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# IRAQI JOURNAL OF MEDICAL SCIENCES

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# Iraqi Journal of Medical Sciences

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# The Electron Microscopes: Concise History and Review

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## Abstract

Since its invention, electron microscope (EM) has been a valuable tool in the development of scientific theory and it contributed greatly to biology, medicine and material sciences. This wide spread use of EMs is based on the fact that they permit the observation and characterization of materials on a nanometer (nm) to micrometer (µm) scale. In this review article, the transmission electron microscope (TEM) and the scanning electron microscope (SEM) were defined and reviewed. The EMs functions and types were discussed, in addition to clarifying the parts and components of TEM, SEM and optical microscopes for neophyte, starting from the sample's preparation through imaging of the samples. Also, this review will point out the limitations and advantages of each type and issues to be considered during experimental design. Advanced EM techniques are listed as well.in this review, Diagrammatically, identify the various parts of a microscopes, what is the differences between TEM, SEM and optical microscopes and finally the methods of samples preparation have been mentioned.

**Keywords** Electron microscopy, SEM, TEM, sectioning, staining

**Citation** Hamid MK. The electron microscopes: Concise history and review. Iraqi JMS. 2022; 20(2): 154-167. doi: 10.22578/IJMS.20.2.1

**List of abbreviations:** EM = Electron microscope, FS = Freeze substitution, HPF = High pressure freezing, LM = Light microscope, STEM = Scanning electron microscope, TEM = Transmission electron microscope

## Introduction

Electron microscopies (EMs) are an instruments for gaining high resolution images of biological specimens and non-biological specimens. It is utilized in biomedical field to examine the structure of tissues, cells, any organized or specialized structures within a living cell and molecules as shown in figure (1), which shows mast cell by EM. At 19$^{th}$ century, physicists realized that to upgrade the light microscope (LM) using shorter wavelengths. Thompson in 1897 found out that the electron has wave-like properties [(1)]. In 1924, de Broglie showed that a beam of electrons moving in a vacuum behaves as a very short wavelength radiation, but Ruska had been used these properties of electrons to build the first EM [(2)]. Improvements are made in forty years within the part of oral biology. The first potential within the development of transmission electron microscopies (TEM) is linked with the next names: Brüche and Johannson (1932) [(3)], Knoll and Ruska (1932) [(4)], Glaser (1933) [(5)], von Borries and Ruska (1938) [(6)]. Significant progression is done, within the technology which come near or nearer to a resolution power of 1 A, and also within the preparation techniques.

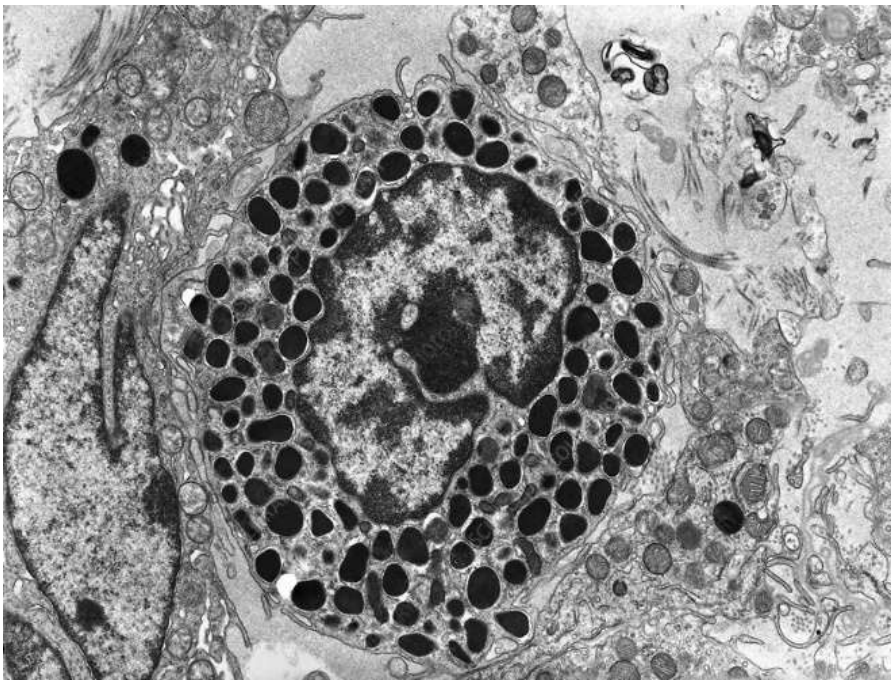


**Figure 1. Mast cell (TEM) [7]**

We could compare two sorts of microscopes light (optical) and EM. The optical microscopes (Figure 2) use beam of light while EMs use electron beams to magnify objects details, which can't be seen by an unaided eye clearly. By wave-like characteristic of electrons, the EMs can magnify an object's image, unlike the LMs that use light beam to magnify images. Checking up virus by EM relies on the detection and identification of virus on the basis of their characteristic morphology. Earlier attempts to visualize viruses with even the foremost powerful optical microscopes of the day was largely unsuccessful. This was so because visible light with a mean wavelength of about 5500 A° were unable to inspect the finer and detailed aspects of virus particles, which are comparatively smaller in size. The light wavelengths are relatively long. Therefore, particles having smaller size can't be properly resolved. This problem was solved with the event of EM by Ruska and Knoll in 1931 [8]. These instruments don't use electromagnetic waves with longer wavelengths. Instead, strong electron beams are projected from a source to resolve the thing under observation. The wavelengths of such electron beams were very small, often but 1A°. On the opposite hand, the space between different atoms during a molecule is more than that. Therefore, it is theoretically possible to get resolutions at the atomic level with the assistance of those. Once resolution at such a fine degree is obtained, it is possible to enlarged and magnified images to the specified extent. We can achieve a magnification of up to 2,000,000X where-as from ordinary LM, it is up to 2000X. The parts of the EM are an electron gun, column, electro-magnetic lenses and a fluorescent screen as shown in figure (3). Electron gun is source of electrons. The gun consists of tungsten filament at 30 KV to 200 KV or more then that potential. It's surrounded by a negative shield with an aperture through which a beam is drawn off.

