABCB proteins help as the protective role of choroid epithelial cells and mediate basolateral efflux of conjugates resulting from CSF drug metabolism into the blood and apical distribution of ABCB1 in apical side of endothelium<sup>(10, 17)</sup>.

Expression of HNF4 $\alpha$  intensity of CP was significantly higher in lateral ventricle (mean of positivity (0.067)) than fourth ventricle (0.032) suggesting that the regulation of drug transporter is more in lateral ventricle, which is not agreed with Suzuki et al who said that CPs of lateral and fourth ventricle similar in activity of drug transporters<sup>(18)</sup>.

HNF4 $\alpha$  binding and expression of many proteins and metabolizing enzymes like multiple transporting factors like the ATP binding cassette ABCB4, ABCC1 in human and rat<sup>(10)</sup> and transthyretin which is one of the proteins secreted by CP cells in cytoplasm<sup>(13,14)</sup> and this protein expressed in CSF-Blood barrier cell<sup>(19)</sup>. These proteins might be stained by anti HNF4 $\alpha$  IHC stain and give cytoplasmic reaction which is detected by the Aperio software which indicates the excessive amount of these

binding proteins that might explain the probability of drugs metabolizing and transporting are more in the lateral ventricle than forth ventricle which is implicated clinically and pharmacologically.

Expression of HNF4 $\alpha$  in the CPs of lateral ventricle was higher than forth ventricle this means higher role in defending programs to prevent entry of xenobiotic drugs into the brain because of this tissue express multiple transporter and drugs metabolizing enzymes<sup>(20)</sup>, also regulates distribution and entry of various compounds between CSF and blood interface and is involved in numerous exchange processes therefore determining the supply of brain by nutrients and hormones<sup>(20)</sup>.

Demonstration of cytoplasmic and intracellular reaction of HNF4 $\alpha$  as shown in fig. 3 by binding with transthyretin in the choroidal cells cytoplasm to regulate the activity of this protein. The presence of numerous endoplasmic reticulum and Golgi apparatus in CPs made their ability to secrete this protein high<sup>(7)</sup>. This protein secretion is specifically in the CP and not in other parts of brain and bind with HNF4 $\alpha$  to control drug transporting, and this protein was the same contents in lateral and forth ventricle<sup>(13)</sup> also can be demonstrating by activity of HNF 4 $\alpha$  in glycolysis process in cytoplasm<sup>(11)</sup>.

Liddelow *et al*, said HNF4 $\alpha$  is expressed in adult CP for regulation of many genes encoding junction –adhesion and tight junction and cytoplasmic regulatory adaptor in lateral ventricle like claudin 2, occludin 6<sup>(21)</sup> that may be demonstrate the positivity of HNF4  $\alpha$  in lateral ventricle more.

Relatively, The expression of HNF4 $\alpha$  in the choroidal cells of the lateral higher than the forth one might indicate the abundant amount of the binding secreted proteins from the endoplasmic reticulum in the cytoplasm of choroid cells, this might suggest that the endoplasmic reticulum content of protein of the CP of the lateral ventricle is more if compared with that of the 4th ventricle.

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## Author contributions

Dr. Farhan conceived and designed the study and preliminary analysis; Dr. Al-Kafagi collected, analyzed and interpreted the data and rewrote the manuscript. Both authors have read and approved the final version of manuscript.

## Conflict of interest

The authors declare no conflict of interest.

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**Correspondence to Dr. Thaer M. Farhan**

**E-mail: [anatomyembryology@gmail.com](mailto:anatomyembryology@gmail.com)**

**Tel. +964 7901611092**

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## Neonatal jaundice with Urinary Tract Infection

Shatha H. Ali *CABP*, Deia K. Khalaf *FICMS*, Sinan A. Ibrahim *FICMS*

Dept. of Pediatrics, College of Medicine, Al-Nahrain University, Baghdad, Iraq

### Abstract

- Background** Urinary tract infection is a common and serious clinical problem in newborns. Renal scarring, hypertension, and even kidney failure can be prevented by early diagnosis and treatment of urinary tract infection. Jaundice is an important and sometimes the presenting feature of urinary tract infection.
- Objectives** To evaluate the frequency and bacterial profile of urinary tract infection in full term and preterm newborns with hyperbilirubinemia in the first two week of life, and the relation with some demographic parameters.
- Methods** Seventy two jaundiced neonates were studied. Hematocrit, white blood cell count, reticulocyte count, blood group and Rh, bilirubin (direct and total), Coombs test, and glucose 6-phosphate dehydrogenase level were evaluated. Septic screen and thyroid function test, urinalysis and culture for all patients.
- Results** Twenty two (30%) out of 72 complain from urinary tract infection. Majority of them were full term 15 (68%), and Body weight > 2.5 Kg 12 (54%), fifteen delivered by normal vaginal delivery (68%). Breast feeding was recorded in 12 (54%), total serum bilirubin level above 20 mg/dl was found in 19 (86%). Gestational age, Normal Vaginal Delivery, Type of feeding, and total serum bilirubin level more than 20 mg/dl had significant correlation with urinary tract infection in jaundiced neonates, while birth weight was not significant. Commonest isolated bacteria was Escherichia coli in 11 (50%), staphylococcus infection in 2 cases (9.1%) have significant correlation with male gender. Ultrasound result was only positive in one (4.5%) neonate and showed pelvi-calceal obstruction.
- Conclusion** Urinary tract infection is common among jaundiced neonates particularly in males. Escherichia coli is the commonest causative bacteria. Gestational age, normal vaginal delivery, type of feeding, and total serum bilirubin level more than 20 mg/dl had significant correlation with urinary tract infection in jaundiced neonates, while birth weight was not significant.
- Key word** Neonatal jaundice, hyperbilirubinemia, UTI

**List of Abbreviations:** CS = Caesarean section, E. coli = Escherichia coli, G6PD = Glucose 6- phosphate dehydrogenase, LBW = low birth weight, NBW = normal birth weight, NICU = neonatal intensive care unit, NVD = normal vaginal delivery, PUJ = pelvi ureteric junction obstruction, TSB = total serum bilirubin, UTI = urinary tract infection, VCUG = voiding cystourethrography.

### Introduction

Urinary tract infection (UTI) is a common and serious clinical problem in newborns. Previous studies have suggested that jaundice may be one of the signs of a UTI in infants<sup>(1)</sup>. It is well known that UTI can occur without apparent signs, and

jaundice is an important and sometimes the presenting feature of UTI<sup>(2)</sup>.

Renal scarring, hypertension, and even kidney failure can be prevented by early diagnosis and treatment of UTI. Symptoms and signs of UTI in infants are nonspecific and prolonged jaundice is more common in breastfed infants than artificially fed infants<sup>(3)</sup>.

Jaundice although it is mostly benign, some cases may have the possibility of having other diseases in combination. There have been some reports regarding the relationship of

idiopathic hyperbilirubinemia and bacterial infections, such as UTI. However, it is still not recommended to perform routine urinary tests in jaundiced infants for such infections. Urinalysis and a urinary culture are only recommended under some certain conditions, such as infants who have an elevation of direct-reacting or conjugated bilirubin, and infant readmitted for phototherapy or exchange transfusion. However, coincidental UTI in jaundiced infants were not uncommon in our clinical practice<sup>(4)</sup>.

The objective of this study was to evaluate the frequency and the bacterial profile of UTI in full term and preterm newborns with hyperbilirubinemia in the first two week of life, and the relation with some demographic parameters.

### **Methods**

A cross sectional prospective study conducted on 72 jaundiced neonates whose age less than 2 week, ranged from 3 to 14 days, admitted to Neonatal care unit or the pediatric ward at Al-Imamain Al-Kadimain Medical City, for the period from 1<sup>st</sup> of October 2013 till June 30<sup>th</sup> 2014, which is the period of collection of data.

A well constructed questionnaire was performed for data collection including: gender, gestational age, birth weight, mode of delivery.

Blood samples were taken from all the cases for hemoglobin, hematocrit, white blood cell count, reticulocyte count, blood group and Rh, bilirubin (direct and total), Coombs test, and Glucose 6- phosphate dehydrogenase (G6PD) level were evaluated. Septic screen and thyroid function test, and urine samples were collected by urine bag for urinalysis and culture for all patients. UTI is positive if there is more than 5 leukocytes per high power field on urine sample, and positive urine culture (There is no colony count done in hospital labs). Renal ultrasound done for all neonates with UTI.

Data analysis was entered into the Microsoft Excel -2010, p value  $\leq 0.05$  was considered to be statistically significant.

### **Results**

Total number of jaundiced patients was 72. Males were 42 (58%) while females were 30 (42%) with male to female ratio of 1.4:1.

Full term neonates were 48 (67%), birth weight < 2.5 kg was found in 38 (52%) of cases, 44 (61%) were delivered by normal vaginal delivery (NVD).

Regarding etiological causes of Jaundice; ABO incompatibility was diagnosed in 12 patients (17%) and 10 (14%) of them were recorded in full term ( $p < 0.05$ ) while Rh- incompatibility and G6PD was found in 2 cases (3%) for each. Septicemia was found in 18 (25%) of cases and 12 (17%) of them were found in preterm ( $p < 0.05$ ). One case was diagnosed as hypothyroidism 1 (1.4%) as shown in Table 1.

As shown in table 2, total number of neonates complain from UTI was 22 (30%), 13 of them were males while 9 were females, majority of them were full term 15 (68%) ( $p < 0.05$ ), and body weight > 2.5 Kg 12 (54%), delivered by NVD 15 (68%). Breast feeding was recorded in 12 (54%), TSB level above 20 mg/dl was found in 19 (86%) of cases. All the studied parameters show significant correlation with gender in jaundiced neonates with UTI except birth weight.

Only in 3 male neonates (14%); the TSB was below 20mg all of them respond to intensive phototherapy with no need to exchange transfusion.

Urine culture revealed that the commonest bacteria was E- coli in 11 (50%), enterobactor in 5 (22%), Proteus in 3 (13%), staphylococcus in 2 (9%), mixed infection of multiple bacteria in 1 (4.5%) as shown in the table 3. Staphylococcus and mixed infection of multiple bacteria have significant correlation with gender.

Ultrasound result was only positive in one (4.5%) neonate and showed pelvi-ureteric junction obstruction (PUJ).

**Table 1. Distribution of 72 jaundiced patients according to different parameters**

Parameter		Full term	Preterm	No. (%)
Birth wt. (Kg)	<2.5	20 (27)	18 (25)	38 (52)
	>2.5	28 (38)	6 (8)	34 (48)*
Gender	Male	27 (37)	15(21)	42 (58)*
	Female	21 (29)	9 (12)	30 (42)
Mode of delivery	Cesarean section	19 (26)	9 (13)	28 (39)*
	Normal vaginal delivery	29 (40)	15 (21)	44 (61)*
Hypothyroidism		1 (1.4)	0 (0)	1 (1.4)
Rh- incompatibility		2 (3)	0 (0)	2 (3)
Glucose 6-phosphate dehydrogenase		2 (3)	0 (0)	2 (3)
ABO incompatibility		10 (14)	2 (3)	12 (17)*
Septicemia		6 (8)	12 (17)	18 (25)*
Total		48 (67)	24(33)	72 (100)*

\* = p &lt; 0.05

**Discussion**

Males were more than females (42 vs 30), which is in agreement with several studies<sup>(3, 5-10)</sup>. Gestational age was found to be statistically significant in this study, while it was not in other studies<sup>(2,7,11)</sup>. This might be related to relatively higher number of preterm neonates

included in this study. This finding was highlighted by high number of LBW neonates in this study. Septicemia showed significant relation with jaundiced preterm neonates. This is related to higher susceptibility of sepsis among preterm's.

**Table 2. Distribution of 22 jaundiced patients with UTI according to gender with different parameters**

Parameter		Male	Female	No. (%)
Birth Weight (Kg)	< 2.5	8	4	12 (54)
	>2.5	6	4	10 (46)
Mode of delivery	Cesarean section	4	3	7 (32)*
	Normal vaginal delivery	10	5	15 (68)
Feeding Type	Breast	8	4	12 (54)*
	Artificial	5	2	7 (32)
	Mixed	2	1	3 (14)
Total serum bilirubin	>20mg	12	7	19 (86)*
	<20mg	3	0	3 (14)
Preterm		4	3	7 (32)*
Full term		9	6	15 (68)*

\* = p &lt; 0.05

The presence of jaundice may be an early sign of sepsis in neonates, especially UTI<sup>(4)</sup>. We need to pay it much attention in clinical practice. Several studies from different regions of the world reported incidence of UTI of 3.6%

- 21% range<sup>(2,3,5-14)</sup>. This wide difference may be due to age selection, different sample size and different methods of urine collection in different studies. In this study the urine was collected with urine bags because it is non-

invasive and easy to perform, although it is known to have high false positive rates<sup>(12)</sup>. UTI was more common in males, which is in agreement with several studies<sup>(2,3,7-11)</sup>. In Study

from Egypt (11), a higher prevalence of UTI was present in full term (77.4%) compared with preterm neonates (22.6%).

**Table 3. The frequency of different bacteria according to urine culture**

Type of bacteria	Male	Female	No. ( %)
Escherichia- coli	7	4	11 (50)
Enterobactor	3	2	5 (22.7)
Proteus	2	1	3 (13.7)
Staphylococci	2	0	2 (9.1)*
Mixed infection	1	0	1 (4.5)*

\* = p < 0.05

In this study, we found a significant relation with UTI in jaundiced babies and mode of delivery; this might be attributed to exposure of bacteria while passing the birth canal. Iranian study<sup>(5)</sup> detected formula feeding as a significant factor for UTI among jaundiced neonates and this is related to the protective function of the breast milk. On the other hand other studies found no significant relation between type of feeding and UTI<sup>(2,3,7,15)</sup>. Birth weight was found to be not significant factor for UTI, which is similar to many other studies<sup>(4,7,8,11,12)</sup>.

Relation of Higher TSB level with UTI was highlighted by Francisco J study who reported increase in the conjugated bilirubin fraction in 2 of 12 infants with a positive urine culture<sup>(7)</sup>. Hyperbilirubinemia associated with UTIs can be unconjugated and related to hemolysis caused by Gram-negative organisms, or conjugated secondary to cholestasis. Possible mechanisms include microcirculatory changes in the liver, direct effects from bacterial products, and/or from endotoxin-induced mediators<sup>(4,7)</sup>.

In agreement with different studies, E.coli was the commonest bacteria isolated by urine culture from 22 neonates with jaundice, enterobacter, klebsiella and proteous and were isolated with different percentages<sup>(2,3,6,7,9-11)</sup>. Since both of these two organisms E.coli and enterobacter are commonly seen in stool and

stool on the diaper may spread over the perineum, hygiene care is very important<sup>(4)</sup>. Interestingly, we detect a significant correlation between staphylococci and male gender; this is mostly related to un-circumcision state of the neonates. This was highlighted in an Iranian study which stated that circumcision was protective factor for UTI<sup>(5)</sup>.

Regarding US results, Nader study found 1/6 renal abnormalities<sup>(3)</sup>, another Iranian study reported 23/ 400<sup>(5)</sup>, Francisco study detected 6/ 11<sup>(7)</sup>. This difference may be related to use of other imaging studies beside the renal ultrasound like VCUG, renal scan

We conclude that UTI is common among jaundiced neonates particularly in males. E.coli is the commonest causative bacteria. Gestational age, NVD, Type of feeding, and TSB level more than 20 mg/ dl had significant correlation with UTI in jaundiced neonates, while birth weight was not significant. Significant correlation between Staphylococci and male neonates with UTI.

We recommend urinalysis and urine cultures to be performed for infants with jaundice less than 2 weeks of age, as UTI might be asymptomatic, only presented with jaundice.

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### Author contribution

Dr. Ali put the idea and protocol of the work; Dr. Khalaf writes the article; and Dr. Ibrahim collects the samples.

### Conflict of interest

The authors declare no conflict of interest.

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**Correspondence to Dr. Shath H. Ali**

**Email: shathah666@yahoo.com**

**Tel.: + 964 7901 47 99 29**

**P. O. Box 70074, Baghdad – Iraq**

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## Study the Effects of Obesity and Body Fat Distribution on the Spirometric Pulmonary Function Tests

Walaa M. Mejbel *MSc*, Abbas F. Abdul-Wahab *MSc PhD*

Dept. of Physiology, College of Medicine, Al-Nahrain University, Baghdad, Iraq

### Abstract

<b>Background</b>	Obesity is one of the most frequently found health risks with increasing in its prevalence all over the world. Several measures of obesity like body mass index, waist circumference and percent of body fat had been used in many studies as predictor of pulmonary function tests.
<b>Objectives</b>	To evaluate the effect of anthropometric measurements on pulmonary function tests, and explore the association between body fat percent and pulmonary function tests.
<b>Methods</b>	A total of one hundred subjects were recruited from both sexes (fifty with normal and fifty with high body mass index). Body mass index, waist circumference and percent of body fat were measured for each subject.
<b>Results</b>	This study shows a significant reduction in spirometric parameters (except for FEV1/FVC ratio) in high body mass index groups compared to those with normal body mass index in both sexes, with a significant negative correlation between percent of body fat and waist circumference with spirometric parameters in high body mass index groups had been identified.
<b>Conclusion</b>	Obesity has a restrictive rather than obstructive pattern of lung impairment. Excess body fat and abdominal obesity have anadverse affect on lung function.
<b>Keywords</b>	Pulmonary function test, BMI, WC, BF%

**List of abbreviation:** BMI = body mass index, PFT = pulmonary function test, WC = wait circumference, WHR = waist to hip ratio, BF% = percent of body fat, FVC = forced vital capacity, FEV1 = forced expiratory volumes in first second, FEF = forced expiratory flow, PEFR = peak expiratory flow rate.

### Introduction

Obesity is a global health hazard and has been linked to numerous metabolic complications such as dyslipidemia, type II diabetes, and cardiovascular diseases and is negatively associated with the respiratory function <sup>(1)</sup>. The mechanism which influences lung function in obesity is still debated and the best marker of adiposity in relation to dynamic pulmonary function is still not clear <sup>(2)</sup>. Body mass index (BMI) can be easily measured and therefore is frequently used in large scale epidemiologic studies to find out the health hazards caused by obesity

<sup>(3)</sup>. Pulmonary function test (PFT) is a basic and essential test for diagnosis and assessment of pulmonary dysfunction, pulmonary diseases, and treatment effects <sup>(4)</sup>.

Obesity is measured using waist circumference (WC) and BMI. Body weight and BMI can be easily measured and therefore are frequently used in large scale epidemiological studies to find out the health hazards caused by obesity <sup>(5)</sup>. The association between BMI or body weight and PFT variables vary in different subpopulations <sup>(2,6,7)</sup>, also the relation of WC and waist to hip ratio (WHR) to pulmonary function parameters revealed a controversial results <sup>(8)</sup>. PFT is a basic and essential test for diagnosis and assessment of pulmonary dysfunction, pulmonary diseases, and treatment effects. Predicted normal values of

PFT is calculated by an equation of regression reflecting gender, age, height, and body weight due to the significant correlation with PFT values<sup>(9)</sup>. Since other body measures have also been reported to have correlations with the result of PFT, studies to find an equation of regression of pulmonary functions values according to other body measures have continued<sup>(10)</sup>.

An important respiratory abnormality in obesity is a decrease in total respiratory system compliance that may relate to the increased pulmonary blood volume seen in obese individuals. However, the primary reason is due to a decrease in chest wall compliance associated with the obese individual's resulted from accumulation of fat in and around the ribs, the diaphragm and the abdomen<sup>(11)</sup>.

Total respiratory compliance is markedly reduced by recumbency in obese individuals compared with non-obese individuals<sup>(12)</sup>. This reduction is almost entirely due to the decreased compliance of the chest wall, although it may also be due to an increase in respiratory resistance<sup>(13)</sup>.

## Methods

A case control study done in the pulmonary function unit in AL-Imamain Al-Kadhmain Medical City during the period from December 2013 to April 2014. A total of one hundred normal healthy persons from 18-45 years old were recruited from both sexes; fifty persons with normal BMI (18.50-24.99 Kg/m<sup>2</sup>), (25 females and 25 males) and fifty persons with high BMI (overweight and obese with BMI ≥ 25 Kg/m<sup>2</sup>), (25 females and 25 males). Any person with lung disease, pregnant women, and smokers will be excluded from this study. Informed consent of all subjects was obtained, with approval of Institute Review Board.

BMI was calculated by measuring weight (kg) and height (cm) using digital height and weight scale, BMI is define as person's weight in

kilograms divided by the square of his height in meters (kg/m<sup>2</sup>)<sup>(5)</sup>. WC was measured to the nearest 0.1 cm using a measuring tape at the midpoint between the last floating rib and the top of the iliac crest<sup>(6)</sup>.

Extremity skinfolds were measured at the triceps and biceps and trunk skinfolds were measured at the supriliac and subscapular areas, the skinfold was picked up between the thumb and the forefinger and the readings were taken 5 seconds after the caliper was applied<sup>(7)</sup>. Three consecutive readings were taken and recorded at each site, the average of the three readings at each site was calculated and the sum of these values was entered into the table given by Durnin and Womersley to estimate body density<sup>(7)</sup> and then percent of body fat (BF%) was calculated using Siri Equation<sup>(8)</sup>.

Spirometric parameters of PFTs were conducted by measuring forced vital capacity (FVC), forced expiratory volumes in first second (FEV1), FEV1/FVC ratio, forced expiratory flow (FEF25%, FEF50% and FEF75%) and peak expiratory flow rate (PEFR) using a spirometer (Jaeger, Germany) after careful explanation of the test to the participant<sup>(9)</sup>.

Statistical analysis was performed with SPSS V. 17 and Excel 2010. Data were expressed as mean±SD. Data analysis was done using unpaired t-test and Pearson correlation. P-value<0.05 was considered statistically significant.

## Results

The present study shows a significant reduction in spirometric parameters FEV1 (L), FVC (L), FEF 25%, FEF 50%, FEF 75% and PEFR in high BMI males and females compared to normal BMI males and females, (p < 0.001), respectively; while no significant changes in FEV1/FVC ratio (L) regarding males and females with normal and high BMI had been identified, (P>0.05), as noticed in tables 1 and 2, respectively.

**Table 1. Pulmonary function tests in males with normal and high body mass index**

Parameter	Body mass index		p value
	Normal mean±SD	High mean±SD	
FEV1 (L)	3.49 ± 0.56	2.27 ± 0.3	<0.001
FVC (L)	4.07 ± 0.57	2.65 ± 0.31	<0.001
FEV1/FVC ratio (L)	0.86 ± 0.06	0.86 ± 0.08	0.743
FEF25% (L/S)	7.2 ± 0.74	5.28 ± 0.44	<0.001
FEF50% (L/S)	4.5 ± 0.54	4.18 ± 0.51	0.035
FEF75% (L/S)	2.57 ± 0.19	1.64 ± 0.32	<0.001
PEF (L/S)	8.26 ± 0.95	5.87 ± 0.48	<0.001

FEV1 = forced expiratory volumes in first second, FVC = forced vital capacity, FEF = forced expiratory flow, PEF = peak expiratory flow

**Table 2. Pulmonary function tests in females with normal and high body mass index**

Parameter	Body mass index		p value
	Normal mean±SD	High mean±SD	
FEV1 (L)	3.36 ± 0.58	2.11 ± 0.21	<0.001
FVC (L)	4.0 ± 0.61	2.3 ± 0.22	<0.001
FEV1/FVC ratio (L)	0.85 ± 0.18	0.92 ± 0.09	0.101
FEF25% (L/S)	6.91 ± 0.86	5.0 ± 0.29	<0.001
FEF50% (L/S)	4.46 ± 0.57	3.78 ± 0.39	<0.001
FEF75% (L/S)	2.41 ± 0.22	1.56 ± 0.27	<0.001
PEF (L/S)	8.15 ± 1.0	5.56 ± 0.33	<0.001

FEV1 = forced expiratory volumes in first second, FVC = forced vital capacity, FEF = forced expiratory flow, PEF = peak expiratory flow

This study shows a significant negative correlation between BF% and spirometric parameters FEV1 (L), FVC (L), FEF 25%, FEF 50%, FEF 75% and PEF in females and males with high BMI (p < 0.001), while no significant correlation between BF% and FEV1/FVC ratio (L) had been observed in both sexes, (p > 0.05) as shown in table 3.

**Table 3. Correlations between percent of body fat and pulmonary function test in high body mass index groups**

Parameters	Percent of body fat			
	Female		Male	
	r value	p value	r value	p value
FEV1 (L)	-0.488	0.013	-0.772	<0.001
FVC (L)	-0.430	0.032	-0.869	<0.001
FEV1/FVC ratio (L)	-0.107	0.609	0.031	0.882
FEF 25% (L/S)	-0.743	<0.001	-0.990	<0.001
FEF50% (L/S)	-0.415	0.039	-0.694	<0.001
FEF75% (L/S)	-0.560	0.004	-0.983	<0.001
PEF (L/S)	-0.853	<0.001	-0.987	<0.001

FEV1 = forced expiratory volumes in first second, FVC = forced vital capacity, FEF = forced expiratory flow, PEF = peak expiratory flow

This study shows a significant negative correlation between WC and spirometric parameters FEV1 (L), FVC (L), FEF 25%, FEF 50%, FEF 75% and PEF in females and males with

high BMI ( $p < 0.001$ ), while no significant correlation between WC and FEV1/FVC ratio (L) had been identified in both sexes, ( $p > 0.05$ ) as demonstrated in table 4.

**Table 3. Correlations between percent of waist circumference and pulmonary function test in high body mass index groups**

Parameters	Waist circumference			
	Female		Male	
	r value	p value	r value	p value
FEV1 (L)	-0.510	0.009	-0.628	0.001
FVC (L)	-0.585	0.002	-0.677	<0.001
FEV1/FVC ratio (L)	0.040	0.851	0.002	0.993
FEF 25% (L/S)	-0.660	<0.001	-0.684	<0.001
FEF50% (L/S)	-0.517	0.008	-0.411	0.041
FEF75% (L/S)	-0.593	0.002	-0.666	<0.001
PEF (L/S)	-0.846	<0.001	-0.732	<0.001

FEV1 = forced expiratory volumes in first second, FVC = forced vital capacity, FEF = forced expiratory flow, PEF = peak expiratory flow

## Discussion

Reduced pulmonary function is an important predictor of mortality in the general population<sup>(14)</sup>. Analysis for the values of lung subdivisions including total lung capacity, residual volume and functional residual capacity demonstrated that standing and sitting height were the best predictors of lung volumes. Lung volumes are also considerably influenced by the amount of body fat; gross obesity decreases the total and chest wall compliance and thus diminishes expiratory reserve volume and functional residual capacity<sup>(15,16)</sup>.

This study shows a significant reduction in spirometric parameters (except for FEV1/FVC ratio) in high BMI groups compared to normal BMI groups among both males and females, tables 1 and 2, respectively. This result agrees with other studies done by Shinde et al<sup>(10)</sup>; Lad et al<sup>(17)</sup> and Soundariya and Neelambikai<sup>(18)</sup> but disagrees with a study done by Ajmani<sup>(2)</sup>. This difference possibly due to small sample size in this study, factors affecting PFTs (environmental conditions, altitude, and socioeconomic status).

The reduction in spirometric parameters with increasing BMI can be explained by the

mechanical resistant to the movement of thorax and abdomen, reduced chest wall compliance and peripheral airway size, while the no difference in FEV1/FVC ratio indicates a restrictive impairment in individuals with higher BMI and rules out any obstructive pattern of lung diseases<sup>(19)</sup>.

This study shows a significant negative correlation between BF% and all studied spirometric parameters (except for FEV1/FVC ratio) in high BMI groups among both females and males (Table 3). This result agrees with a study done by Lad et al<sup>(17)</sup>. A study done by Steele et al<sup>(20)</sup> shows a significant negative correlation between BF% and [FEV1 (L) and FVC (L)] in high BMI groups; another study done on female subjects by Kim et al<sup>(4)</sup> shows a significant negative correlation between BF% with FEV1 and FVC.

The present study shows no correlation between BF% and FEV1/FVC ratio (L). This result agrees with other studies done by Kim et al<sup>(4)</sup> and Behera and Pradhan<sup>(20)</sup>.

There are several mechanisms by which excess body fat might lead to reduced lung function, broadly categorized into mechanical and inflammatory<sup>(21)</sup>. With increasing obesity,

intra-abdominal fat deposition and accumulation may impede the descent of the diaphragm during inspiration<sup>(22)</sup>. In addition, the deposition of fat on the chest wall may impede expansion and excursion of the rib cage, through a direct loading effect or by altering intercostal muscle function<sup>(23)</sup>.

This study shows a significant negative correlation between WC and spirometric parameters (except for FEV1/FVC ratio) in both females and males with high BMI (Table 4). This result is concordance with other studies done by Chen et al<sup>(3)</sup> and Khan<sup>(23)</sup>. Abdominal adiposity (central fat distribution) may restrict the descent of the diaphragm, limit lung expansion and increase the thoracic pressure, leading to restrictive respiratory impairment<sup>(23)</sup>.

We conclude that obesity has a restrictive rather than obstructive pattern of lung impairment. Excess body fat (increase BF%) and abdominal obesity (as measured by WC) have adverse effect on lung function, suggesting use of these indices in evaluation of lung function in high BMI individuals.

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### **Conflict of interest**

No conflict of interest

### **Author contribution**

Dr. Abdul-Wahab put the setting, computes the parameters and participates in writing the article; Mejbel collects the data, did the statistical analysis and write the article.

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**Correspondence to Dr. Abbas F. Abdul-Wahab**

**E-mail: [abbasalhashimi04@colmed-alnahrain.edu.iq](mailto:abbasalhashimi04@colmed-alnahrain.edu.iq)**

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## The value of Oesophageogastroduodenscopy (OGD) in Assessment of Anemic Patients

Waseem F. Al-Tameemi<sup>1</sup> CABMS FIBMS FICMS Ali S. Mahdi<sup>2</sup> MBChB

<sup>1</sup>Dept. of Medicine, College of Medicine, Al-Nahrain University, <sup>2</sup>Al-Imammian Al-Kadhimian Medical City

### Abstract

- Background** Endoscopic evaluations are commonly included within work up of unexplained anemia.
- Objectives** Defining the value of the oesophageogastroduodenscopy as routine investigation in anemic patients in concern with gross pathological findings, as well as determining its importance in state of anemia in relation to age and gender.
- Methods** It is a retrospective study reviewed 89 anemic patient reports, at the Oesophageogastroduodenscopy clinic, Al-Imammian Al-Kadhimian Medical City throughout the period between Oct 2011-Jan 2013. The following data had been reported from the patients files and included; gender, age, referral indication (which should be anemia with or without accompanying symptom) and their gross endoscopic pathological findings.
- Results** The mean age was  $46 \pm 17.36$  years with range of 13-80 years. Male to female ratio (1:1.23). Majority of patients 50.6 % were referred due to lack of obvious cause to their anemia in absence of gastrointestinal symptoms. The most frequent finding is normal report in 33.7% followed by gastritis in 18%. Almost 82.5% of male patients were reported to have abnormal oesophageogastroduodenscopy in comparison to female patients (53.06%) ( $p = 0.003$ ). Male patients at or under 45 years is statistically highly significant to undergo screening for the cause of anemia by endoscopy in comparison to same age matched counterpart females ( $p = 0.015$ ).
- Conclusion** It is important to apply practical algorithm in deciding the indication and value of referral anemic patients for oesophageogastroduodenscopy clinic considering young male patients and those with gastrointestinal tract symptoms as priorities after attempting to exclude all other possible causes, otherwise there will be no further yield by endoscopy in addition to exhaustion of resources.
- Keywords** Unexplained anemia, oesophageogastroduodenscopy

**List of abbreviation:** OGD = oesophageogastroduodenscopy, IDA = iron deficiency anemia, GI = gastrointestinal, GIT = gastrointestinal tract.

### Introduction

Anemia diagnosis may be simply proved clinically, however; tasks must be directed toward confirmation of state and type of anemia to start with, and then directed to define the underlying possible etiology. Endoscopic procedures like oesophageogastroduodenscopy (OGD) is labeled as an essential investigation to determine the cause of anemia like in iron

deficiency anemia (IDA) or megaloblastic anemia..etc<sup>(1)</sup>

The commonest cause of anemia world wide is iron deficiency and chronic blood loss is reported to be the most possible underlying reason<sup>(2)</sup>. Chronic gastrointestinal (GI) blood loss whether overt or occult<sup>(3)</sup> may be the earliest feature of gastrointestinal (GI) malignancy especially at old age men and post menopausal women up to 40 % and must be always excluded during work up<sup>(4)</sup>

IDA comprises approximately 4-13% of referrals to endoscopic clinic, with a nearly



equal prevalence in men and post-menopausal women 2-5%<sup>(5)</sup>. The indication of this referral is evaluate the GI tract (GIT) for bleeding lesions<sup>(6)</sup> however up to 35% of these referral may be inappropriate<sup>(7)</sup>.

Studies had demonstrated that advanced age, male gender, previous non-steroidal anti-inflammatory drugs (NSAIDs) use, diarrhea or positive fecal occult blood test were considered clues to look for endoscopic lesions in patients with IDA with and without GI symptoms<sup>(8,9)</sup>. Studies have concluded that prevalence of endoscopic lesions in patients with IDA without GI symptoms is between 48-71%<sup>(10,11)</sup>.

The objective of this study is to defining the value of the OGD as routine investigation in anemic patients in concern with gross pathological findings, as well as determining its importance in state of anemia in relation to age and gender.

### Methods

This retrospective study had reviewed the data of 89 patient reports, throughout the period between Oct 2011-Jan 2013, who referred for OGD Clinic, Al-Imammian Al-Kadhimain Medical City to define the possible explanations for their anemia presentation. They were selected randomly depending on their registration files. The gender, age, referral indication (which should be anemia with or without accompanying symptom) and their gross endoscopic pathological finding on OGD reports were reported from the patients' files. Procedure of endoscopy had performed by different gastroenterology specialists with different endoscopic tools.

The files which not contain some of these data had been excluded. This study had been followed the guidelines and approved by the Institute Review Board of the College of Medicine, Al-Nahrain University.

Statistical package for the social sciences (SPSS) program had been used for statistical analysis and included student t test, ANOVA, Fisher

Exact test; p value of < 0.05 considered the least significant level.

### Results

Eighty nine patients were enrolled in this retrospective study. Female patients constituted 55% (49/89) as male to female ratio (1:1.23)

The mean age was 46±17.36 years with range of 13-80 years. The distribution of patients in term of different age interval demonstrated that 20.2% were presented between 51-60 years while only 6.7% within group of 71-80 years (Table 1).