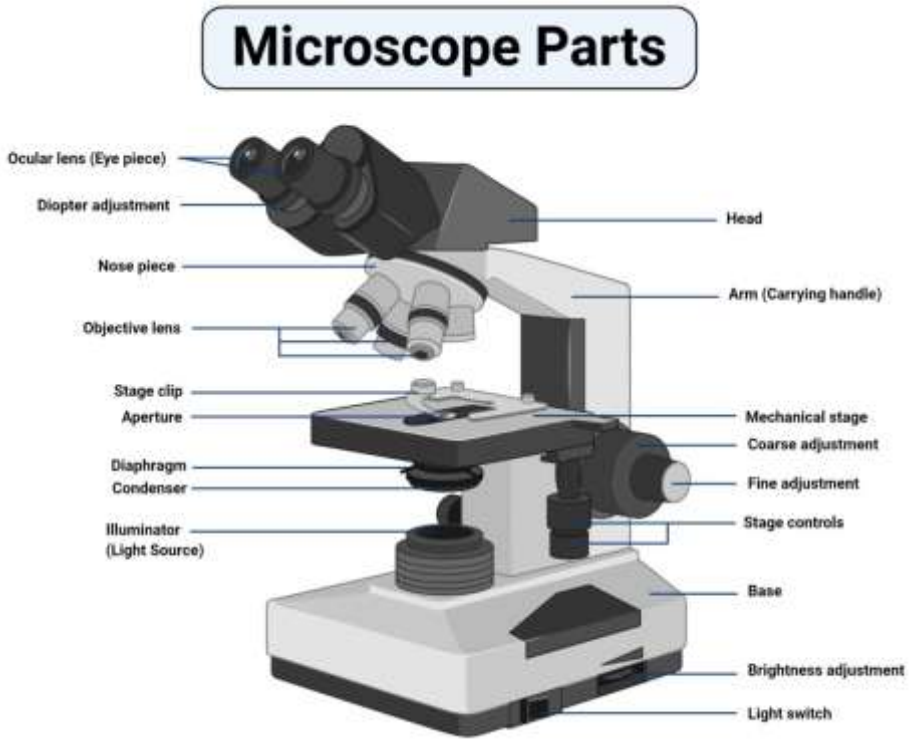



**Figure 2. Parts of a microscope [9]**

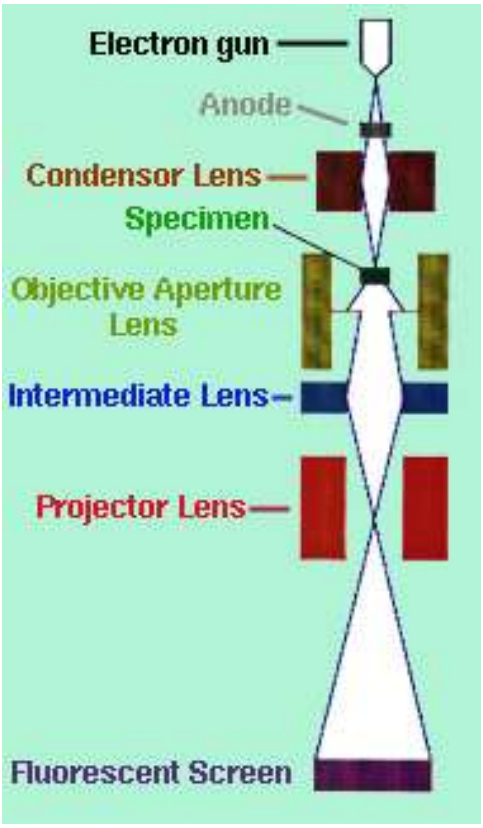



**Figure 3. Parts of electron microscope [10]**

We can brief the differences between optical microscope and EM by 35 differences as shown in table (1).

**Table 1. The differences between optical microscope and electron microscope [11]**

| Character | Light microscope | Electron microscope |
|---|---|---|
| Famed as | Optical microscope | Electron beam microscope |
| Developed by | Dutch Zacharius Jansen and Hans were the first to invent the compound microscope in the 16th century | In 1931 physicist Ernst Ruska and German engineer Max Knoll |
| Source | By light (wavelength 400-700 nm) to illuminate the objects under study | By a beam of electrons (equivalent wavelength 1 nm) |
| Precept | The images created by absorption of light waves | The image created by scattering or transmission of electrons |
| Size | Smaller and lighter | Heavier and larger |
| Lenses | Glass lenses | Electromagnets lenses |
| Vacuum | No vacuum used | Need a high vacuum |
| Specimen | Fixed, unfixed, stained, unstained, living and non-living | Fixed, stained and non-living |
| Specimen | Both live and dead specimens can be examined. | Only dead specimens can be examined |
| Specimen preparation | Less tiresome and simple | It involves hard processes, e.g. Using corrosive chemicals and skill required to prepare specimens |
| Time of preparation | Takes a few minutes to hours | Takes a few days |
| Specimen thickness | 5 µm or thicker | Ultra-thin, 0.1 µm or below |
| Specimen dehydration | No need dehydrated before viewing | Used only dehydrated |
| Coating | Stained by colored dyes | Coated with heavy metals |
| Specimen | Mounted on the glass slide | Mounted on the metallic grid |
| Focusing adjustment | Done mechanically | Adjusting by electromagnetic lenses |
| Magnification limit | Low magnification of up to 2000X | High magnification of up to 1,000,000X |
| Resolving power | Low power of resolving, below 0.3 µm | The high resolving power about 0.001 µm |
| Viewing the image | Images are viewed directly. Images are viewed by the eyes through the eyepiece | Images are viewed on a photographic plate or zinc sulfate fluorescent screen |
| Nature of image | Poor surface view | Good surface view and internal details |
| Color of the image | Colored images | Grayscale or black and white |
| Dimension of the image | Plane (2D) | 2D only in a transmission electron microscope, 3D images in scanning electron microscope |
| Living processes | Visualization of living processes and even cell division is possible. | Living processes cannot be viewed. |
| Room | No special settings required. | Used in a room where humidity, pressure, and temperature are controlled |
| Simplicity | Simple to use | Users must have technical skills |
| Voltage requirement | No high voltage is need | High voltage is needed (50,000 v or above) |
| Filaments | No filaments used | Tungsten filaments |
| Cooling system | No cooling or chiller system | Cooling system need to cool down the |