**Table 1. Patients distribution according to their age interval**

Interval (year)	Frequency (N %)
11 - 20	7 (7.9)
21 - 30	14 (15.7)
31 - 40	16 (18.0)
41 - 50	14 (15.7)
51 - 60	18 (20.2)
61 - 70	14 (15.7)
71 - 80	6 (6.7)
Total	89 (100)

Majority of patients were referred to endoscopic clinic due to unexplained anemia in absence of GIT symptoms 50.6% (45/89). Those were labeled as pallor, IDA or megaloblastic anemia, while the presence of abdominal pain on the top of diagnosis of anemia showed to be as a second reason for endoscopic screening (Table 2).

Gross endoscopic finding were listed in table 3. The most frequent finding is normal report in 33.7% (30/89) followed by gastritis in 18% (16/89). Only 3 patients had reported as atrophied duodenal mucosa with suspicion of celiac disease while malignancy documented in 4.5% (4/89). Combination of different finding as most likely gastroesophageal reflux disease and gastritis, gastrodeudentis or gastric ulcer were seen at 5/89 patients.

**Table 2: Causes of referral and indications of oesophageogastroduodenscopy**

Indications (presentation of anemia at time of referral)	Frequency (N %)
Unexplained anemia	32 (36)
Iron Deficiency anemia	10 (11.2)
Megaloblastic anemia	3 (3.4)
Abdominal pain	13 (14.6)
Weight loss	9 (10.1)
Diarrhea	7 (7.9)
Organomegally	5 (5.6)
Dyspepsia	4 (4.5)
Jaundice	3 (3.4)
Dysphagia	3 (3.4)
Total	89 (100)

**Table 3. Gross finding on oesophageogastroduodenscopy**

Gross Finding	Frequency (N %)
Normal OGD	30 (33.7)
Gastritis	16 (18)
Gastroduodenitis	8 (9)
GERD	7 (7.9)
Hiatal hernia +/- Lax cardia	6 (6.7)
Erosions	4 (4.5)
Gastric Malignancy	4 (4.5)
Gastric Ulcers	3 (3.4)
Atrophied duodenal mucosa (suspicion of celiac sprue)	3 (3.4)
Varicies	2 (2.2)
Polyps	1 (1.1)
Combinations of above findings	5 (5.6)
Total	89 (100)

Those fifty nine patient out of 89 who identified to have abnormal endoscopic finding, twenty two (37.3%) of them were denied any GIT manifestation during referral and there were no obvious cause of anemia, while the rest 62.7% had been suffered from different GIT symptoms in addition to anemia. In contrary those 30 patients who proved to have normal endoscopy report, unexplained anemia was the only reason beyond referral despite absence of other abdominal symptoms

in 33.3% of them which is of no statistical significance ( $p = 0.713$ )

However, almost 82.5% of male patients were reported to have abnormal OGD in comparison to female patients who discovered to have any form of abnormalities at endoscopy (53.06%) and this is statistically highly significant ( $p = 0.003$ ). Concerning age distribution, patients who aged 45 year or less were presented as 55.05% (49/89). It is found that 61.22% of them showed abnormal endoscopic reports which are less than what discovered in patients aged

more than 45 years where 72.5% had abnormal gross endoscopy for both genders but in non statistical significance ( $p = 0.263$ ) as shown in table 4.

**Table 4. Relationship between endoscopic findings and both gender and age**

OGD findings	Male		Female		Total	
	No.	%	No.	%	No.	%
Abnormal	33	82.5	26	53.06	59	66.29
Normal	7	17.5	23	46.94	30	33.71
<b>Total</b>	<b>40</b>	<b>100</b>	<b>49</b>	<b>100</b>	<b>89</b>	<b>100.00</b>
<b>p value</b>	0.003					
OGD findings	≤45 years		>45 years		Total	
	No.	%	No.	%	No.	%
Abnormal	30	61.22	29	72.5	59	66.29
Normal	19	38.78	11	27.5	30	33.71
<b>Total</b>	<b>49</b>	<b>100</b>	<b>40</b>	<b>100</b>	<b>89</b>	<b>100.00</b>
<b>p value</b>	0.263					

OGD = oesophageogastroduodenscopy

Analysis the role of both factors age and gender simultaneously with results of OGD reveals that male patients at or under 45 years is statistically highly significant to undergo screening for the cause of anemia by

endoscopy in comparison to same age matched counterpart females ( $p = 0.015$ ) but this significance is lost above this age between them ( $p = 0.273$ ) as shown in table 5.

**Table 5. Significance of age and gender in relation to endoscopy results**

Parameter			Male		Female		Total		p value
			No.	%	No.	%	No.	%	
≤45 years	OGD	Abnormal	13	86.67	17	50.00	30	61.22	0.015
		Normal	2	13.33	17	50.00	19	38.78	
		Total	15	100.00	34	100.00	49	100	
>45 years	OGD	Abnormal	20	80.00	9	60.00	29	72.5	0.273
		Normal	5	20.00	6	40.00	11	27.5	
		Total	25	100.00	15	100.00	40	100	

## Discussion

Anemia is public health problem and reported as one of commonest presentation in clinical presentation<sup>(12)</sup>. Evaluation of the gastrointestinal tract is indicated in anemic patients, even in the absence of GI symptoms<sup>(13)</sup>. Wang et al<sup>(14)</sup> as well as Rocky<sup>(6)</sup> had considered that upper endoscopy (OGD) more important than lower endoscopy (30% and 6.7%) in evaluation of anemia respectively.

Normal endoscopy was demonstrated in 33.7% (as the most predominant finding) and this is around 2 times higher than Çetinkaya et al<sup>(15)</sup> report (18.75%). While other contributing causes of anemia were gastritis and gastroduodenitis in 27% which were similar to Wang et al<sup>(14)</sup> finding who did report the most common etiology as gastritis. However; gastric carcinoma or polyp are not so common causes in both studies.

Surprisingly hiatus hernia and gastroesophageal reflux disease had reported significantly in this study (14.6%) which differs than what found by Wang et al <sup>(14)</sup>. These findings may not explain the actual cause of anemia and therefore a thorough search for the proper cause is highly indicated in these examples.

Celiac disease was suggested to be possible underlying cause of anemia according to characteristic gross features of atrophied duodenal mucosa in only 3.4% of patients which is definitely need to be confirmed by serological and histopathological manifestation but it is lower than Corazza et al <sup>(4)</sup> report who identify this cause in 10% of anemia cases celiac; although other authors had described that in only 2%-3% <sup>(15,16)</sup>.

British Society of Gastroenterology guidelines recommend that a minimum of 90% of patients with asymptomatic IDA (other than menstruating women) should be screened for coeliac disease (by serology) and should undergo an upper GI endoscopy <sup>(7)</sup>.

One third (33.7%) of revised endoscopic reports had confirm no obvious abnormality and this can indicate earlier referral for endoscopy even before exclusion of other causes unlike other authors conclusion <sup>(8,14-16)</sup> that showed high prevalence of GI findings in patients who diagnosed already as cases of IDA at time of referral and therefore in presence of unexplained IDA, endoscopic evaluation of the GIT may be mandatory even when GI symptoms are absent <sup>(8,16)</sup>, as well as lower endoscopy must be complementary to non revealing upper endoscopy.

When taking the age and gender as landmarks for endoscopic screening to identify the anemia possible cause, it may be concluded that anemic young male patients are more likely to be considered for this investigation according to this study that identified 86.6% of them will reveal the possible cause.

Unlike anemic female patients with equivalent age patient where definite abnormality

detected in only 50% in statistical significance ( $p = 0.015$ ).

There were several limitations in this study like being retrospective and depending on referral letters taking in consideration of upper endoscopy only and registering the gross features rather than histopathological manifestations.

This study shed a light that many referral of anemia cases to OGD clinic may be either unnecessary or at least being requested before attempting to search for other causes of anemia which is also reported by other authors as well as at other countries <sup>(7,17)</sup>.

In conclusion it is important to apply practical algorithm in deciding the indication and value of referral anemic patients for OGD clinic considering young male patients and those with GIT symptoms as priorities after attempting to exclude all other possible causes, otherwise there will be no further yield by endoscopy in addition to exhaustion of resources.

The advances in knowledge from this work is to improve evaluation of anemia, define the need of referring anemic patients to endoscopy clinic and identify the importance and the drawback of considering endoscopy as routine investigation in anemia in relation to age and gender.

In conclusion, this paper improve the indications for referring patients to endoscopy clinic from health and economic point of view and helping in planning algorithm for investigations priorities in case of unexplained anemia.

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### **Author contribution**

Dr. Al-Tameemi makes the design, analyze the study in addition to patient care; and Dr. Mahdi reviewed and reports the records of endoscopy.

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The authors declare no conflict of interest.

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Correspondence to Dr Waseem F. Al-Tameemi

E-mail: [drwaseem72@hotmail.com](mailto:drwaseem72@hotmail.com)

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## Immunological Response to Hepatitis B Vaccine in End Stage Renal Diseases

Jawad K. Manuti *FICMS*

Dept. of Medicine, College of Medicine, Al-Nahrain University, Baghdad, Iraq

### Abstract

- Background** End stage renal diseases patients have lower seroconversion rates compared with the subjects with intact renal function. Moreover, even after the completion of vaccination schedule anti-hepatitis B titers of responder who kept on dialysis, patients are low and decline logarithmically with time.
- Objectives** To determine the response of patients with end stage renal disease undergone hemodialysis to hepatitis B virus vaccination and to identify the factors that could affect this response.
- Methods** One hundred patients with an age range from 21 to 75 years complaining of chronic renal failure on regular hemodialysis. Patients negative for hepatitis B antigen and anti-hepatitis C were vaccinated with 40µg of Euvax B intramuscularly in the deltoid muscle by following a schedule of 0, 1 and 2 months. The antibody titer was tested at third month and if titer was <10 or 10-100IU/mL (patients whom regarded as non-responded or poor responded). Then they were given another fourth dose (40µg) of vaccine at sixth month.
- Results** The rate of seroconversion to hepatitis B vaccine among individuals with end stage renal disease is 63%. Thirty one (31%) patients were anti hepatitis C virus positive. Eighteen (58%) were responsive to hepatitis B vaccination and 13 (42%) did not response to Hepatitis B vaccination. Advanced age, sex and diabetes mellitus show no effect on response to vaccination. The response to hepatitis B vaccine is significant in patient's well control of hemoglobin, calcium, albumin and long duration on hemodialysis.
- Conclusion** Patients on maintenance dialysis typically show a suboptimal immune response to hepatitis B virus vaccine compared with the non-uraemic population.
- Keyword** Hemodialysis, chronic renal failure, HBsAg, vaccination.

**List of abbreviation:** Anti HCV = anti-hepatitis C virus, DM = diabetes mellitus, CRF = chronic renal failure, CKD = chronic kidney disease, ESRD = end stage renal disease, HD = hemodialysis, HBV = hepatitis B virus, HLA = human leukocyte antigen, RRT = renal replacement therapy, rHuEPO = recombinant human erythropoietin, Hb = hemoglobin.

### Introduction

In haemodialysis (HD) patients, hepatitis B virus (HBV) infection has higher mortality and morbidity rate is more likely to result in carrier state. Although Hepatitis B vaccine is effective in producing protection against HBV infection, the antibody response may be variable<sup>(1)</sup>.

Blood is a major vehicle for the transmission of the HBV. Therefore, patients undergoing regular HD are at particularly high risk of exposure to HBV infection, with a wide variation in endemicity between the countries. In addition, immunodeficiency renders patients with end stage renal disease (ESRD) susceptible to infection and subsequent disease<sup>(2)</sup>. Uremia impairs not only the clearance of the virus but also antigen presentation, T-cell activation, and subsequent antibody production. HBV vaccination is recommended for all predialysis and dialysis patients, but the seroconversion rate of anti-hepatitis B surface

antigen (anti-HBs >10 IU/l) and adequate responses (anti-HBs >100 IU/l) are markedly lower, quite variable, and shorter lasting than in healthy immunocompetent subjects. Therefore, patients with chronic kidney disease (CKD) should undergo vaccination in the early stages of the disease when the primary immune response is still intact<sup>(3)</sup>.

Despite the use of HBV vaccines and preventive measures, infection with HBV remains a major global health problem. Patients with CKD are at an increased risk of acquiring HBV infections from shared dialysis equipment, increased exposure to blood products, and immune-deficiency associated with CKD. In addition, they may be more likely to develop chronic infections on exposure to HBV<sup>(4,5)</sup>.

HBV infection remains a concern in dialysis populations, because the vaccination programs have been less successful in these populations than in the general population. The causes of poor seroconversion in CKD patients include malnutrition, uremia, and immunosuppression due to renal failure<sup>(6)</sup>.

Various approaches have been adopted to improve the response rate to hepatitis B vaccine in ESRD including the increased vaccine dose<sup>(7)</sup>, additional vaccine inoculations and the use of intradermal route rather than intramuscular vaccine route<sup>(8)</sup>.

Ineffective vaccination is predictive for prevalence and incidence of HBsAg positivity and anti-hepatitis C (anti-HBc) positivity<sup>(9)</sup>. At present, the attention of nephrologists is focused on CKD patients, who are currently non-dialyzed, but as their kidney disease progresses, it is likely to lead to renal replacement therapy (RRT) in the future. Hepatitis B vaccination of such patients is thought to decrease a number of HBV susceptible patients on RRT<sup>(10)</sup>.

Moreover, an anti-HBs titer tends to fall with time in persons who mounted an antibody response. In dialysis patients, the loss of hepatitis B immunity seems to be quicker than in healthy subjects<sup>(11)</sup>.

Numerous inherited and/or acquired factors are implicated in diminished immunization following hepatitis B vaccination. However, at first, in this study should exclude variables such as improper storage or administration that is not compatible with a manufacturer instruction. Involvement of genetic factors in the anti-HBs development is continuously examined. Already in the seventies of the past century, immune response to HBsAg in HBV infected HD patients was linked to human leukocyte antigens (HLA)<sup>(12)</sup>.

Immune response to HBsAg in HBV infected HD patients was linked to HLA, possession of major histocompatibility complex haplotype HLA-B8, SCOI, DR3, interleukin genotypes (i.e., IL-10, IL-12, IL-18) were associated with the anti-HBs development in response to HBsAg in HD patients<sup>(13)</sup>.

## Methods

Prospective study involved one hundred patients (56 male and 44 female) of different age groups range from 22-75 years. Their mean age was 47±21 years were complaining of chronic renal failure (CRF) on regular HD in Al-Imamain Al-Kadhmain Medical City for the period from June 2014 to July 2015.

Demographic and clinical variables including age, gender, cardiovascular disease, cancer, infection, HD vintage, use of fistula or central vascular access were analyzed. Therapeutic and laboratory variables, erythropoietin dose, the levels of blood urea, creatinine, serum calcium, total protein, serum albumin, hemoglobin (Hb) level, Kt/V (adequacy of dialysis) and anti-HBs titer were monthly analyzed.

Screening for HBsAg and total antibody to HBsAg and Anti-HCV were performed by ELISA method. Patients negative for HBsAg and anti-HBs were vaccinated with 40µg of Euvax B (LG Life Sciences, Korea) intramuscularly in the deltoid muscle by following a schedule of 0, 1, 2 months. Seroconversion was defined as an antibody titer equal to or more than 10IU/mL. The antibody titer was tested at third month and if the titer was <10 (non-responded) or 10-

100IU/mL (partial responded) respectively they were given another fourth dose (40µg) of vaccine at sixth month.

Statistical analysis was performed using chi-square test. At level of significance  $p \leq 0.05$  regarded as statistical significant.

**Result**

The etiology of ESRD was DM in 37%, hypertension in 30%, obstructive uropathy in 7%, glomerular disease in 4%, autosomal dominant polycystic kidney disease in 4%, vasculitis in 3%, and interstitial nephritis in 3% and unknown causes in 12% (Table 1).

**Table 1. Primary renal diseases causing end stage renal disease and the response to HB vaccine related to number of doses.**

Causes of chronic renal failure	Number	Response to hepatitis B vaccine					Total	p value
		1 <sup>st</sup> dose	2 <sup>nd</sup> dose	3 <sup>rd</sup> dose	4 <sup>th</sup> dose	≥4 doses		
Diabetes mellitus	37	4	3	5	6	4	22	0.0221
Hypertension	30	3	6	4	3	2	18	1.745
Obstructive uropathy	7	-	2	1	-	1	4	4.757
Glomerular disease	4	1	1	1	-	1	3	6.016
ADPK	4	1	2	-	-	-	3	6.016
Vasculitis	3	-	-	-	1	1	2	1.370
Interstitial nephritis	3	-	-	2	-	-	2	1.0259
unknown	12	1	4	2	-	2	9	5.4228
<b>Total</b>	<b>100</b>	<b>9%</b>	<b>18%</b>	<b>15%</b>	<b>10%</b>	<b>11%</b>	<b>63%</b>	

ADPK=autosomal dominant polycystic kidney disease

Responded to 1st dose vaccination 9% of patients with end stage renal disease, 18% responded to the second dose, 15% responded to the third dose, 10% responded to fourth dose of vaccination and 11% response to vaccination for more than four doses of vaccination.

In the current study, thirty one percent (31%) of patients were anti- HCV positive; 18 (58%) were responding to hepatitis B vaccination and 13 (42%) not response to Hepatitis B vaccination. There were variations in response to hepatitis B vaccine in relation to other parameter; age, sex, HCV status and DM, have no effect on response to hepatitis B vaccine ( $p = 0.1072, 0.1711, 0.4932, 0.2388$ , respectively) while the Hb level, serum calcium, serum albumin and duration of dialysis showed significant effect on the response to hepatitis B vaccine in ESRD ( $p = 0.0323, 0.0076, 0.0002, 0.0013$ , respectively) as mention in table 2.

**Discussion**

With the introduction of hepatitis B vaccine in the 1980, it was hoped that HBV would be eliminated from dialysis population. Although HBV has not been eradicated yet, the vaccine has helped to reduce the incidence further, but with suboptimal efficacy in patient with CRF. Currently available hepatitis B vaccines have an excellent safety and immunogenicity profile, conferring seroprotection in more than 95% of the vaccinated population<sup>(14)</sup>. The rate of seroconversion to hepatitis B vaccine among individuals with CRF on HD in current study is 63%, near to other study by Hashim et al<sup>(15)</sup> in Iran who was found 78%. Bel'eed et al<sup>(16)</sup> also found that seroconversion rates were similar in HD patients (66%; 90/136). In another local study in Iraq, it was found that 77.7% of vaccinated subjects were apparently healthy after receiving the full course of vaccination and had protective titer of anti-HBS<sup>(17)</sup>.



**Table 2. Hepatitis B vaccine responses to clinical and laboratory parameters**

Characteristic		Response to vaccination		p value
		Yes	No	
Gender	Male	32	24	0.171
	Female	31	13	
Age (years)	> 40	44	20	0.107
	< 40	19	17	
Hepatitis C virus	Positive	18	13	0.493
	Negative	45	24	
Serum calcium	≥ 8.5 mg	22	20	0.007
	≤ 8.5 mg	50	17	
Serum albumin	≥ 3.5 mg	55	20	0.000
	≤ 3.5 mg	8	17	
Hemoglobin level	≥ 10 g/dL	15	22	0.032
	≤ 10 g/dL	13	15	
Duration of dialysis (months)	≥ 6	46	15	0.001
	< 6	17	22	
Diabetes mellitus		22	17	0.238

Lower responsiveness to hepatitis B vaccination occurs despite recommendations to use higher vaccine doses (40µg) in HD patients than in general population. There are many explanation for this may be duo to immunocompromised patients, delaying vaccination until ESRD, genetic factor, poor complain with dialysis, route of administration of the vaccine by intramuscular rather than intradermal and generation of vaccine.

Elderly and male patients show similar response to the young age and female patients ( $p = 0.171$  not significant) in contradiction to the study of McNulty<sup>(18)</sup> who noticed lower rate of seroconversion in elderly and male patients.

In the current study, patients with HCV-infection did not show a significant decrease in their response rates among HCV-infected versus non-infected patients ( $p = 0.493$ ). Most of the patients who responded to vaccination of hepatitis B vaccine have controlled Hb (above 10g/dL) in comparison to others who not responded to vaccination ( $p = 0.032$ ) duo to the treatment with rHuEPO that increases antibody titers after hepatitis B vaccination in dialysis patients. There is much evidence

suggesting that rHuEPO may influence the immune response because of its effects on the cells of the humeral and cellular immune system<sup>(19)</sup>.

The patients who responded to vaccination have well controlled calcium level ( $p = 0.007$ ). This is supported by other study that showed vitamin D deficiency is associated with poor response to active hepatitis B immunization in patients with CKD<sup>(20)</sup>.

Normal serum albumin was found to enhance the response to hepatitis B vaccine in HD patients ( $p = 0.0002$ ) duo to good nutritional state. This sis also noticed by Fabrizi<sup>(6)</sup>.

Long duration of HD showed good responses to hepatitis B vaccine ( $p = 0.001$ ) because patients are receiving more doses of vaccination (more than five doses), good nutritional state and control on other parameter such as albumin, calcium and hemoglobin.

In conclusion, viral hepatitis continues to be a relevant topic for HD centers, although the number of infected dialysis patients is declining in most countries. Immune response to hepatitis B vaccine affected by well control of Hb, calcium, albumin and long duration on HD.

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E-mail: drjawadkadhemi@yahoo.com

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## Effects of Metformin alone, Metformin with Flaxseeds Oil on Serum 1,5 anhydroglucitol, Adiponectin and Insulin Resistance in Patients with Type 2 Diabetes Mellitus

Shatha H. Mohammad<sup>1</sup> MSc, Ahmed R. Abu-Raghib<sup>2</sup> PhD, Nabeel N. Fadhil<sup>3</sup> FRCP

<sup>1</sup>Dept. of Pharmacology, <sup>3</sup>Dept. of Internal Medicine, College of Medicine, Nineveh University <sup>2</sup>Dept. of Pharmacology, College of Medicine, Al-Nahrain University

### Abstract

**Background** Adiponectin is an amino acid collagen-like protein that is secreted by adipocytes to acts as a hormone with anti-inflammatory and insulin-sensitizing properties. 1,5 anhydroglucitol, is 1-deoxy form of glucose, is a validated marker of short-term glycemic control. Flaxseed oil is a colorless to yellowish oil obtained from the dried, ripened seeds of the flax plant (*Linum usitatissimum*, L).

**Objectives** To investigate the effects of metformin alone, metformin with flaxseed oil on fasting serum 1,5 anhydroglucitol, adiponectin and insulin resistance.

**Methods** Newly diagnosed ( $\leq 1$ year) male and female patients with type 2 diabetes mellitus aged 25 to 70 years were enrolled and divided into two groups; group 1 consisted of 32 patients, treated by oral metformin alone over a period of 12 weeks and group 2 consisted of 30 patients treated by oral metformin with flaxseed oil. Fasting serum 1,5 anhydroglucitol, adiponectin, fasting plasma glucose and fasting serum insulin were estimated. All parameters were measured initially, before any intervention, and later on at two steps, the 6th and the 12<sup>th</sup> week of the study.

**Results** After 12 weeks of treatment with metformin alone, metformin with flaxseed oil there was significant improvement in both 1,5 anhydroglucitol ( $p = 0.010$  and  $0.013$ , respectively) and adiponectin ( $p = 0.041$  and  $0.037$ , respectively). For insulin resistance,  $p$  value with metformin, metformin with flaxseed oil was  $0.105$  and  $0.110$ , respectively. Both results showed an apparent improvement only which was statistically insignificant.

**Conclusion** Metformin, metformin with flaxseed oil associated with statistically significant improvement in 1,5 anhydroglucitol and adiponectin. Insulin resistance in both treatment groups showed only insignificant apparent improvement. Metformin with flaxseed oil group was more effective in insulin resistance improvement and elevation of adiponectin hormone.

**Key words** Adiponectin, 1,5 anhydroglucitol, Homeostasis model assessment for insulin resistance.

**List of abbreviation:** T2DM = type 2 diabetes mellitus, FSO = flaxseed oil, 1,5AG = 1,5 anhydroglucitol, ADP = adiponectin, HOMA-IR = Homeostasis model assessment of insulin resistance.

### Introduction

Type 2 diabetes mellitus (T2DM) is a metabolic disorder characterized by high blood glucose in the context of insulin resistance and relative insulin deficiency<sup>(1)</sup>. T2DM accounts for ~90-95% of cases of

diabetes<sup>(2)</sup>. Metformin has been the most recommended monotherapy of T2DM<sup>(3,4)</sup>.

Maida et al<sup>(5)</sup> and Zhou et al<sup>(6)</sup> reported that the activation of adenosine monophosphate - activated protein kinase by metformin in the liver, and probably in other tissue provides a unified explanation for the pleiotropic beneficial effects of this drug.

Flaxseed oil (FSO), also known as linseed oil, is a colorless to yellowish oil obtained from the

dried, ripened seeds of the flax plant (*Linum usitatissimum*, L) <sup>(7)</sup>. Many of flaxseed's health effects are attributed to its alpha-linolenic acid lignan and fiber components <sup>(8)</sup>. The anti-diabetogenic property of flaxseed is the active fraction: LU6.

Obesity is a major public health problem and it increases insulin resistance, reactive oxygen species (ROS) generation and nuclear factor NF- $\kappa$ B activation <sup>(9)</sup>. The increase in NF- $\kappa$ B activation leads to low grade inflammation and contributes to the development of diabetes <sup>(10)</sup>. 1,5 anhydroglucitol (1,5AG) was first discovered in the plant family *Polygala senegain* 1888. The presence of the compound in human blood <sup>(11)</sup> and cerebrospinal fluid was established in 1972 and 1973, respectively. 1,5-AG, is 1-deoxy form of glucose, is a major metabolically inert circulating polyol arising primarily from ingestion and excreted competitively with glucose <sup>(12)</sup>. Research studies have shown that 1,5AG originates mostly from foods with a mean daily intake of ~4.4 mg/day. The rate of intake is matched by the rate of daily excretion with a bodily pool of about 500–1000 mg of 1,5AG is constantly maintained <sup>(13)</sup>.

1,5AG is a validated marker of short-term glycemic control and its levels in blood respond within 24 hour as a result of glucose's competitive inhibition of 1,5AG reabsorption in the kidney tubule <sup>(14)</sup>.

Adiponectin (ADP) is a 244–amino acid collagen-like protein that is solely secreted by adipocytes to acts as a hormone with anti-inflammatory and insulin-sensitizing properties <sup>(15)</sup> and to be involved in cardiovascular tone. Obesity caused down-regulation of adiponectin which is the mechanism whereby obesity could cause insulin resistance and diabetes <sup>(15)</sup>. The high molecular weight oligomer of ADP has been implicated as the major active form responsible for the insulin-sensitizing effects of ADP in the liver and peripherally than total ADP levels <sup>(16)</sup>.

ADP may decrease the risk of T2DM, including suppression of hepatic gluconeogenesis,

stimulation of fatty acid oxidation in the liver, stimulation of fatty acid oxidation and glucose uptake in skeletal muscle, and stimulation of insulin secretion <sup>(15,17)</sup>.

ADP receptors have been cloned in the skeletal muscle (AdipoR1) and liver (AdipoR2), AdipoR1 and AdipoR2 have been shown to mediate adenosinmonophosphate-activated protein kinase, peroxisome proliferator-Activated receptor-alpha (PPAR- $\alpha$ ) ligand activities, glucose uptake and fatty-acid oxidation by adiponectin <sup>(18)</sup>.

T2DM is caused by a combination of progressive  $\beta$ -cell dysfunction, relative insulin deficiency, and variable degrees of insulin resistance that lead to dysregulation of glucose homeostasis <sup>(19)</sup>. Homeostasis model assessment of insulin resistance (HOMA-IR) is a mathematical model which can estimate an individual's degree of insulin sensitivity (HOMA % S) and level of beta cell function (HOMA % B) from simultaneous measurements of fasting plasma glucose and insulin or C-peptide concentrations.

IR is a condition in which normal amounts of insulin are inadequate to produce normal responses from fat, muscle (promote glucose uptake) and liver (inhibit glucose output) cells <sup>(20)</sup>. It is a major hallmark in the development of T2DM <sup>(21)</sup>. In non-diabetic individuals the best HOMA-IR cut-off levels ranged from 1.85 in men to 2.07 in women. A lower cut-off value for diabetic than non-diabetic individuals ranged from 1.60 in men to 2.05 in women probably because in the diabetic population there is an increased prevalence of hypertension, obesity, and dyslipidemia <sup>(22)</sup>.

The objective of the study is to investigate the effects of metformin alone, metformin with FSO on fasting serum 1,5AG, adiponectin and HOMA-IR.

## **Methods**

This study is a randomized, single blinded interventional, dose escalation study of 12 weeks treatment duration, comparative and prospective study. The study was conducted on

adult patients with T2DM attending the Diabetic and Endocrine Diseases Clinic in Mosul city, Iraq over the period from March 3, 2014 through February 15, 2015.

The study concept and design were approved by the Institute Review Board of the College of Medicine, Al-Nahrain University. The study included newly or recently diagnosed ( $\leq 1$  year) male and female patients ( $N = 62$ ) with T2DM whose ages ranged between 25 and 70 years.

The study excluded patients who were known to have hepatobiliary diseases, hypothyroidism, chronic kidney diseases or nephrotic syndrome, cigarette smoking, the use of any glucose altering medications, such as oral contraceptive pills, diuretics, steroids and neuroleptics during the last month. In addition, pregnant or lactating women, patients with hematological abnormalities such as hemolytic anemia.

A 30, apparently healthy, volunteers whose age matched enrolled patients were involved. All the enrolled participants were informed about the aim of the study and an written consent was obtained from each of them. Thereafter, the patients were divided into two groups as follows:

**Group 1:** consisted of 32 patients, treated by oral metformin alone (merk-Germany), 500 mg b.d initially and the dose was adjusted according to the glycosylated hemoglobin and fasting plasma glucose readings over a period of 12 weeks, which is the period of the study.

**Group 2:** consisted of 30 patients treated by metformin plus FSO in a form of soft gel capsules obtained from a local source in Mosul city (Al-Emad Factory). Each flaxseed capsule contains 500 mg of FSO. The dose of flaxseed was two capsules given in a single dose (1 g) after lunch.

Thereafter, each patient instructed to have fasting serum 1,5AG, ADP, fasting plasma glucose and fasting plasma insulin. Standard kits were used to measure biochemical profiles suggested in this study using double-sandwich ELISA technique for both 1,5AG and ADP. Both fasting plasma glucose and fasting plasma

insulin that required in HOMA-IR equation ( $\text{HOMA-IR} = \text{fasting plasma glucose (mg/dl)} \times \text{fasting plasma insulin } (\mu\text{U/L})/405$ ) was measured by an enzymatic immunoassay technique. An initial physical examination was conducted and all the above parameters were assessed initially, before any intervention, and later on at two steps, the sixth and the twelfth week of the study time. The data were recorded in specially preformed case record.

### Statistical Analysis

The statistical analysis was carried out using Statistical Package for the Social Science (SPSS); version 21. Descriptive statistic; mean  $\pm$  standard deviation ( $\pm\text{SD}$ ), was used to describe numerical values<sup>(23)</sup>. The differences between the means were considered significant at the 5% confidence level and the level of significance was set at  $p < 0.05$ ,  $p < 0.01$  and  $p < 0.001$  as significant, highly significant and very highly significant respectively.

The inferential statistics; one way analysis of variance (ANOVA) followed by T test comparison *t*-test for one sample was used to compare between parameters within treatment groups and to compare the same parameter with its analogue in other treatment groups. Independent *t*-test was used to compare between the results of studied parameters obtained at the 12<sup>th</sup> week from treatment for each group with their corresponding at the controls.

### Results

The patients were 62; 33 males and 29 females. Their ages ranged between 33 and 70 years with a mean age  $\pm\text{SD}$  ( $49.5 \pm 7.93$ ) for females and ( $46.5 \pm 11.57$ ) for males. The BMI on initial visit in general was ( $33.51 \pm 5.85$ ).

After 12 weeks of treatment with metformin alone, there was significant improvement in 1,5 AG with mean  $\pm\text{SD}$  at base line level and at week 12 was ( $7.4 \pm 1.5 \rightarrow 10.5 \pm 0.7$ ). A significant rise in ADP with mean  $\pm\text{SD}$  before and after treatment was ( $9.0 \pm 3.6 \rightarrow 18.3 \pm 4.3$ ). HOMA-IR improved, mean  $\pm\text{SD}$  was ( $14.7 \pm$

8.4 → 3.9 ± 1.8) however, and these alterations were statistically insignificant (Table 1).

**Table1. Effect of treatment by metformin on 1,5 anhydroglucitol, adiponectin, and HOMA-IR from baseline and at the 6<sup>th</sup> and 12<sup>th</sup> week of treatment**

Duration	HOMA	1-5AG(µg/ml)	ADP (ng/ml)
Base line	14.7 ± 8.4	7.4 ± 1.5	9.0 ± 3.6
Week 6	8.1 ± 4.3	9.2 ± 1.5	12.1 ± 3.9
Week 12	3.9 ± 1.8	10.5 ± 0.7	18.3 ± 4.3
<i>p</i> value	0.105	0.010	0.041

1-5AG = 1-5 anhydroglucitol, ADP = adiponectin

On comparing the results of metformin at the 12<sup>th</sup> week with the control group, the parameters didn't approach the control group values (Table 2).

**Table 2. Comparison between metformin group and control group in regard to HOMA-IR, 1-5 anhydroglucitol, adiponectin at the 12<sup>th</sup> week of treatment**

Group	HOMA	1-5AG (µg/ml)	ADP (ng/ml)
Treated	3.9 ± 1.8	10.5 ± 0.7	18.3 ± 4.3
Control	3.7 ± 1.5	25.9 ± 8.6	23.8 ± 4.3
<i>p</i> value	0.000	0.000	0.000

1-5AG = 1-5 anhydroglucitol, ADP = adiponectin

After 12 weeks of treatment by metformin with flaxseed, there was significant improvement in 1,5AG with a mean ±SD before and after treatment was (6.9 ± 1.5 → 10.4 ± 0.8). A significant rise in ADP (9.4 ± 4.1 → 18.4 ± 4.9)

However, HOMA-IR (mean±SD) was (20.2 ± 10.2 → 5.1 ± 3.2), showed an apparent improvement only which was statistically insignificant (Table 3).