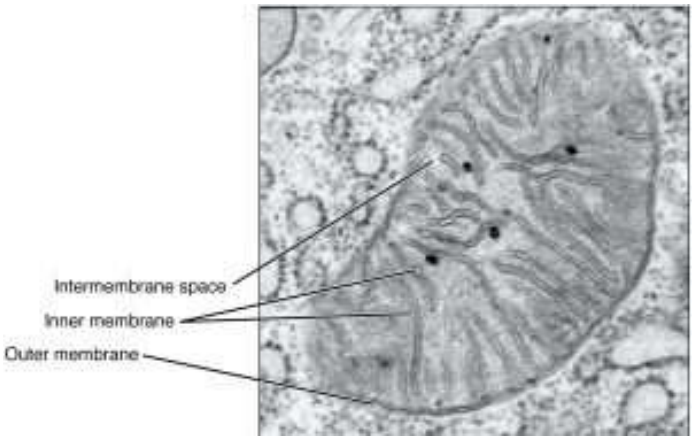
| | | |
|---|---|---|
| | | heat generated due to high voltage electric current |
| Radiation risk | No risk | Risk of radiation leakage |
| Complexity | Less complex | Complex |
| Cost | Cheap and low maintenance costs | Very expensive as well as to maintain |
| Convenience | Suitable for schools and for learning institutions | Is for limited to specialized use such as research |
| Advantages | • Easy<br>• Cheap<br>• Real color and sometimes require staining<br>• Live specimens | • More resolution<br>• Give images of surface and interior structures<br>• More magnification<br>• 3d images |
| Disadvantages | • Low resolution (0.2 nm)<br>• Low magnification<br>• The specimen used is thin | • Expensive<br>• Requires extensive training<br>• Sample must be dead<br>• Black and white |
| Types | • Dark-field microscope<br>• phase-contrast microscope<br>• Fluorescent microscope<br>confocal microscope<br>polarized microscope<br>• Differential interference contrast microscope | • Transmission electron microscope (TEM)<br>• Scanning electron microscope (SEM) |

## Types of EM

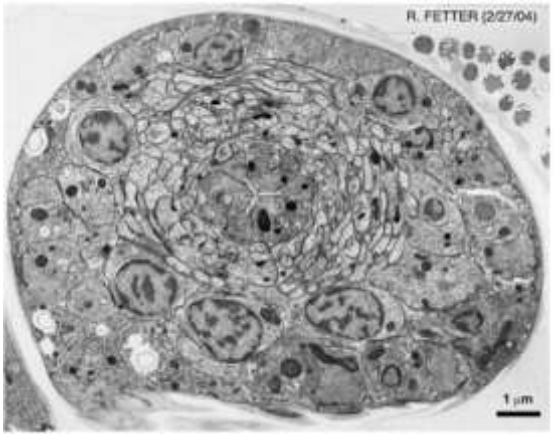
There are two kinds of EM [12]:

**(a) Transmission Electron Microscope (TEM)**

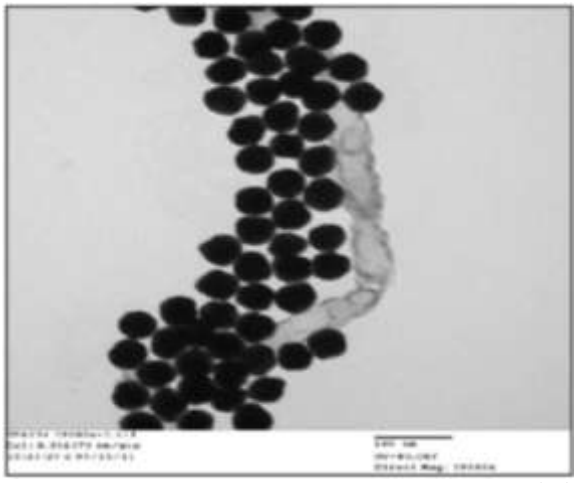
This EM is often compared with a LM [11]. It uses transmitted electrons which will penetrate the thin sample (usually no more than 100 nm thick). TEM is employed to look at very thin specimens (tissue, molecules, etc.) through which electrons can pass after the sample, the electrons hit a fluorescence screen that forms an image with the electrons that were transmitted. Figure (4) shows some images obtained in TEM. TEM is analogous to the conventional (compound) LM. TEM is used also to image the interior of cells.




**This transmission electron micrograph shows a mitochondrion as viewed with an electron microscope [13]**




**TEM section through an embryo fixed using High pressure freezing (HPF) [14]**

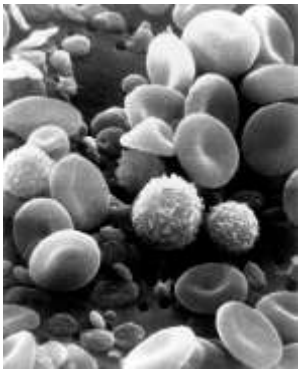


**TEM image of 50 nm gold nanoparticles [15]**

**Figure 4. Transmission electron microscopy (TEM) images of some samples**

**(b) Scanning Electron Microscope (SEM)**

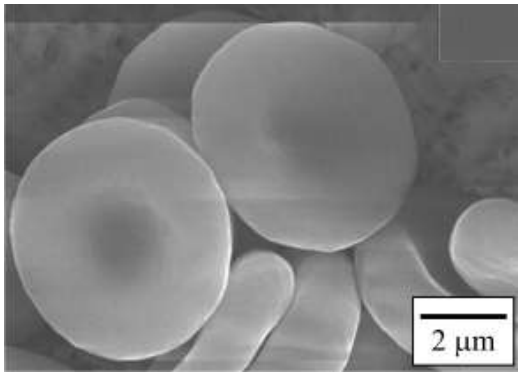
SEM utilize scattered electrons and secondary or back scattered from the surface of the sample, thus showing a three-dimensional image. SEM depends on the secondary electrons that emitted from the surface of a specimen. It gives detailed images of the surfaces of samples like cells and organisms that aren't possible by TEM as shown in figure (5). It is also use to count particles and measure particles size. It is named SEM because the image is made by scanning a focused beam of electrons onto the surface of the sample during a raster pattern. The interaction of this beam with the atoms near the surface causes emission of electrons at each point within the raster such as low energy secondary electrons, high energy back scattered electrons, X-rays and photons. These are often collected with a spread of detectors, and converted to brightness at each equivalent point on a cathode ray tube (CRT). Because the dimensions of the raster at the specimen is smaller than the viewing screen of the cathode ray tube, equipped SEMs with secondary, backscatter and X-ray detectors are often use to study the topography and composition of specimens. Figure (6) shows the SEM and figure (7) shows part of SEM.