**Table 3. Effect of treatment by metformin with flax seed oil on HOMA, 1-5 anhydroglucitol, adiponectin from baseline and at 6<sup>th</sup> and 12<sup>th</sup> week of treatment**

Duration	HOMA	1-5AG (µg/ml)	ADP (ng/ml)
Base line	20.2 ± 10.2	6.9 ± 1.5	9.4 ± 4.1
Week 6	11.0 ± 5.1	9.0 ± 1.4	12.3 ± 4.2
Week 12	5.1 ± 3.2	10.4 ± 0.8	18.4 ± 4.9
<i>p</i> value	0.110	0.013	0.037

1-5AG = 1-5 anhydroglucitol, ADP = adiponectin

On comparing effect of therapy by metformin with FSO after 12 weeks of treatment with the control group values, it was shown that the parameters didn't approach the control group values (Table 4).

The effects of metformin alone, metformin with flaxseed on 1,5 AG showed statistically significant improvement (Table 5).

**Table 4. Comparison between metformin and flax seed oil group with control group in regard to HOMA, 1-5 anhydroglucitol, adiponectin at the 12<sup>th</sup> week of treatment**

Group	HOMA	1-5AG ( $\mu\text{g/ml}$ )	ADP (ng/ml)
Treated	5.1 $\pm$ 3.2	10.4 $\pm$ 0.8	18.4 $\pm$ 4.9
Control	3.7 $\pm$ 1.5	25.9 $\pm$ 8.6	23.8 $\pm$ 4.3
<i>p</i> value	0.036	0.000	0.000

1-5AG = 1-5 anhydroglucitol, ADP = adiponectin

**Table 5. Effect of treatment by metformin, metformin with flaxseed oil on 1-5 anhydroglucitol from baseline and at the 6<sup>th</sup> and 12<sup>th</sup> week of treatment**

Duration	Metformin	Metformin + flaxseed oil	<i>p</i> value (t-test)
Base line	7.4 $\pm$ 1.5	6.9 $\pm$ 1.5	0.000
Week 6	9.2 $\pm$ 1.5	9.0 $\pm$ 1.4	0.000
Week 12	10.5 $\pm$ 0.7	10.4 $\pm$ 0.8	0.001
<i>p</i> value (t-test)	0.010	0.013	(F test) 0.000

HOMA-IR among both groups did not show significant improvement although there were marked reduction by using metformin alone, metformin with flaxseed (14.7  $\pm$  8.4  $\rightarrow$  3.9  $\pm$  1.8), and (20.2  $\pm$  10.2  $\rightarrow$  5.1  $\pm$  3.2) respectively.

However, this apparent improvement was most marked in metformin with flaxseed group (improvement of HOMA-IR by 15.2) and least in metformin alone group (improvement of HOMA-IR by 10.8) as shown in table 6.

**Table 6. Effect of treatment by metformin, metformin with flaxseed oil on HOMA from baseline and at the 6<sup>th</sup> and 12<sup>th</sup> week of treatment**

Duration	Metformin	Metformin + flax seed oil	<i>p</i> value (t-test)
Base line	14.7 $\pm$ 8.4	20.2 $\pm$ 10.2	0.009
Week 6	8.1 $\pm$ 4.3	11.0 $\pm$ 5.1	0.011
Week 12	3.9 $\pm$ 1.8	5.1 $\pm$ 3.2	0.010
<i>p</i> value (t-test)	0.105	0.110	(F test) 0.001

Metformin and metformin with flaxseed significantly increased ADP level (9.0  $\pm$  3.6  $\rightarrow$

18.3  $\pm$  4.3) and (9.4  $\pm$  4.1  $\rightarrow$  18.4  $\pm$  4.9) respectively (Table 7).

**Table 7. Effect of treatment by metformin, metformin with flaxseed oil on ADP from baseline and at the 6<sup>th</sup> and 12<sup>th</sup> week of treatment**

Duration	Metformin	Metformin + flax seed oil	<i>p</i> value (t-test)
Base line	9.0 $\pm$ 3.6	9.4 $\pm$ 4.1	0.001
Week 6	12.1 $\pm$ 3.9	12.3 $\pm$ 4.2	0.007
Week 12	18.3 $\pm$ 4.3	18.4 $\pm$ 4.9	0.003
<i>p</i> value (t-test)	0.041	0.037	(F test) 0.001

ADP = adiponectin

## Discussion

After 12 weeks of treatment with metformin alone, there was significant elevation in 1,5AG. To the best of our knowledge, no comparable data exist on the effects of metformin on 1,5AG. In this ADP was increased after treatment with metformin for 12 weeks. Adamia *et al*<sup>(24)</sup>, reported an increment in the ADP after 6 months of metformin therapy together with significant reduction in HOMA-IR and the magnitude of the change in ADP levels positively correlated with the magnitude of IR reduction.

On the contrary, Fujita *et al*<sup>(25)</sup> found that after 4 weeks of treatment with metformin, the serum ADP levels were not significantly elevated in metformin-treated patients, which might indicate that the 4 weeks period might not be enough for metformin to exert a change in ADP serum level.

Moreover, Cannon *et al*<sup>(26)</sup> observed significant lowering of ADP levels following 4 months treatment with metformin.

Metformin-mediated improvements in insulin sensitivity may be associated with several mechanisms, including increased insulin receptor tyrosine kinase activity, enhanced glycogen synthesis, and an increase in the recruitment and activity of GLUT4 glucose transporters<sup>(27)</sup>.

In our study, HOMA-IR was apparently reduced; however, this attenuation was statistically insignificant. In accordance to our study, Moghetti *et al*<sup>(28)</sup>, Ponssen *et al*<sup>(29)</sup> and Freemark and Bursey<sup>(30)</sup> demonstrated that metformin induces significant reduction in fasting plasma insulin and increased insulin sensitivity; and hence a significant reduction in HOMA-IR. In contrast, Pau *et al*<sup>(31)</sup> where metformin did not improve insulin sensitivity but just improve glucose effectiveness. Furthermore, Shaker *et al*<sup>(32)</sup>, after three months treatment with metformin, showed a significant reduction in serum concentrations of insulin and HOMA-IR.

After 12 weeks of treatment by metformin with FSO, there was significant improvement in 1,5

AG. To the best of our knowledge, no comparable data about the effect of flaxseed oil on fasting serum 1,5 AG exists.

In the current study, HOMA-IR was insignificantly increased. In congruence with our results, Barre *et al*<sup>(33)</sup> showed that flaxseed oil addition (10g/day) had no impact on insulin levels. Likewise, Taylor *et al*<sup>(34)</sup> studied the effect of 12 weeks treatment with metformin and FSO and found that fasting plasma insulin was unchanged. In agreement with our study, Viguiliouk *et al*<sup>(35)</sup> found that flaxseed oil diet produced no significant effect for fasting insulin and HOMA-IR.

Nelson *et al*<sup>(36)</sup>, studied the effect of metformin with FSO for 8 weeks; they disclosed no significant changes in fasting insulin, or quantitative insulin sensitivity check index values as a result of this intervention.

On the contrary, Rhee and Brunt<sup>(8)</sup> determined the antioxidant activity of flaxseed and its role in inflammation and insulin resistance in obese glucose intolerant people. They used a randomized crossover design, with 12 weeks treatment with FSO and metformin. HOMA-IR decreased and plasma insulin concentration significantly reduced compared to baseline data.

After 12 weeks of treatment by metformin with flaxseed, there were significant rise in ADP level. In agreement with our result, Sekine *et al*<sup>(37)</sup> suggested that  $\alpha$ -linolenic acid-rich FSO intake might exhibit beneficial effects through an increase of the ADP level. The experimental period with metformin and FSO was 4 weeks. Pan *et al*<sup>(38)</sup> in their study using flaxseed found that ADP level was associated with significant increases as well. However, Paschos *et al*<sup>(39)</sup> using flaxseed oil rich in  $\alpha$ -linolenic acid (8.1 g/day) for 12 weeks found the ADP plasma levels did not changed after the increase in dietary intake of  $\alpha$ -linolenic acid in the flaxseed oil supplementation group. On contrary, Nelson *et al*<sup>(36)</sup> who studied the effect of metformin and FSO for 8 weeks disclosed significant decreases in ADP after the intervention.



As a conclusion, metformin, metformin with flaxseed oil associated with statistically significant improvement in 1,5 AG and ADP. HOMA-IR in both treatment groups showed only insignificant apparent improvement. Metformin with flaxseed oil group was more effective in HOMA-IR improvement and elevation of ADP hormone.

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### Author contribution

All authors contribute equally in the literature review and drafting this paper.

### Conflict of Interest

We declare no conflict of Interest

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None.

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**Correspondence to Shatha H. Mohammad**

**E-mail: shth\_mohamad@yahoo.com**

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## Association of ATG16L1 T300A Genetic Variant with *H. pylori* and None *H. pylori* Atrophic Gastritis

Haider F. Ghazi PhD

Dept. of Microbiology, College of Medicine, Al-Nahrain University, Baghdad, Iraq.

### Abstract

- Background** *Helicobacter pylori* persistence may develop atrophic gastritis, gastric ulcer or cancer.
- Objectives** To determine the association between the presence of autophagy related gene 16 like 1 and threonine 300 alanine mutation and *H. pylori* infection among atrophic gastritis patients.
- Methods** Gastric biopsy was taken from eighty patients and tested for urease, and blood samples were taken for serum separation for detection of Anti-*H. pylori* IgG by ELISA and DNA extraction from whole blood were used for sequence specific primer – polymerase chain reaction for autophagy related gene 16 like 1 and threonine 300 alanine mutation allelic discrimination.
- Results** Among 40 *H. pylori* positive cases, the carriers of mutated allele were 62.5% compared with 36.25% in *H. pylori* negative cases ( $p = < 0.001$ , OR = 1.72, CI = 1.23-2.42).
- Conclusions** Among Iraqi atrophic gastritis, there is an association between *H. pylori* infection and mutated autophagy related gene 16 like 1 and threonine 300 alanine mutation allele. Threonine 300 alanine may confer higher risk for infection with *H. pylori*.
- Key words** Autophagy, *H. pylori*, atrophic gastritis.

**List of abbreviations:** *H. pylori* = *Helicobacter pylori*, Vac-A = Vacuolating cytotoxin-A, SNP = single nucleotide polymorphism, ATG16L1 =, OR = odd ratio, CI = Confidence interval.

### Introduction

*Helicobacter pylori* (*H. pylori*) infection remains the most common cause for chronic gastritis in humans that may develop to gastric ulcer or cancer<sup>(1)</sup>. The precise mechanisms by which *H. pylori* exploit host cell machineries for intracellular survival are poorly understood<sup>(2)</sup>. However, *H. pylori* able to enter and embed in the mucus, attach to gastric epithelium, evade immune responses, and persistently colonize there<sup>(3,4)</sup>. Among several virulence factors, Vacuolating cytotoxin-A (Vac-A) can modulate disease pathogenesis. It has been found that Vac-A can contribute to increase persistence of infection

through inactivation of cellular autophagy and promoting *H. pylori* survival inside gastric epithelia and macrophage<sup>(3)</sup>. Studies described that Vac-A can induce the formation of large autophagic-like vesicles. Over the last decade, several research groups have independently reported that infection by *H. pylori* can inhibit autophagy<sup>(5)</sup>.

Autophagy is an intracellular mechanism by which host cells can eliminate microbes<sup>(6)</sup>. However, some pathogens have the capacity to escape from autophagy processes as a strategy for increasing intracellular survival<sup>(7)</sup>. It has been proposed that *H. pylori* once internalized and sequestered in double-membrane autophagosomes, can use these compartments as a replicative niche. Genetic studies have confirmed that a single nucleotide

polymorphism (SNP) in the autophagy related gene 16 like 1 (ATG16L1) confer an increasing risk for intracellular survival of several microbes due to impaired phagosome-lysosome fusion and secretion of antimicrobial peptides<sup>(8)</sup>.

Considering the capacity for colonization, and the persistence in gastric tissue, it is theoretically plausible that ATG16L1 threonine 300 Alanine (T300A) genetic variant, that the resultant protein reduce autophagic responses to Vac-A and increased susceptibility to infection with an *H. pylori* Vac-A suggesting that it facilitates chronic inflammation.

This study aims to investigate the association between the presence of mutated ATG16L1 allele and infection with *H. pylori* among gastritis patients.

## Methods

### Study subjects

This cross-sectional study involved eighty patients suffering from gastritis were recruited from the gastroenterology units in Gastroenterology and Hepatology Teaching Hospital and Al-Emamain Al-Kadhemain Medical City during the period of September 2013- August 2014. Patients were undergone gastroscopy due to clinical indications and the IgG anti-*H. pylori* antibody examination and rapid urease test was performed in order to identify the presence of *H. pylori*.

### Genotyping of ATG16L1 T300A by Sequence Specific Primer-Polymerase Chain Reaction (SSP-PCR)

The steps of DNA extraction and genotyping were done in molecular biology laboratory / department of Microbiology/ AL-Nahrain University. DNA was extracted from 300µl peripheral blood EDTA containing tubes using DNA isolation kit (Wizard®, Promega, USA) following manufacturer information. The DNA concentration ranged from (85-120) and purity (1.7-1.84). Allelic discrimination of substitution mutations of Adinin with Guanine was checked by SSP-PCR. DNA from study groups individuals were amplified by using two sequence specific

primers in two separated reaction mixtures, to give a PCR products of 201bp in positive reaction for allele A or allele G, allowing discrimination of homozygous or heterozygous genotype.

The sequence of primers customized as Forward allele A: 5'-CCCCAGGACAATGTGGATA<sup>3</sup>, Forward allele G 5'-CCCCAGGACAATGTGGATG<sup>3</sup> and common reverse 5'-AGGTGGAAAGGCTTGATATAAG<sup>3</sup>. Detection of β-globin gene considered as internal control. For each reaction of allele A or G or internal control 0.3 µl of each primer (forward and reverse) added to pre-mix PCR tube (Promega, USA) and 0.5-3 µl of genomic DNA and complete reaction volume to 20 µl by DNase free water (Fig. 1).

PCR reaction tubes were transferred into thermal cycler (eppendroff-thermal cycler, Germany), that was programmed as following in (separated PCR-runs-for each allele): 96°C for 1min (X1), (96°C 20s, 72°C) for 1min 10s (X5), 96°C for 25s, 69°C for 50s, 72°C for 30s (X21), 96°C for 30s, 59°C for 1min and 72°C for 1 min and 30s (X4) then PCR products were electrophoresed in 2% agarose gel.

### Statistical analysis

The statistical analysis was done by using Graphpad PRISM® version 6. Crosstab model used to estimate association of allelic variant among study groups and relative risk (RR) and corresponding 95% confidence intervals were estimated.

## Results

### Demographic, anthropometric and serologic data of enrolled patients

As shown in Table 1, the age and gender type were distributed between patients from two groups without statistical significant difference ( $p > 0.05$ ). The results in Table 1 showed that there are no statistical significant difference in the mean of age Likely, smoking habit, vomiting, antibiotic therapy, anti-acid therapy, H<sub>2</sub> blocker and NSAID all of then does not reach the statistical significance value. Proton pump inhibitor showed a statistical significance in

which *H. pylori* negative cases were 21(52.5%) using PPI ( $p < 0.05$ ).

#### Association ATG16L1 Thr300Ala allelic variant with *H. pylori* infection

The allelic frequencies were presented in Table 2. The carriage of mutated allele was

statistically significant higher in *H. pylori* positive cases 62.5% compared with 36.25% in *H. pylori* negative ( $p = < 0.001$ , OR = 1.72, CI = 1.23-2.42) and it was associated with the increased risk for *H. pylori* gastritis.

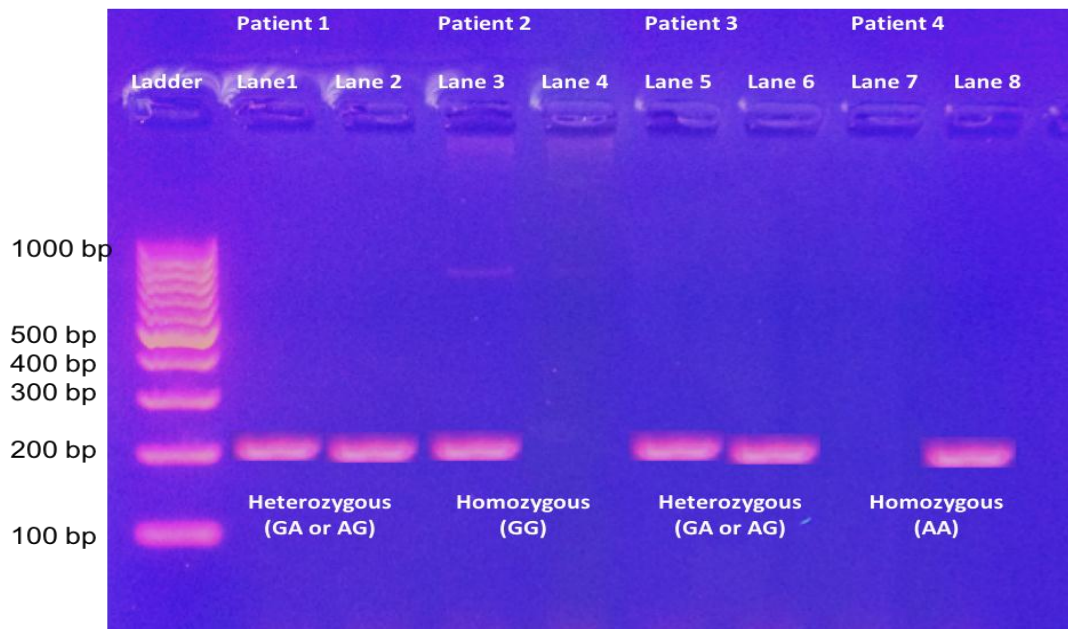


Fig. 1. Agarose gel electrophoretic profiles corresponding to SSP-PCR products, Lane 1 and Lane 2: heterozygous genotype (GA). Lane 3 and Lane 4: homozygous for allele G, Lane 5 and Lane 6: were heterozygous for allele GA, Lane 7 and Lane 8: Homozygous genotype AA. Molecular size of PCR product of allele A or allele G=201bp. Lane 1,3,5 and 7 represents allele G reactions, Lane 2,4,6 and 8 represents allele A reactions.

Table 1. Summary of demographic and clinical description for study groups

Feature	<i>H. pylori</i>	
	Negative N = 40	Positive N = 40
Age Mean±SD	38.7±9.2	41.3±12.4
Gender (Male)	15 (37.5)	17 (42.5)
Fresh blood*	4 (10)	12 (30)
Smoking habit	12 (30)	9 (22.5)
Vomiting	19 (47.5)	18 (45)
Antibiotic therapy	10 (25)	10 (25)
Anti-acid therapy	6 (15)	12 (30)
H <sub>2</sub> blocker	11 (27.5)	18 (45)
NSAID	6 (15)	6 (15)
Proton Pump Inhibitor*	21 (52.5)	12 (30)
Steroid therapy	0 (0)	3 (7.5)
Anti- <i>H. pylori</i> (IgG)	32 (40)	48 (60)

\* = Significant difference ( $p < 0.05$ ), NSAID = non-steroidal anti-inflammatory drugs..

### Discussion

The chronic atrophic gastritis state could contribute to the development of gastric ulcer as well as gastric cancer<sup>(9)</sup>. Several lines of evidence support that *H. pylori* can invade, survive and multiply in both epithelial cells and professional phagocytes *in vitro* and *in vivo*<sup>(10)</sup>. However, after induction of autophagy by *H. pylori*, it can evade autophagy by down-regulating autophagic proteins; alternatively the bacterium can exploit autophagosomes as their intracellular niche or be degraded in autolysosomes<sup>(5,11,12)</sup>.

**Table 2. Allelic frequencies of ATG16L1 T300A in atrophic gastritis patients**

<i>H. pylori</i>	Allele G	Allele A	p value
Positive	50 (62.5%)	30 (37.5%)	0.001
Negative	29 (36.25%)	51 (63.75%)	
Relative risk		1.72	
Confidence interval		1.23-2.42	

The study presents an association between *H. pylori* incidence with the presence of T300A allelic variant (Table 2). However, the substitution mutation in the ATG16L1 gene at the position 300 considered to be as a loss of function mutation with reduced selective autophagy against invading microbes<sup>(8)</sup>. Results in this study have been supported by Raju et al. 2012; when they found that after 24 hrs of VacA exposure to the gastric epithelia would disrupt autophagy by impairing of autophagy and reduction of tissue lysosomal enzymes production<sup>(13)</sup>. Studies demonstrated that susceptibility to *H. pylori* infection were increased in the presence of T300A genetic variant<sup>(14)</sup>. So, the presence of risk allele will modulate autophagy<sup>(15)</sup> resulting in reduced clearance of bacterium in the mucosa or phagocytic cells.

The impaired autophagy could exacerbate chronic gastritis by different mechanisms. First, the microenvironment undergoes secondary

necrosis in the gastric mucosa due to release pro-inflammatory cytokines as a compensatory mechanism<sup>(8)</sup>. These events will release of pro-inflammatory cytokines, including TNF- $\alpha$ , by mononuclear phagocytes, which may further affect tissue pathology. It can be explored by the fact that virulent bacterium could impair dendritic cell response against it<sup>(12)</sup>. This impairment occurs due to autocrine effect and paracrine effect of pro-inflammatory cytokine<sup>(16)</sup>.

The up-regulation of autophagy has been an attractive approach in treatment and prevention of *H. pylori* infection. Several papers have highlighting this address, the using of combination sialic acid and catechins in dose dependent manner by prevention of infection and reducing bacterial number after treatment and reducing bacterial number after treatment<sup>(17)</sup>. Thus, identification of patients whom have mutated ATG16L1 allele may benefit from the using of these compounds in addition to antibiotics. It may be helpful to treat *H. pylori* gastritis and reducing the risk of development to ulcer or even cancer of stomach.

### Acknowledgment

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### Conflict of Interest

There is no conflict of interest.

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**E-mail: [dr.haider.ghazi@colmed-alnahrain.edu.iq](mailto:dr.haider.ghazi@colmed-alnahrain.edu.iq)**

**Mobile: + 964 7706900320**

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## Conventional Methods for the Diagnosis of *Pneumocystis jirovecii* in Immunocompromised Iraqi Patients

Isa S. Touhali<sup>1</sup> MSc, Azhar A.F. Ibrahim<sup>1</sup> PhD, Haider N. Dawood<sup>2</sup> FIBM FABM

<sup>1</sup>Dept. of Microbiology, College of Medicine, Al-Nahrain University, <sup>2</sup>Dept. of Medicine, Al-Imammian Al-Kadhmain Medical City, Baghdad, Iraq

### Abstract

<b>Background</b>	<i>Pneumocystis jirovecii</i> is the causative agent of <i>pneumocystis</i> pneumonia, one of the most frequent and severe opportunistic infections in immunocompromised patients.
<b>Objectives</b>	To determine the possible implication of <i>pneumocystis jirovecii</i> in immunocompromised Iraqi patients with pulmonary infections, and investigate the efficiency of indirect qualitative immunofluorescence diagnosis in compared with conventional stains for the detection of this agent.
<b>Methods</b>	A total of 200 clinical samples from 100 immunocompromised patients (70 bronchoalveolar lavage, 21 sputum samples and 9 pleural fluids). One hundred samples from immunocompetent individuals (50 bronchoalveolar lavage, 30 sputum samples and 20 pleural fluids). Detection of pneumocystosis was done by conventional satins and indirect qualitative immunofluorescence technique.
<b>Results</b>	Fourteen samples gave positive results by indirect immunofluorescence monoclonal antibody test. Twelve out of 100 samples were positive by each Gomori methenamine silver, modified toluidine blue O stains and Diff-Quik stain (modified Giemsa).
<b>Conclusion</b>	<i>Pneumocystis jirovecii</i> is the fundamental opportunistic infection among immunocompromised patients. The indirect qualitative immunofluorescence method gives a promise for use as a primary method for diagnosis of <i>pneumocystis jirovecii</i> pneumonia or as rapid screen to exclude the presence of <i>pneumocystis jirovecii</i> in sputum and bronchoalveolar lavage samples.
<b>Keywords</b>	<i>Pneumocystis jirovecii</i> ( <i>carinii</i> ), <i>pneumocystis jirovecii</i> pneumonia, immunocompromised, Iraqi patients.

**List of abbreviation:** *p. jirovecii* = *pneumocystis jirovecii*, PCP = *Pneumocystis* pneumonia, BAL = bronchoalveolar lavage, GMS = Grocott-Gomori methenamine silver stain, DQS = differential Quik stain, TOB = Toluidine blue, PE = pleural effusion, IS = induced sputum, IFA = indirect Immunofluorescence antibody.

### Introduction

**P***neumocystis jirovecii* (*p. jirovecii*), previously known as *Pneumocystis carinii*) is an unusual opportunistic organism. *P. jirovecii* most commonly causes *Pneumocystis* pneumonia (PCP) in patients with acquired immune deficiency syndrome and patients receiving intensive or prolonged immune suppressive treatment for malignancy, transplantation and immune disorders<sup>(1-3)</sup> which causes a severe and often fatal

pneumonia in immunocompromised individuals<sup>(4)</sup>.

The organism has a unique tropism for the lungs, where it exists primarily as an alveolar pathogen. Individuals with intact immunity control this primary infection, there are no apparent clinical manifestations of primary infection in immunocompetent individuals, and the organism likely remains latent in the lungs for long periods of time, clinically apparent pneumonia occurs when cellular or humoral immunity becomes severely deficient, the organisms proliferate, evoking a mononuclear cell response, alveoli become filled with



proteinaceous material and intact and degenerating organisms<sup>(5,6)</sup>.

*P. jirovecii* inability to culture suggests that it has evolved to require a very specific environment that is not easy to reproduce outside its host<sup>(7)</sup>. The diagnosis of *P. jirovecii* disease requires the demonstration of cysts or trophozoites within tissue or body fluids via colorimetric or immunofluorescent stains since the human organism cannot be cultured *in vitro* and *vivo*<sup>(8)</sup>.

The aims of this study was to determine the possible implication of *P. jirovecii* in a sample of immunocompromised Iraqi patients, and investigate the efficiency of indirect qualitative immunofluorescence diagnosis in comparing with conventional stains for the detection of this agent.

## Methods

### Patient's selection

This study included a total of 200 clinical samples from 100 immunocompromised patients (43 men, 57 women; average age 16-90 years) as 70 bronchoalveolar lavage (BAL), 21 sputum samples and 9 pleural fluids, with different underlying immunocompromised diseases including 22 (22%) leukemia, 17(17%) solid tumor, 15(15%) lymphomas, 12(12%) chronic obstructive pulmonary disease, 10(10%) asthma (steroid therapy), 10(10%) rheumatoid arthritis (cytotoxic therapy), 8(8%) solid-organ transplantation and 6(6%) Multiple myeloma with suspected of pneumocystosis. Control group included 100 samples (50 BAL, 30 sputum samples and 20 pleural fluids) from immunocompetent individuals were collected from in-and out patients who attended of Al-Imammian Al-Kadhmain Medical City, Baghdad teaching Hospital, Baghdad, and Al-Zahra Teaching Hospital, Wasit province and other private laboratories, during the period from May 2014 to March 2015. The ethical aspects of this study have been approved by the Institute Review Board of the College of Medicine, Al-Nahrain University.

### Samples collection

**Bronchialveolar lavage (BAL)** was performed by a bronchofibroscope (STORZ, Germany) wedged in segmental orifice of sedated spontaneous breathing patients or intubated patients, in most cases, 20-50 ml warmed saline was infused into targeted segment followed by gentle suction by specialist physician. BAL fluids were directly collected by sterile syringe. About 10-15 ml were dispensed into sterile test tube and immediately placed on ice then transmitted to the laboratory for processing.

**Induced sputum samples (IS)** were obtained by induction in patients involved in the study. Sputum induction was done using an ultrasonic nebulizer (serial No. 2000, England). This was done in an open space using a 3ml saline as an inducing fluid, from each induced patient by nurse practitioner; this sputum sample (10-15ml) was directly collected by sterile screw cup bottles and immediately placed on ice then transmitted to the laboratory for processing. Induced sputum were divided into two portions and treated with either 0.1% Dithiothreitol or with 0.9% NaCl alone.

**Pleural effusion samples (PE)** was done by aspirating pleural fluid with 25G needle after marking, cleaning the suspected area with antiseptic and then local anesthetic (5-10 ml of 2% lidocaine) was injected locally; this method was done by specialist physician. Ten-15ml pleural fluid was aspirated into sterile test tube and immediately placed on ice then transmitted to the laboratory for further processing.

### Samples processing

BAL, sputum and pleural fluid containing mucous martial were added to a 2-fold volume of 0.9% NaCl and were mixed vigorously vortexes for 5 minutes. Samples centrifuged at 3000 rpm for 5 minutes, supernatants were discarded and the precipitated pellets were placed into a 1.5 ml microcentrifuge tubes according to Alexander *et al*<sup>(9)</sup> several slides

were prepared simultaneously, depending on the number of stains to be employed, with a few spare slides prepared for any repeat stains which might be needed.

A portion of precipitate pellets (100µl) was used to prepare smears for each Diff-Quik stain (SYRBIO®, Syria), Grocott-Gomori methenamine silver stain (abcam®, UK), Toluidine blue stain (BHD, England) and for fluorescent monoclonal antibody test (IF: Detect IF PC, Axis-Shield Diagnostics, Dundee, UK).

## Results

### Descriptive data on study subjects

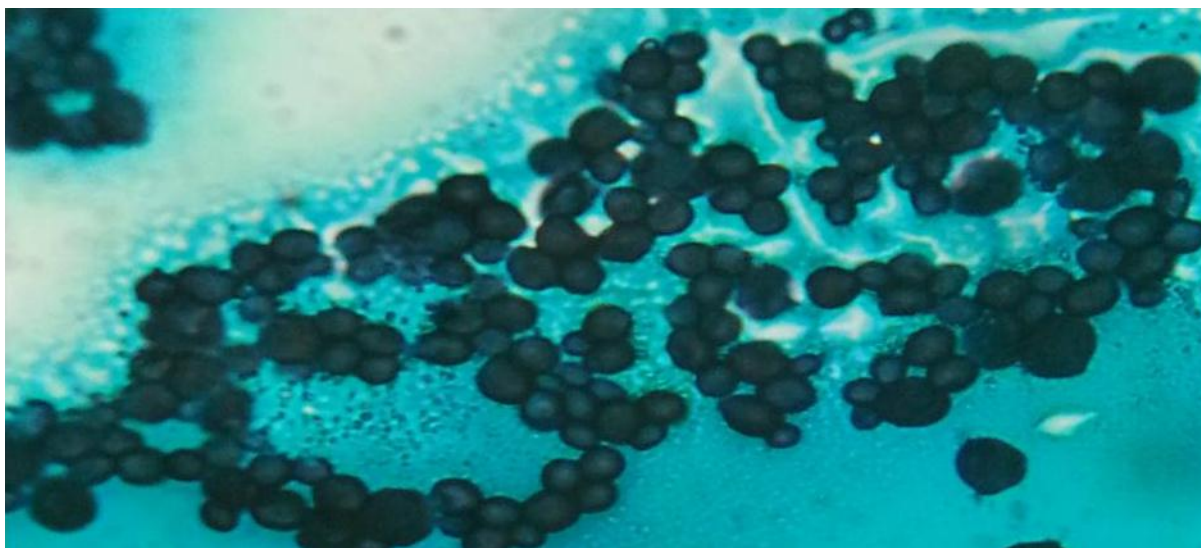
Among those 100 immunocompromised patients, 43 were males and 57 were females whereas in the control group each group comprised of 50 subjects. The age of the

patients ranges from 16-90 years (Mean± S.D = 54.56±16.46 years) as compared to 16-66 years (Mean± S.D = 37.33±13.24 years) of the control group ( $p < 0.001$ ).

### Laboratory Diagnosis of *P. jirovecii*

#### Gomori's Methenamine Silver stain (GMS)

Under light microscope, cysts of *P. jirovecii*, the diagnostic form, had a characteristic appearance as a spherical, cup-shaped, or crescent-shape object stained by GMS which usually crowded in foamy alveolar casts in BAL. GMS used for staining carbohydrates producing gray to black and green background counterstained with light green, this stain is useful for identifying cyst form 12(12%) out 100 samples were positive (Fig. 1).

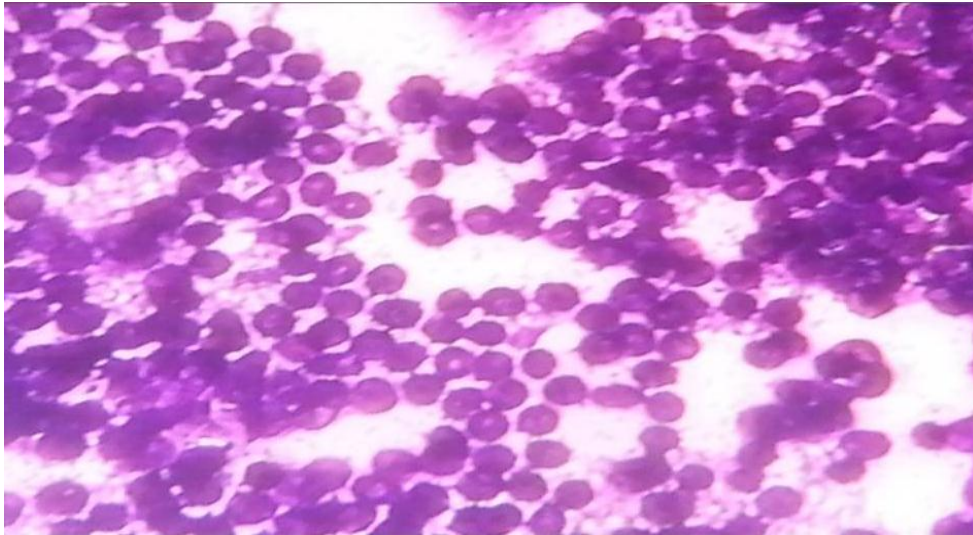


**Fig. 1. Gomori's methenamine silver (GMS) stained smear of BAL from leukemia gray to black color of the fungus is seen against a light green background, the cysts of *P. jirovecii* were crowded in foamy alveolar casts in BAL. The cyst appears as a spherical, cup-shaped, or crescent-shaped object. Some cysts are empty and collapsed; others contain dark bodies or dots, which acquire different positions in relation to the cyst depending on the angle of visualization (X1000).**

#### Modified toluidine blue O Stain (MTolB)

Cyst forms of *P. jirovecii* are cup-shaped appear as violet to purple. The cyst outline is distinct, and the internal region stains uniformly. The

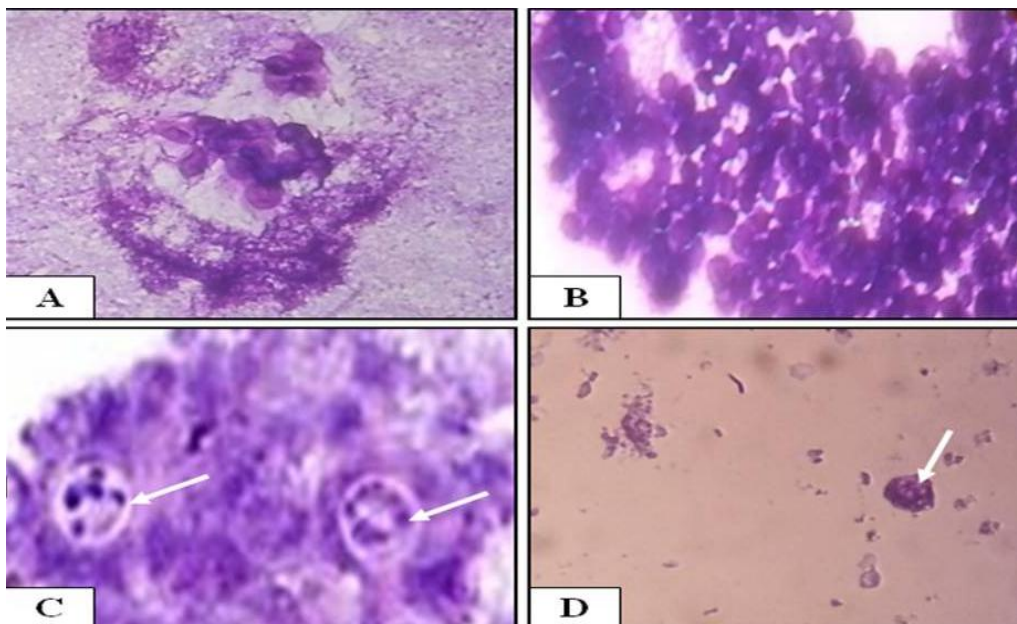
cysts were frequently observed in clusters, while trophic form cannot be stained 12(12%) out 100 samples were positive (Fig. 2).