**A**

**SEM blood cells conventional pretreatment**

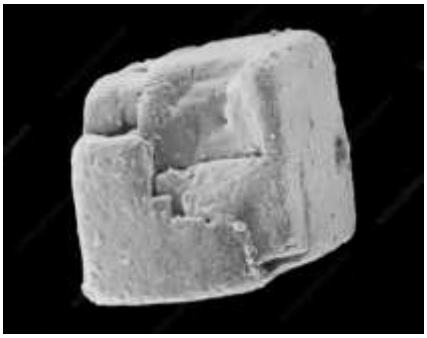
**(16)**




**B**

**SEM images of RBCs**

**(16)**



**C**

**Table salt crystal (NaCl) [17]**

**Figure 5. A, B and C scanning electron microscopy images**

**A**
**Q250 Analytical SEM for materials science**

**B**
**SU3500 Scanning electron microscope**

**Figure 6. A and B models of scanning electron microscopes [18]**

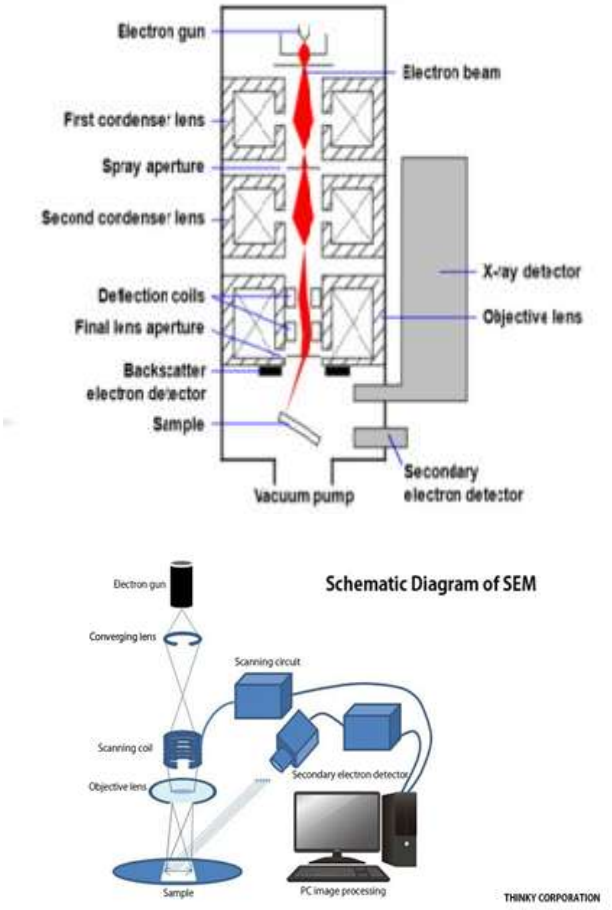



**Figure 7. Schematic and parts of scanning electron microscope (SEM)** [19]

## Technique

The first plant virus to be observed under the microscope was tobacco mosaic (Williams and Wykoff, 1943) [20]. Since then, microscopy technology has improved considerably. Many techniques associated with EM have brought out reasonably clear pictures and these techniques are:

### Ultrathin sectioning

This technique is useful in studying the particle within the host cell. It is also useful for studying the crystal structure. Thin sections (25-90 nm) are created from fixed, dehydrated and embedded biological materials, using special microtomes thermal or mechanical advance microtomes and glass or diamond knives with very sharp and hard cutting edges.

### *Negative Staining*

Negative staining is a simple way for observe the structures of isolated organelles, individual macromolecules and viruses by Hall (1955) [21] was the first to explain the effectiveness of negative staining during a study in which panicles were being positively stained with phosphotungstic acid. Instead of looking like dark on a light background, they would be seen light on a dark background. Huxley (1956) [22] also observed a same effect with tobacco mosaic Virus. Brenner and Horne (1959) [23] noticed an equivalent event and named its negative staining. Some negative stains with normal pH are:

Sodium or Phosphotungstate (PTA) 5 to 8
Uranyl acetate 4.2 to 4.5.
Ammonium molybdate 5 to 7
Methylamine tungstate 5 to 7

The significance of negative staining is to surround or embed the biological samples within electron dense material which gives high contrast.

***Positive staining***

In positive staining method, significant metal salts attach to the various organ or macromolecules inside the sections to rise their electron density to be dark against a lighter background. Some positive stains are: chemical group acetate (UA), Reynold's lead citrate. Uranyl ions react with phosphate and amino groups, in order that the nucleic acids and certain proteins are extremely stained [24].

***Comparison between negative and positive staining***

Negative staining may be a technique utilized in preparing specimens for microscopical examination. The sample is mixed with an electron dense material that penetrates the interstices of the sample but not the material of the sample itself. The specimen then appears transparent against an opaque background. The positive stain-stick with specimen and provides its color where-as negative stain doesn't mix with the specimen but settle around its outer boundary and forming a silhouette (outline). The negative stain produces a dark background round the cell.

**Freeze drying**

To pull out the moisture (e.g., from food) by freezing firstly then subjecting to a high vacuum used as a way for drying foods and chemicals while causing little decomposition is named freezing drying or process of drying food or blood plasma or pharmaceuticals or tissues without damage their physical structures, material is frozen and then after that warmed in a vacuum in order that ice sublimes for biochemical the term lyophilized is usually used. Freeze drying technique helps in getting an accurate idea about the shape of the particles [25].

***Method***

The freezing dying technique has been described intimately by Williams (1952) [26]. This technique includes the rapid freezing of the specimen and thus the layer of water covering it on the grid to which it had adsorbed followed by sublimation of the ice around it. A thin layer of heavily metal is then precipitate on the dehydrated surface to provide contrast.

***Carbon replica***

Replication is a means of depicting the topography of an object, such as a tissue surface. Even before the introduction of the SEM replication techniques had been used for light and TEM [27,28]. Preparation of carbon replica almost like plaster of Paris molds are prepared in many cases to bring out the surface characteristics of virus particles.

## 4- Samples preparation

Commonly, cells are fixed with chemicals using glutaraldehyde, followed by (Osmium) Os tetroxide. Glutaraldehyde primarily cross-links proteins. Os tetroxide reacts powerfully with membrane lipids and collectively with proteins. As a result of this methodology and due to the diffusion of the fixative into the cell, slow infiltration of the fixative and extraction of cellular contents. Both of these problems will cause fixation artifacts, like malformed cellular membranes or organelles, and to loss of material, making the cell appear less dense than it is really. A premium resource for protocols and procedures for EMs sample preparation are generally found among the sensible ways (practical method) for microscopy series (e.g., Glauert 1975) [29]. When fixation with liquid fixatives, samples dehydrated in increasing concentrations of a solvent. General solvents used are acetone or ethanol, usually followed by propene oxide. There are wondrous reviews on the principles, practice, and utility of high-pressure freezing sample preparation (Moor, 1987) [30] and (McDonald et al., 2007) [31]. Most freeze substitution (FS) protocols include a progressive warming to temperatures that let fixation chemistry to occur at a reasonable rate. Embedding is that the strategy of

infiltrating the specimen with resins that is ready to be polymerized into a tough plastic applicable for thin sectioning. A variety of embedding resins can be offered. Epoxy resins such as Epon are the best to section and permit for wonderful post staining. Epoxy resins are typically polymerized at 60-70°C and don't seem to be contributing to immune labeling. Methacrylate resins similar to the Lowicryls can infiltrate into dehydrated specimens and be polymerized at temperature by ultraviolet light. Combined with HPF and FS, embedding in these resins retains antigenicity, making HPF/FS samples acceptable for post-embedding immunolabeling. In any event, embedding and curing in any resin have to be compelled to yield a tough block where the sample within it.

**4-1 Sectioning and staining**

To examine the sample among the EM, thin sections (~60–80 nm) ought to be cut from the block [32]. The face of the block ought to be cut with an instrument with a sharp blade or combination of blades or glass microtome knife to a neat trapezoid, usually <1 mm on an aspect. Cutting tiny sections allow the loading of dozens of serial sections on an EM specimen support known as a grid as shown in figure (8). Once cut, the block is mounted in associate ultramicrotome - a specialized machine that cuts sections by slowly advancing the block face by very little, specifically controlled increments over a diamond or glass knife edge to supply sections of a given thickness chosen by the operator. Sections are so small and fragile to be directly manipulated with forceps or different tools. Sections float off the ultramicrotome knife edge onto a little, water-filled reservoir built into the knife, and so the sections ought to be strictly transferred onto metal grids. Grids go along with varied mesh patterns or open slots through that the sections are generally imaged. To hold up the sections over these holes, grids square measure coated with a thin plastic (Formvar is customary) that is in a position to be strengthened with carbon coating. The final step in sample preparation before imaging is post sectioning staining, |this is usually often finished uranylacetate, followed by lead citrate to strengthen contrast, and is well done by floating the grids, sections side down, on droplets of stain, followed by distilled water rinses type of stained structures in varied cells and samples, followed by checking for background with secondary antibody–only controls. An example of this approach square measure found in Rout et al. (2000) [33].

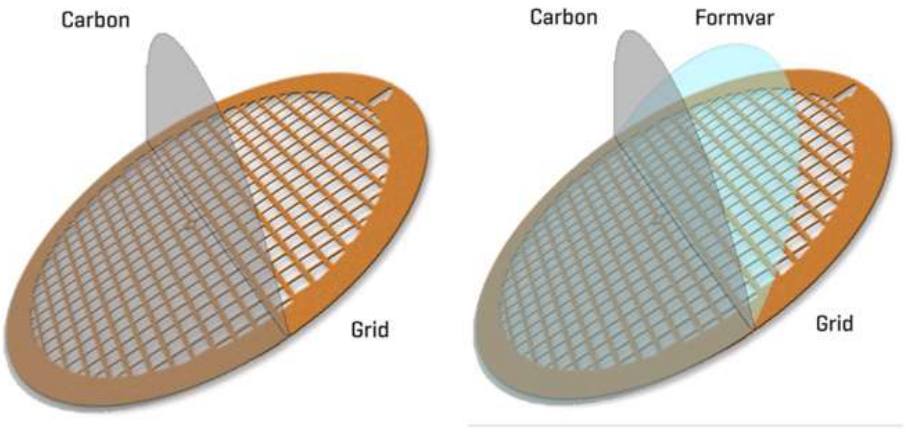



**Figure 8. Carbon film supported copper grid and formvar/Carbon supported copper grids [34]**

## 5- Imaging

The currently stained sections on grids are prepared for examination within the EM. By using a holder, a grid is inserted into the column of the microscope where the grid is in the beam. Modern EM have a PC interface creating it easy to search out and operate the instruments. Digital cameras also are used for locating areas of interest, focusing, and correcting astigmatism besides record the images and have almost universally replaced film. Figure (9) shows samples of the standard of cell structure as viewed among the TEM from samples prepared by typical chemical fixation, similar to the elaborate cytoskeletal arrays in cultured myocytes (Figure 9A) to the detailed ultrastructure of cellular organelles (Figure 9B). Wonderful ultrastructural preservation, notably of membrane organelles similar to the Golgi, are typically obtained using HPF/FS samples (Figure 9C).

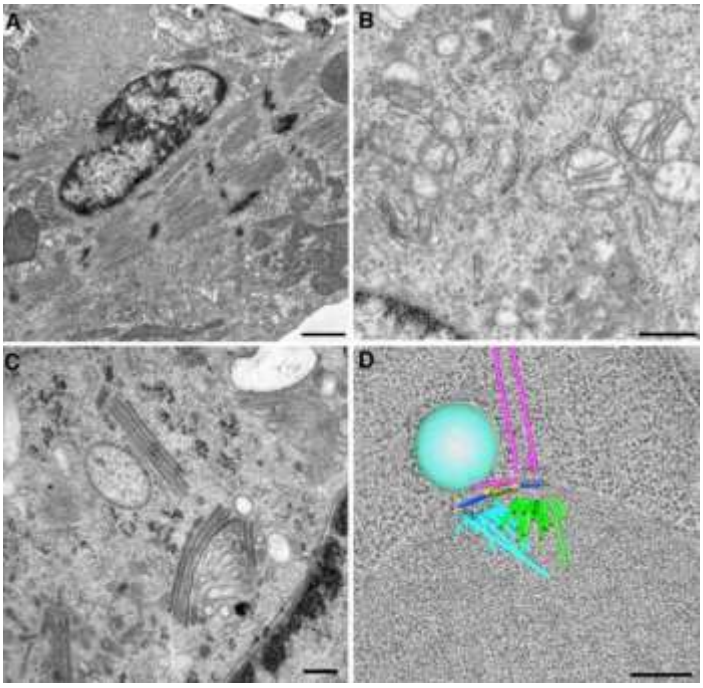



**Figure 9. Cell structure as visualized by EM. (A) Actin-myosin cytoskeleton revealed in a cultured cardiomyocyte ready by standard chemical fixation. Scale bar (1 µm). (B) cytoplasmic organelles in a mouse macrophage prepared by standard chemical fixation. Scale bar (700 nm). (C) Golgi membranes in a cultured 3T3 cell prepared by HPF and freeze substitution. Scale bar (200 nm). (D) Three-dimensional tomographic model of a forming mitotic spindle from budding yeast. Scale bar (200 nm) [32]**

## 6- Main parts of EM

EM is in the form of a vacuum column, which is vertically mounted, and it has the following components [35].