**Fig. 2. Toluidine blue (TOB) stained smear from BAL of solid tumor, the cyst of *P. jirovecii* forms appear as violet to purple.(X1000).**

**Differential Quik Stain Kit (Modified Giemsa)**  
Specimens were collected and stained with Diff-Quik stain (modified Giemsa). By this technique, it was possible to demonstrate cystic and trophic forms of *P. jirovecii* and

confirm the diagnosis of *Pneumocystis pneumonia* in this patient. Stained slides were examined using a light microscope 12(12%) out 100 samples were positive (Fig. 3).



**Fig. 3. Diff-Quik stain (modified Giemsa). Stained sputum smear from leukemia, the cysts of *P. jirovecii* appear as spherical dark blue (A). Stained BAL smear from asthma, the cysts of *P. jirovecii* appear as aggregated spherical dark blue forms (B). Stained BAL smear from solid tumor (intracystic bodies were arrowed) (C). Stained sputum smear lymphoma, (intracystic bodies were arrowed) (D) X1000).**

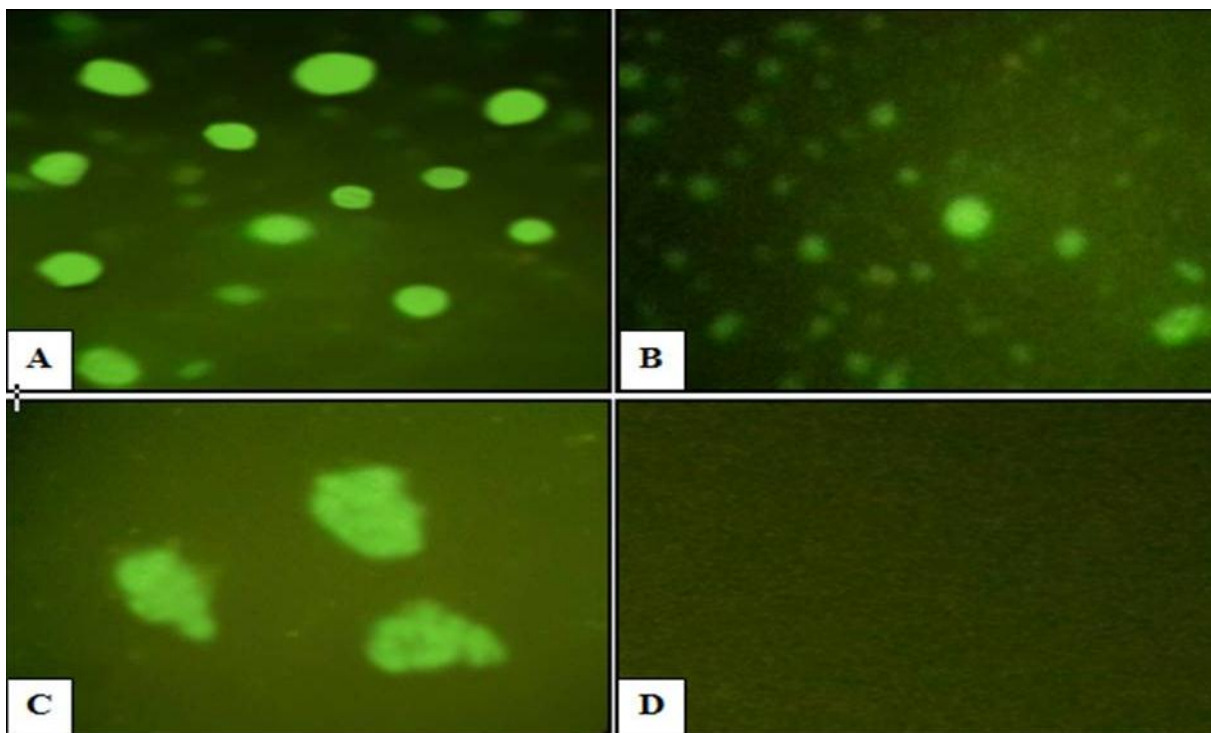
#### **Indirect qualitative immunofluorescence**

The pellets from (BAL, IS or PE) specimens were placed on slides and fixed for the detection of

*P. jirovecii* by a monoclonal antibodies technique. This technique was performed according to manufacturer's instructions.

Stained slides were examined by a fluorescence microscope, oocysts show as medium bright to bright apple green color they may be evenly or

unevenly labeled 14(14%) out 100 samples were positive (Fig. 4).



**Fig. 4. Immunofluorescent staining using monoclonal antibodies against *P jirovecii*. (A) stained smear from BAL of lymphoma disease, the cyst of *P. jirovecii* appears bright to bright apple green (X1000). (B) stained smear from BAL of leukemia disease showing bright to bright apple green cysts with different size (X400). (C) stained smear from sputum of kidney transplantation, many aggregated cysts of *P jirovecii* which appear bright to bright apple green (X1000). (D) Negative control (X1000).**

#### Overall results of conventional methods

The organism was detected in BAL, and/or sputum of immunocompromised patients only. Fourteen samples gave positive results by indirect immunofluorescence monoclonal antibody test within this number only 12 (12%) were positive by Gomori methenamine silver (GMS), 12 (12%) were positive by modified toluidine blue O stains (TOB), and 12 (12%) were positive by Diff-Quik stain (DQS) modified Giemsa (Fig. 5).

These samples were obtained from 14 immunocompromised patients including, four with leukemia, three with solid tumor, two with lymphoma, and only one for each chronic pulmonary obstructive disease, asthma (steroid therapy), rheumatoid arthritis (cytotoxic

therapy), solid-organ transplantation and multiple myeloma disease. Eighty six immunocompromised patients were initially negative by both indirect immunofluorescence and staining methods (GMS, TOB and DQS), while only 12 samples were positive by both methods.

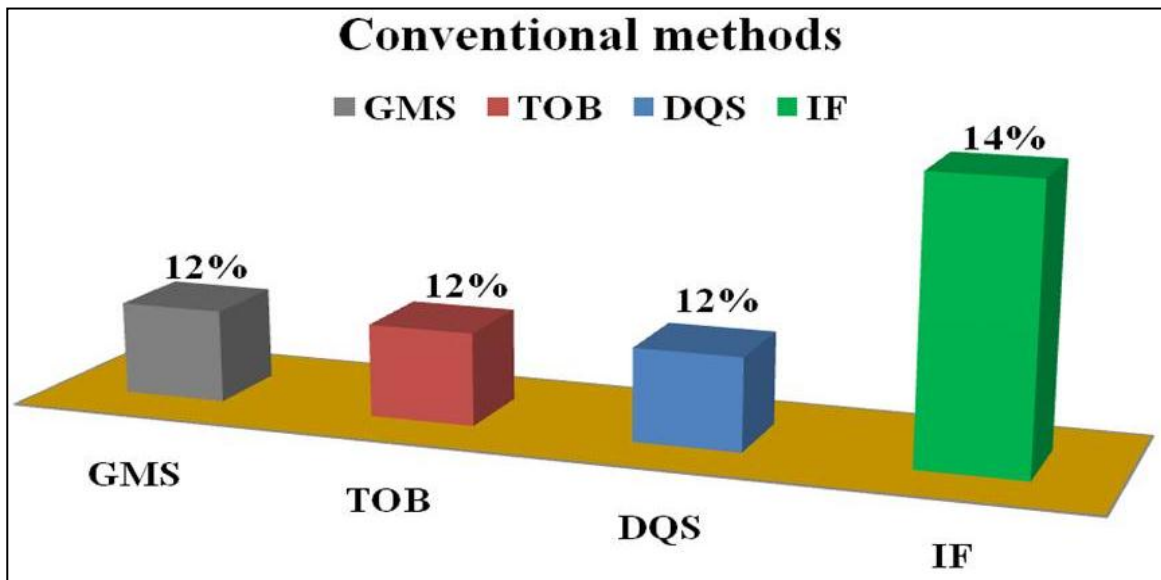
#### Discussion

*P. jirovecii* is the causative agent of *Pneumocystis* pneumonia, one of the most frequent and severe opportunistic infections in immunocompromised patients. As *P. jirovecii* cannot be grown in culture from clinical specimens<sup>(10,11)</sup>. The current laboratory diagnosis of *Pneumocystis* pneumonia has relied mainly upon microscopic techniques, for

detection of cysts and trophozoites of the organism by cytological staining or by immunofluorescent assay (IFA) with monoclonal or polyclonal antibodies<sup>(12,13)</sup>.

In this study the positive results of 12 samples by three staining methods (GMS, TOB and DQS), is in consistent with Ng *et al.*<sup>(14)</sup> who proved, that the specimens were considered to

contain *P. jirovecii* (i.e., a truly positive specimen) if this organism was detected by two or more of the staining methods. Conversely, specimens were considered to not contain the organism (i.e., a truly negative specimen) if all stains were negative or if only one stain was positive that could not be corroborated.



**Fig. 5. *Pneumocystis jirovecii* detection methods using Gomori's methenamine silver, Diff-Quik stain (modified Giemsa), Toluidine blue and Immunofluorescent staining in bronchoalveolar lavage and sputum samples.**

LaRocque *et al.*<sup>(15)</sup> found that the choice of the optimal staining method for the detection of *Pneumocystis* was also important for patients with other immune-compromising conditions who were at risk for infection. In fact, the choice of the optimal staining method may be more important for the detection of *Pneumocystis* in the non-HIV-infected, immunocompromised patients, since it has been shown that respiratory specimens from these patients have a lower burden of organisms than those from HIV-infected, immune-compromised patients.

Results of the current study is in consistent with Yehia *et al.*<sup>(16)</sup> who diagnosed *P. jirovecii* infection by conventional methods only from lower respiratory tract of both immune-competent and immunocompromised Iraq patients. The organism was detected in

alveolar lavage and/or sputum of immune-compromised patients only. *P. jirovecii* was diagnosed by direct microscopical examination with different stains included calcofluor stain, Giemsa and TOB was identified in 8 cases out 150 samples with immune-compromised patients with malignant diseases under radiation and/or cytotoxic therapy

In this study the results of the detection of *P. jirovecii* in respiratory samples BAL and sputum agree with this by Turner *et al.*<sup>(17)</sup> who diagnosed the diseases by using three different staining techniques, included silver stain, Diff-Quik (a modified Giemsa stain) modified toluidine blue, and found that Induced sputum (IS) has been shown to be a reliable tool in terms of sensitivity and specificity comparable to BAL sample in diagnosing PCP.

In another study, John *et al*<sup>(18)</sup> who used direct immunofluorescence monoclonal antibody (DFA) method for identification of *P. jirovecii* in induced sputum and BAL specimens was compared in a blinded study with an established Giemsa stain method, for the 67 patients (64%) infected with the human immunodeficiency virus 49 were initially negative by both the DFA and Giemsa methods, none were negative by DFA and positive by Giemsa, 6 were positive by DFA and negative by Giemsa, and 12 were positive by both methods, were indicates that the DFA method represents an advance as a sensitive, simple, and rapid way to identify *P. jirovecii* in induced sputum and BAL specimens from HIV-infected patients and suggests greater sensitivity of the DFA than the Giemsa method in this patient population. This result in line with the present study concerning immunofluorescence mono-clonal method compared with staining methods.

Procop *et al*<sup>(19)</sup> who used four staining methods on replicate smears of respiratory specimens submitted for *Pneumocystis jirovecii* examination, he found that the indirect immunofluorescent antibody stain is the more sensitive than silver stain (GMS), Diff-Quik stain and Merifluor *Pneumocystis* stain. Baughman *et al*<sup>(20)</sup> who described the sensitivity of an indirect immunofluorescent antibody stain it was the superior in comparison with a modified Wright stain and GMS stain. In another studies when compare the feasibility of different stain methods were applied in respiratory secretions to stain the *P. jirovecii* (Methenamine Silver stain, TOB, Acridine Orange, Diff-Quik, Gram-Weigert, etc.) they found the low sensitivity although these stains are cheap and easily applicable<sup>(21-24)</sup>. However, commercial immunofluorescence (IF) kit which contains monoclonal antibodies has, subsequently, increased the specificity and sensitivity for diagnosis<sup>(25-27)</sup>.

It is concluded from this study that the *P. jirovecii*, is the fundamental opportunistic infection among immunocompromised

patients. GMS staining may have the best overall predictive values for routine clinical use when monoclonal antibody staining is not available, Diff-Quik is a good diagnostic tool in the diagnosis of, *P. jirovecii* because of its cost-effectiveness and because of its rapid diagnosis of severe pneumocystosis. In the present study showed the indirect qualitative immunofluorescence method give a promise for use as a primary method for diagnosis of *P. jirovecii* pneumonia or as rapid screen to exclude the presence of *P. jirovecii* in sputum and BAL samples.

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### Author contributions

Touhali conducted the sampling, isolation, and staining, the molecular work and writing the manuscript. Dr. Ibrahim and Dawood supervised the work, edit and finalize the writing of the study.

### Conflict of interest

The authors declare no conflict of interest.

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Correspondence to Isa S. Touhali

E-mail: isaswadi@yahoo.com

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## The Significance of Hyperglycemia in the First 24 Hours of Stroke

Muhanad A. Kadhim<sup>1</sup> FIBMS, Hasan A. Al-Hamadani<sup>2</sup> FICMS, Munther T. Hamzah<sup>1</sup> FIBMS

<sup>1</sup>Dept. of Neurology, Al-Imamain Al-Kadhymian Medical City, <sup>2</sup>Dept. of Medicine, College of Medicine, Al-Nahrain University, Baghdad, Iraq.

### Abstract

<b>Background</b>	The study focused on acute stage of stroke patients and investigated which parameters of glucose in acute phase of stroke are significant in nondiabetic stroke patients.
<b>Objective</b>	To evaluate hyperglycaemia in acute phase of stroke.
<b>Methods</b>	Cross sectional study was conducted in Al-Imamain Al-Kadhimiyan Medical City in Baghdad city from October 2013 to September 2014. We studied glucose levels and glycosylated haemoglobin in 100 consecutive patients with acute stroke admitted within 24 hours after onset of symptoms.
<b>Results</b>	One hundred consecutive patients (65 men and 35 women ) were included in this study, 76 patients with diagnosis of ischemic stroke and 18 of them with diagnosis of intracerebral haemorrhage and 6 patients with transient ischemic attack. Hyperglycemia was found in 44 patient. High glycosylated haemoglobin levels were found in 29 patients, 16 patients of them have history of diabetes. The other 13 with elevated glycosylated haemoglobin levels were considered as prediabetes. The remaining 15 patients had normal glycosylated haemoglobin.
<b>Conclusion</b>	There is significant relationship between idiopathic hyperglycemia and prediabetes with acute stroke.
<b>Keywords</b>	Stroke, diabetes mellitus, hyperglycaemia

**List of abbreviation:** ICH = intracerebral hemorrhage, HbA1c = glycosylated haemoglobin, DM= diabetes mellitus, RBS = random blood sugar, FBS = fasting blood sugar.

### Introduction

Stroke is classically characterized as a neurological deficit attributed to an acute focal injury of the central nervous system by a vascular cause, including cerebral infarction, and intracerebral hemorrhage (ICH) and is a major cause of disability and death worldwide <sup>(1)</sup>. Ischemic strokes are caused by interruption of the blood supply, while hemorrhagic strokes result from the rupture of a blood vessel or an abnormal vascular structure. About 87% of strokes are ischemic, the rest are hemorrhagic. Some hemorrhages develop inside areas of ischemia (hemorrhagic transformation). It is unknown how many

hemorrhagic strokes actually start as ischemic stroke <sup>(2)</sup>.

The origin of the term hyperglycaemia is Greek, hyper-, meaning excessive, glyc-, meaning sweet and -emia, meaning of the blood, is a condition in which an excessive amount of glucose circulates in the blood plasma <sup>(3)</sup>. Hyperglycaemia was defined as a random glucose of 200 mg/dl or greater or a fasting glucose of 126 mg/dl or greater. Glycosylated haemoglobin (HbA1c) was determined by high-performance liquid chromatography (BioRad) on a cation-exchange column. The upper level of the nondiabetic reference range was less than 6.30% <sup>(4)</sup>.

Chronic hyperglycemia that persists even in fasting states is most commonly caused by diabetes mellitus (DM). In fact, chronic



hyperglycemia is the defining characteristic of the disease. Intermittent hyperglycemia may be present in prediabetic states. Acute episodes of hyperglycemia without an obvious cause may indicate developing diabetes or a predisposition to the disorder <sup>(5)</sup>.

A high proportion of patients suffering an acute stress such as stroke or myocardial infarction may develop hyperglycemia, even in the absence of a diagnosis of diabetes. Or perhaps stroke or myocardial infarction was caused by hyperglycemia and latent diabetes <sup>(6)</sup>. Certain medications increase the risk of hyperglycemia <sup>(7)</sup>.

Stress-induced hyperglycemia typically described as blood glucose concentrations above 200 mg/dl, has been described in the literature for almost 150 years. The causes of stress-induced hyperglycemia can be attributed to the impact of integrated endogenous hormonal, cytokine, and counterregulatory nervous system signals on glucose metabolic pathways <sup>(8)</sup>.

The detection of abnormal metabolic milieu is a window of opportunity for aggressive management in persons with stroke as this will improve outcome. Routine screening for hyperglycaemia in persons with stroke using HbA1c tests and blood glucose may uncover previously undiagnosed DM <sup>(9)</sup>.

## Methods

**Design and Setting:** cross sectional study was conducted in the wards of internal medicine and neurology of Al-Imamian Al-Kadhimiyyain Medical City in Baghdad city. The period of data collection was one year started from October 2013 to September 2014.

**Study population:** We studied 100 consecutive patients who were admitted to the hospital. This centre has no specific selection criteria for the admission of stroke patients. All patients were screened according to a strict protocol consisting of a full neurological examination, standardized blood tests, at least one and usually one computed tomographic scans, magnetic Resonance imaging of the brain,

duplex scanning of the carotid arteries, and cardiac analysis including standard 12-lead electrocardiography in all patients and 24-hour electrocardiographic monitoring and echocardiography on indication. Patients were excluded if the neurological signs onset more than 24 hours.

**Data collection and analysis:** The population was divided into the following subgroups:

- (1) Diabetics with hyperglycaemia
- (2) Nondiabetics with hyperglycaemia and increased HbA1c levels
- (3) Idiopathic hyperglycaemia; hyperglycaemia and normal HbA1c levels
- (4) Normoglycemia.

In all patients, at least one glucose level was obtained within 24 hours after onset: a random glucose level on admission, a fasting glucose on the morning after the stroke. Glucose was measured in hemolyzed whole blood using the hexokinase method.

**Statistical analysis:** The data were analyzed by means of statistical package for social sciences (SPSS) software programs. Values were expressed as mean  $\pm$  SD. A comparison of continuous variables was performed by unpaired two-tailed student's t test. A level of  $p < 0.05$  (two-sided testing) was considered statistically significant.

## Results

One hundred consecutive patients (65 men and 35 women) were included in this study, 76 patients with diagnosis of ischemic stroke and 18 of them with diagnosis of primary intracerebral haemorrhage and 6 patients with transient ischemic attack. The mean  $\pm$  SD age of the 100 patients was  $65.14 \pm 3.848$  min 55 and max 78.

Random blood sugar (RBS) and fasting blood sugar (FBS) were measured in all patients. Hyperglycemia was found in 44%. None of the patients with hyperglycemia had received glucose infusions during the first day after admission. 29 patients had elevated HbA1c levels, 16 patients with a history of diabetes and 13 were prediabetes.

The remaining 15 patients had normal HbA1c but with elevated blood sugar. Table 1 shows the mean $\pm$  SD values of glucose on admission, fasting glucose, and HbA1c, in patients with known diabetes, prediabetes,

idiopathic hyperglycemia, and normoglycemia. In patients with a history of diabetes and prediabetes, all glucose levels were significantly higher ( $p < 0.01$ ) than in all other patients.

**Table 1. Illustrates the random blood sugar on admission, fasting blood sugar and glycosylated haemoglobin in the diabetic, prediabetes, idiopathic hyperglycemia with normoglycemic group using t test.**

Parameters	Diabetes (type 1 & 2) (n=16) mean $\pm$ SD	Prediabetes (n=13) mean $\pm$ SD	idiopathic hyperglycaemia (n=15) mean $\pm$ SD	Normoglycemia (n=56) mean $\pm$ SD
RBS	264.44+66.52	207.0+7.09	205.27+15.48	111.23+12.24
FBS	146.88+23.43	130.62+3.45	131.67+7.4	93.39+6.57
HbA1c	7.41+0.49	7.01+0.03	4.53+0.52	4.93+0.66

RBS = random blood sugar, FBS = fasting blood sugar, HbA1c = glycosylated haemoglobin.

The mean RBS of patient with idiopathic hyperglycemia was significantly higher than normal range (205.27 $\pm$ 15.48;  $p = 0.001$ ) as shown in fig. 1.

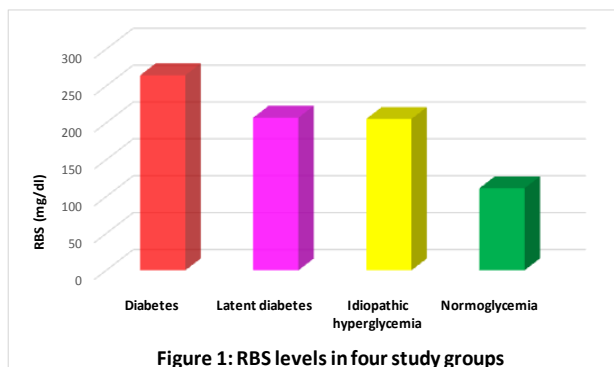


Figure 1: RBS levels in four study groups

The mean FBS of patient with idiopathic hyperglycemia was significantly higher than normal range (131.67 $\pm$ 7.4;  $p = 0.001$ ) as demonstrated in fig. 2.

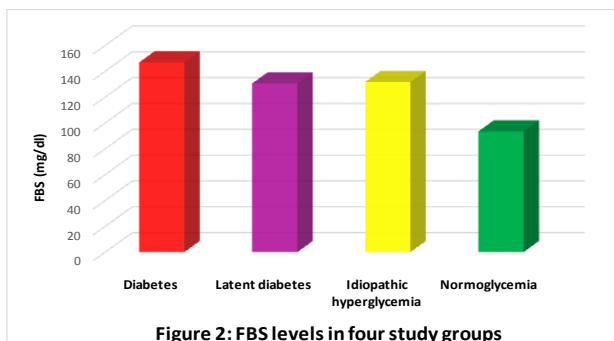


Figure 2: FBS levels in four study groups

The mean HbA1c of patient with idiopathic hyperglycemia was significantly lower than diabetes and prediabetes (4.53 $\pm$ 0.52 versus 7.41 $\pm$ 0.49 and 7.01 $\pm$ 0.03, respectively) as illustrated in fig. 3.

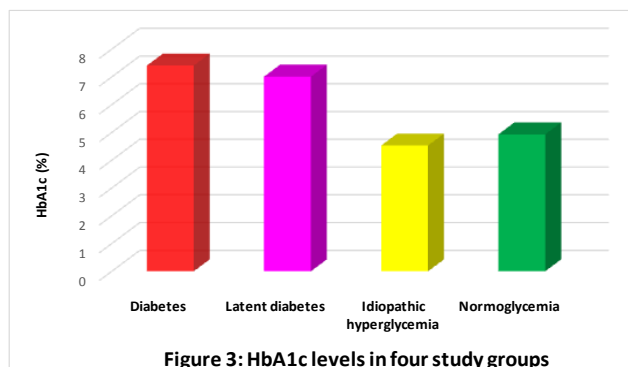


Figure 3: HbA1c levels in four study groups

### Discussion

In this study, the relation of hyperglycaemia in acute phase of stroke was evaluated. We have found that 44% of patients with hyperglycemia, 29% with history of DM or previously undiagnosed DM. Hyperglycaemia is a strong risk factor for poor outcome after stroke<sup>(10)</sup>. Diabetic patient is liable for ischemic stroke and for intercerebral haemorrhage due to macrovascular disease which is the process of atherosclerosis, which leads to narrowing of arterial walls throughout the body.

Atherosclerosis is thought to result from chronic inflammation and injury to the arterial wall<sup>(11)</sup>. Ogbera et al<sup>(9)</sup> reported that 47% of patient with hyperglycaemia (24% known DM and 8% previously undiagnosed DM) this nearly the same of our study as this study sample slightly larger than our sample.

However a higher incidence of previously undiagnosed DM in stroke (18%) was noted in a Japanese report but this may be ascribed to the fact that oral glucose tolerance test was employed in making the diagnosis<sup>(12)</sup>. In our study we found 15% of patient with idiopathic hyperglycaemia (table 1). This may ascribe acute stress results in a raised concentration of counter-regulatory hormones (catecholamines, cortisol, glucagons, and growth hormone) that promote pathways opposite to the action of insulin in the liver and peripheral tissues.

Catecholamine, cortisol, and growth hormone antagonize insulin action, which decreases peripheral glucose uptake. In addition, increased catecholamine and cytokines promote triglyceride breakdown (lipolysis) to free fatty acids increased release of cytokines (i.e., tumor necrosis factor, interleukin-1, and interleukin-6) also contributes to insulin resistance through direct effects on insulin receptors<sup>(13)</sup>. Słowik et al<sup>(14)</sup> found transient hyperglycaemia in 31.9% of patient and this differ from our study, this may ascribe to large sample involved in this study. Kooten et al<sup>(15)</sup> suggest the transient hyperglycaemia is not related to the stress and this differ from our study as it takes the measurement plasma catecholamine with RBS, fasting blood sugar and HbA1c. In our study we found HbA1c elevated in patient with DM and previously undiagnosed DM but in patient with idiopathic hyperglycaemia we found HbA1c was normal. HbA1c can reflect pre-stroke glycaemia status and is one of the criteria for diagnosing diabetes, Roquerj et al<sup>(16)</sup> suggested HbA1c determination detected both previously undiagnosed DM and prediabetes in acute stroke patients and HbA1c determination should be included in the systematic screening

of all acute stroke patients. He found new DM in cases (11.5%) and detected patients with prediabetes (36.2%) this differ from our study since he not study idiopathic hyperglycaemia we not involve oral glucose tolerance test in our study but we find HbA1c of patient with idiopathic hyperglycaemia was significantly lower than diabetes and prediabetes.

We conclude that there is significant relationship between idiopathic hyperglycaemia and prediabetes with acute stroke.

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### Author contribution

Dr. Kadhim did the acquisition of the data, analyse, and interpret the data and statistical analysis; Dr. Al-Hamadani revises the manuscript and study concept and design; and Dr. Hamzah collect the data.

### Conflict of interest

No potential conflicts of interest.

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**Correspondences to Dr. Hasan A. Al-Hamadani**  
**E-mail:hah\_hamdani@yahoo.com**

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## Pseudomonas aeruginosa: Uncommon Cause of Bacterial Meningitis

Yasser W. Al-Mula Abed MBChB, MRCP, MRCP

Dept. of Medicine, Sultan Qaboos University Hospital, Muscat, Sultanate of Oman

### Abstract

**Introduction** Gram negative bacillary meningitis is uncommon disease that needs proper diagnosis and urgent treatment to avoid serious complications. Escherichia coli, Klebsiella pneumonia and Pseudomonas aeruginosa are the most frequent gram negative bacilli. Use of third generation cephalosporins has made a significant therapeutic change with significant reduction in mortality. Ceftriaxone should always be the drug of choice until the full sensitivity report is available. Here, we report a case of bacterial meningitis caused by *Pseudomonas aeruginosa* which is thought to be secondary to previous ear infections. The diagnosis was confirmed by gram stain and culture of the cerebrospinal fluid. The patient was successfully treated with intravenous antibiotic for total of three weeks.

**Key words** Pseudomonas aeruginosa, meningitis, ear, infection.

### Introduction

*Pseudomonas aeruginosa* is increasingly recognized as an emerging opportunistic organism of clinical significance. One of its very worrying features is its increasing antibiotic resistance and high mortality<sup>(1)</sup>. It is not very common pathogen of adult gram negative meningitis<sup>(2)</sup>. It may occur as a spontaneous infection or as a complication of neurosurgical events like penetrating trauma, haemorrhage, craniotomy, extra-ventricular drain, clipping of arterio-venous malformation, ventriculo-peritoneal shunt, spinal anaesthesia and brain or nasopharyngeal tumours with invasion of the base of skull<sup>(3-5)</sup>. Recurrent otitis media is another reported risk factor. Immune suppressed patients and recipients of cochlear implants are at higher risk<sup>(6)</sup>.

### Case Report

A 38-year-old gentleman was admitted to the hospital with 5 days history of headache and fever. The pain was of sudden onset, frontal,

described as worst ever headache and associated with high grade fever and neck stiffness. He denied abnormal respiratory, genitourinary or ear-nose-throat symptoms. He was an ex-smoker, having 10 pack year smoking history. He had no significant past medical or surgical history apart from recurrent right ear infection few years ago which resulted in reduced hearing on the same side but he never required admission for such. He had no history of trauma, seizure or altered sensorium. No history of recent travel or contact with sick patient. Initial clinical examination was significant for high grade fever with temp of 38.9°C, mild tachycardia with heart rate of 110 beat per minute and some neck rigidity but no other meningeal or neurological signs. No papilloedema on fundoscopy. Ears were full of wax so it was difficult to assess the tympanic membranes but no discharge was noted. His C-reactive protein was significantly elevated with mild neutrophilic leucocytosis. Rest of his basic blood

investigations were normal including coagulation profile, renal profile, bone profile and liver function test. He had a CT brain which was reported as normal after which he immediately had a lumbar puncture which showed a turbid fluid with high opening pressure of 25 cm. The cerebrospinal fluid (CSF) analysis showed 450 WBC (polymorph predominant, 90%) with high protein and low glucose levels. He was covered initially with empirical antibiotic and anti-viral as per hospital's policy which include ceftriaxone 2 gm twice a day, vancomycin 1 gm twice a day and acyclovir 10 mg/ kg three times a day intravenously. The empirical therapy was given to the patient less than 1 hour after the lumbar puncture which was done on the day of admission. *Pseudomonas aeruginosa* was suspected few hours later by gram stain and further confirmed by culture after 48 hours. Accordingly; the acyclovir got stopped and the antibiotic got changed to ceftazidime as advised by microbiologist based on laboratory sensitivity report. He had MRI brain and spines which showed leptomeningeal enhancement but no other abnormality. CSF cytology showed no malignant cells. The patient showed a dramatic recovery within 72 hours and got discharged from the hospital on day 5 and antibiotic was continued in the community via a peripherally inserted central line for total of 21 days. It is worth to mention that his immunological work up was normal and that his retroviral screen was negative.

### **Discussion**

Meningitis should always be excluded, if suspected, and treatment should be initiated as soon as possible. Lumbar puncture for CSF analysis is the gold standard test to confirm or exclude this condition which could be life threatening. Gram stain examination of CSF allows a rapid and accurate identification of the causative bacterium in 60-90% of patient and has a specificity of > 97%. However, this correlates with the CSF concentration of bacteria which can be improved by use of

cyto-spin technology which is also useful if the submitted specimen is too small <sup>(7)</sup>. It also depends on the causative pathogen that had been 90% for *Streptococcus pneumoniae*, 86% for *Haemophilus influenzae*, 75% for *Neisseria meningitidis*, 50% for gram negative bacilli and approximately 33% for *Listeria monocytogenes* <sup>(8)</sup>. The gram stain result correlates also with timing of initiation of antibiotics and could also be affected by human factors like operator contamination or observer mis-interpretation <sup>(9)</sup>. Other important and promising tests of CSF in addition to the basic biochemical tests include culture, latex agglutination, Limulus lysate assay, enzyme linked immunosorbent assay, polymerase chain reaction and others. Once a bacterial pathogen is identified and sensitivity test is reported then antimicrobial treatment should be modified for optimal therapy. Despite administration of third generation cephalosporins, mortality rate had been reported to be as high as 83% in the spontaneous gram negative bacillary meningitis versus 35% in the post neurosurgical patients <sup>(4)</sup>.

Our patient was treated with the proper antibiotic according to sensitivity report for total of 3 weeks with which he had a full and dramatic recovery.

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**E-mail: mulaabed@yahoo.com**

**Tel. + 968 96283141**

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عنوان المراسلات إلى المجلة العراقية للعلوم الطبية، صندوق بريد 70044 بغداد، العراق. تلفون(+964 7717516090).

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