### 1- Electron gun

The electron gun is a tungsten filament, which generates electrons.

### 2-Electromagnetic lenses

- Condenser lens, which focuses the electron beam on the specimen. A second condenser lens make the electrons into a thin tight beam.
- Objective lens, which has high power and make the intermediate magnified image.

- Projector (ocular) lenses, the third set of magnetic lenses which produce the final further magnified image.

**3- Specimen holder**

The specimen holder is a thin film of carbon held by a metal grid.

**4- Image viewing and recording system.**

- The final image is projected on a fluorescent screen.
- There is a camera below the fluorescent screen where the image is recorded.

## 7- Advantages

- Very high magnification.
- High resolution.
- Material rarely damage or distorted by preparation.
- Can investigate a greater depth of field.

## 8- Limitations

- The live specimen cannot be determined.
- As the penetration power of the electron beam is low, the object ought to be ultra-thin. For this reason, the specimen is dried and take ultra-thin sections before observation.
- Since the EM works in a vacuum, the specimen should be completely dry.
- Very expensive to build and maintain.
- Need training.
- This microscope is a large, cumbersome and sensitive to vibration and external magnetic fields.

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# Evaluation of Paraoxonase 1 Oxidative Stress Enzyme in Cord Blood of Newborn to Patients Delivered with Oxytocin Induced Labor

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## Abstract

**Background** The use of labor induction to shorten the duration of a pregnancy has increased steadily during the last few decades. Paraoxonase 1 levels were observed to be higher in the oxytocin-induced group than in the spontaneous labor group.

**Objective** To compare the influence of induced labor on fetal Apgar score and birthweight, and to measure the level of oxidative stress (paraoxonase 1) experienced during labor by the neonates of pregnant women undergoing induced or spontaneous birth.

**Methods** A case control study that was conducted in the Department of Obstetrics and Gynecology, Azadi Teaching Hospital, Kirkuk during a period of nine months from 10th of February till 10th of November 2019. It included 60 healthy pregnant women with singleton pregnancy and viable fetus attending the labor room of the hospital. They were divided into two groups: Case group included 30 pregnant women who underwent oxytocin-induced labor and control group included 30 pregnant women underwent spontaneous vaginal delivery in latent phase of first stage labor without oxytocin induction. A five ml of blood was drawn from the umbilical artery to measure Paraoxonase 1 level. Pregnancy outcome was also monitored.

**Results** The mean of Paraoxonase 1 enzyme was significantly higher in women who underwent oxytocin-induced labor than that in women who underwent vaginal delivery without oxytocin induction. No significant differences between study groups regarding birthweight, Apgar score 1 min, Apgar score 5 min, packed cell volume, and hemoglobin F.

**Conclusion** Serum Paraoxonase 1 as a marker was elevated in neonates of pregnant women who underwent oxytocin induced labor without a significant effect on Apgar score of neonates or its birthweight.

**Keywords** Paraoxonase 1, labor induction, oxidative stress, pregnancy outcome

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**List of abbreviations:** BMI = Body mass index, C/S = Cesarean section, HbF = Fetal hemoglobin, PCV = Packed cell volume, PON = Paraoxonase

## Introduction

Labour is a physiological process, in which the fetus, membranes, umbilical cord, and placenta are ejected from the uterus [(1)]. A spontaneous vaginal delivery occurs when the baby is born without the need for doctors to utilize instruments to help pull the baby out [(2)]. Induction of labor is the procedure of stimulating the uterus artificially to start labor. It's usually accomplished by administering oxytocin or prostaglandins to the pregnant mother, or by manually rupturing the amniotic membranes. The use of labor induction to shorten the duration of a pregnancy has increased steadily during the last few decades

[3]. In developed countries, the proportion of infants born at term after induction of labor can be as high as one out of every four births [4]. The most commonly used pharmacologic drug for labor induction and augmentation is oxytocin. Oxytocin regimens can be classified as high-dose or low-dose based on the initial dose and the amount and rate of subsequent dose increases [5]. Paraoxonase (PON) is a hydrolytic enzyme with a broad substrate range and the capacity to protect lipids from oxidation. They are a group of mammalian enzymes that act as aryl-di-alkyl phosphatases. PON isozymes are enzymes that are involved in the hydrolysis of organophosphates. There are three types of PON isozymes. The majority of research on the PON family has focused on the PON 1 type, leaving much to learn about the other two [6]. PON have been discovered to perform a variety of biological roles, while the basic function of this family of enzymes is yet unknown. Anti-inflammatory, anti-oxidative, anti-atherogenic, anti-diabetic, anti-microbial, and organophosphate-hydrolyzing capabilities have been discovered in some of the identified roles [7]. Although the specific antioxidant mechanism of PON is unknown, it is known that it is neither mediated by copper ion chelation or possible lipid transfer from low density lipoprotein (LDL) to high density lipoprotein (HDL) [8]. Intrauterine oxidative stress was reported to develop in women who gave delivery or experienced induction of labor [9]. There are studies in the literature that use cord blood to measure oxidative state. Nitric oxide, asymmetrical dimethylarginine, PON, total oxidative state, and total antioxidative status, on the other hand, have yet to be cited in the literature as indicators. Labor triggered by oxytocin raises stress markers but has little effect on Apgar scores. Antioxidative systems may be activated in pregnant women as a result of oxidative stress [10]. For a good pregnancy, the mother's vascular anatomy and vessel activity within the placental bed should be normal. At this stage, PON's antioxidant properties become more essential. PON has potent antioxidant properties and is found at 3- to 4-fold higher levels in females than males, providing increased protection against oxidative stress [11].

This study aimed to compare the influence of induced labor on fetal Apgar score and birthweight, and to measure the level of oxidative stress (PON 1) experienced during labor.

## Methods

A case control study conducted at Department of Obstetrics and Gynecology at Azadi Teaching Hospital, Kirkuk during a period of nine months from 10th of February till 10th of November 2019. This study was approved by the Council of Iraqi Board of Medical Specialization and the Department of Obstetrics and Gynecology at Azadi Teaching Hospital.

The study included 60 healthy pregnant women with singleton pregnancy and viable fetus attending the labor room of the hospital. They were informed about the nature of the study and verbal consent was obtained from them. Women with history of hypertensive disorder of pregnancy, diabetes (gestational or pre-gestational), history of intrauterine growth retardation, history of cardiac diseases, aberrant complete blood count and blood biochemistry test results, pregnancies that occurred as a result of assisted reproductive procedures, history of smoking and using of antioxidant drugs, history of cesarean section (C/S) or uterine surgery and abnormal presentation, and congenital anomalies of the fetus were excluded from the study. They were divided into two groups:

1. Case group: Included 30 pregnant women who underwent labor induced by oxytocin.
2. Control group: Included 30 pregnant women without a previous history of C/S nor uterine surgery with cephalic presentation underwent vaginal delivery without oxytocin induction after matching for age and gestational age with case group. All in latent phase of first stage labor.

Assessment and estimation of gestational age was done depending on the date of last

menstrual cycle, and/or early ultrasound scan. Detailed history by questionnaire, obstetrical history, past medical and surgical histories were taken in both groups. General examination and vital signs also monitored. Abdominal examination was done to assess uterine contraction, also we assessed fundal height, fetal heart. Fundal grip, lateral grip and pelvic grip techniques to assess engagement of fetal head.

Internal examination (per vaginal examination) for consistency, length, dilatation and effacement of cervix and engagement of presenting part in both groups. Labor was followed by partogram, fetal monitoring done by cardiotocography (CTG). Investigation as complete blood count, liver and renal function tests, and serum uric acid were done. Postnatal assessment was done by calculating the Apgar score for all newborns at the first and fifth minute' systemic physical examination. Birthweight was measured by a pediatric scale and blood sent for packed cell volume (PCV) and fetal hemoglobin (HbF).

**Induction of labor in case group**

Five IU/ml of synthetic oxytocin liquid ampoule was prepared by adding it into 500 ml of 0.9% NaCl fluid and given as initial dose of 2 mIU/mint. Intravenously until effective contractions were achieved, then increasing infusion dose by 2 mIU/mint. Every 20 mints. highest dose average was 20 mIU/min. The induction agent dose and contractions formed during labor were recorded [12].

**Sample collection and oxidative stress marker test procedure**

After delivery of fetus, from all study participants, the fetal cord was clamped. Then a five ml of blood was drawn from the umbilical artery and put into a plain tube and left for two hours at room temperature to clot. Then centrifugation was done at 5000 rpm for five minutes. Then the serum was stored at –80° C until the analysis time [12]. Human PON1 was measured by a kit using enzyme linked immune sorbent assay (ELISA) based on sandwich technology. The detection range is 0.16-10 ng/ml.

**Statistical analysis**

Statistical Package for Social Sciences (SPSS) version 26 was used to analyze the data. The information is displayed in the form of a mean, standard deviation, and ranges. The continuous variables were compared using a two-tailed independent t-test. Pearson's correlation test (r) was used to assess correlation between continuous variables accordingly. A level of P-value less than 0.05 was considered significant.

## Results

In this study, study participants age was ranging from 19-41 years with a mean of 29.16±6.0 years. Mean of human PON1 was significantly higher and mean of serum uric acid was significantly lower in women who underwent oxytocin-induced labor than that in women who underwent vaginal delivery without oxytocin induction (10.14 versus 8.08 mg/ml, P=0.045; and 3.5 versus 2.87 mg/dl, P=0.009 respectively). No significant differences (P≥0.05) between study groups regarding age, gestational age, body mass index (BMI), parity, and all other biochemical parameters (Table 1).

No significant differences (P≥0.05) between study groups regarding birthweight, Apgar score 1 min, Apgar score 5 min, PCV, and HbF as shown in table (2).

No significant correlations (P≥0.05) between human PON1 and all of maternal age, BMI, birthweight, Apgar score 1 min, and Apgar score 5 min as shown in table (3).

**Table 1. Comparison between study groups by general biochemical parameters, and human PON1 marker**

| Variable | Case Mean±SD | Control Mean±SD | P - Value |
|---|---|---|---|
| Age (Year) | 29.1±6.3 | 28.72±4.9 | 0.804 |
| GA (Week) | 38.5±1.1 | 38.1±0.7 | 0.132 |
| BMI (kg/m$^2$) | 23.5±2.5 | 23.7±2.1 | 0.694 |
| Parity | 1.33±0.5 | 1.42±0.4 | 0.791 |
| SGOT (U/L) | 37.33±11.8 | 35.78±7.4 | 0.549 |
| SGPT (U/L) | 35.6±8.6 | 36.25±8.4 | 0.771 |
| ALP (IU/L) | 108.0±25.4 | 113.5±16.8 | 0.332 |
| B. Urea (mg/dl) | 31.0±7.1 | 31.3±8.9 | 0.891 |
| S. Creatinine (mg/dl) | 0.48±0.2 | 0.46±0.2 | 0.705 |
| S. Uric acid (mg/dl) | 2.87±1.0 | 3.5±0.7 | 0.009 |
| Human PON1 (mg/ml) | 10.14±4.5 | 8.08±3.1 | 0.045 |

GA = Gestational age, BMI = Body mass index, ALP = Alkaline phosphatase, SGOT = Serum glutamic-oxaloacetic transaminase, SGPT = Serum glutamate-pyruvate transaminase

**Table 2. Comparison between study groups by pregnancy outcome characteristics**

| Variable | Case Mean±SD | Control Mean±SD | P - Value |
|---|---|---|---|
| Birthweight (kg) | 3.44±0.4 | 3.29±0.3 | 0.131 |
| Apgar 1 min | 6.06±1.2 | 6.46±1.3 | 0.237 |
| Apgar 5 min | 8.26±1.1 | 8.03±1.5 | 0.487 |
| PCV of baby (%) | 57.35±7.6 | 60.89±7.8 | 0.089 |
| HbF (gm/dl) | 16.71±1.0 | 17.21±1.4 | 0.141 |

PCV = Packed cell volume, HbF = Fetal hemoglobin

**Table 3. Correlation between human PON1 marker and certain parameters**

| Variable | Human PON1 (mg/ml) | |
|---|---|---|
| | r | P - Value |
| Maternal age (Year) | 0.104 | 0.431 |
| BMI (kg/m$^2$) | 0.105 | 0.427 |
| Birthweight (kg) | 0.03 | 0.765 |
| Apgar 1 min | - 0.2 | 0.125 |
| Apgar 5 min | - 0.197 | 0.131 |

BMI = Body mass index

## Discussion

Maintaining the fetus's proper development during intrauterine life and achieving birth with minimal maternal-fetal trauma has become two of obstetrics' key goals. The birth process involves a sequence of cellular, molecular, and hormonal activities [11]. Induction to start labor is required in about 20-30% of all births.

Intravenous oxytocin is the most extensively used and acknowledged method for inducing labor in third-trimester pregnancies at the moment [12]. The current study is part of a small body of work that looks at the impact of oxytocin induction on oxidative stress and subsequent maternal and fetal adverse effect. This study showed that human PON1 was significantly higher in case group than that in control one and no correlation detected between human PON1 and maternal age, BMI level, birthweight, Apgar score 1 and 5 min. This result agreed with a study done by Karaçor et al. in 2017 who concluded that PON1 has an antioxidant function; thus, the presence of a defensive mechanism against the stress experienced by the mother is indicated by high levels of PON1. These findings suggest that using oxytocin to induce labor increases oxidative stress, and that anti-oxidative mechanisms are activated as a result [12]. PON1 deficiency promotes the oxidation of both high- and low-density lipoproteins, and is consequently linked to a variety of cardiac vascular diseases, including atherosclerosis [13]. Both the mother's vascular structure and vessel activities within the placental bed should be normal for a successful pregnancy. The antioxidant effect of PON1 becomes more important at this time [14]. In this study, mean of serum uric acid was significantly higher in control group than that in case group and this is agreed by Karaçor et al. study in 2017. Uric acid possesses antioxidant properties and is an important determinant of total plasma antioxidant capacity [12]. Oxytocin is the drug most frequently linked to avoidable perinatal complications. Indications, timing, dose, and monitoring of maternal and fetal effects are all ambiguous in recommendations for this drug's use. Precise, evidence-based guidelines for the intrapartum administration of oxytocin can be generated based on a review of current clinical and pharmacologic data. If implemented, such processes may reduce the risk of patient harm [15]. This study showed no statistically significant differences between study groups regarding birthweight, Apgar score 1 min, Apgar score 5 min, PCV, HbF. This is agreed with a result found by Karaçor et al. study in 2017 [12], Hidalgo-Lopezosa et al. study in 2016 [16], and Heimstad et al. study in 2007 [17]. Different result obtained in Gülmezoglu et al. study in 2018 as Apgar score is less than seven at five minutes in the induction groups compared with expectant management [18]. The variations in results might attributed to the sample size enrolled in each study, gestational age (since post-term is associated with higher perinatal morbidity and mortality), type and dosage of stimulants, associated co-morbid diseases, and parity (In fact, because labor is frequently longer, more painful, and can present more difficulties in primiparous women) [19]. Interventions with oxytocin, especially at high doses, have the potential to harm both the mother and the fetus, such as uterine tachy-systole and fetal heart rate disruption [15]. During contractions, blood flow to the intervillous space is reduced or interrupted, resulting in this condition [20]. The majority of fetuses tolerate contractions in typical births; but, if the contractions are particularly frequent and/or lengthy, there is a danger of fetal hypoxemia and acidemia [21]. Interventions during the physiological birth process, according to some authors, increase the risk of alterations for the mother and the fetus in the absence of problems. They argued that evidence-based clinical practice should be used to promote physiological delivery, as well as the avoidance of unnecessary labor inductions and regular care interventions, as well as unnecessary limits [22].

In conclusion, neonates of pregnant women who underwent oxytocin induced labor showed elevated serum human PON1 as antioxidant marker without a significant effect on Apgar score of neonates or its birthweight.

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## Author contribution

Dr. Ghalib: Put the research plan. Dr. Ahmed: Did the sampling, wrote the manuscript and did the statistical work.

## Conflict of interest

Authors declare no conflict of interest.

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# Blink Reflex study in Patients with Migraine

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## Abstract

**Background** The pathogenesis of migraine is thought to include activation of the trigeminovascular system. The blink reflex (BR) test is a well-known method of studying the trigeminal system.

**Objective** To look into the differences in BR response in migraine patients using the standard approach and to compare the results with matched control subjects, looking for a possible difference in BR ictally and interictally, and clarify whether migraineurs with aura differ from those without aura.

**Methods** A case-control study of 80 subjects; 40 patients were diagnosed with migraine, with or without aura, and 40 matched healthy volunteers. Disease duration ranges from 2 to 82 months. Both groups were submitted to medical history, clinical neurological examination, and binocular BR study of both eyes.

**Results** BR data were not different between male and female patients. Right cR2 latency and left iR2 were prolonged in the migraineurs group patients. Right iR2 and cR2 latencies recorded interictally were longer than those obtained ictally. No difference was observed between those with and without aura. The pain location side is not associated with the stimulation side.

**Conclusion** Patients suffering from episodic migraine may have altered interneuronal brainstem circuits. BR data would not change whether migraine was with or without aura. Interictal changes in BR suggest trigeminovascular dysfunction in migraineurs patients is not a transient phenomenon.

**Keywords** Migraine, Blink Reflex

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**List of abbreviations:** BR = Blink reflex

## Introduction

It is widely recognized that migraine implicates activation and sensitization of trigeminovascular pathways, as well as trigeminal nuclei in the brain stem, which play an important role in its pathophysiology [1,2].

It has been proposed that migraine may be considered a brain state of altered excitability (overactive nociceptive and antinociceptive systems) both ictally and interictally [3,4]. In chronic migraine, studies have found a loss of inhibition in pain transmission regulation and an anomaly in cortical pain control [5,6].

The blink reflex (BR) is an electrophysiological test that is achieved by electrically stimulating the supraorbital nerve and is used to evaluate the trigeminovascular system [7].

The ocular branch of the trigeminal nerve serves as an afferent conduit for BR, whereas the facial nerve serves as an efferent pathway [8,9]. Upon stimulation, two responses are recorded; an early ipsilateral (R1) response with an onset latency of 9-12 ms designated, and two late bilateral ipsi- and contralateral responses with a latency of 25-35 ms designated iR2 and cR2, respectively [10].

According to research, the R2 component is influenced by segmental and suprasegmental

pathways. As a result, R2 recordings can be used to examine the excitability of brainstem reticular formation and corticoreticular pathways. Furthermore, changes in R2 latency are caused by aberrant synaptic transmission in the brainstem and interneuronal excitability; thus, changes in R2 latency have been largely related to changes in the control of higher central structures [9,11].

The purpose of this study was to look into the differences in BR response in migraine patients using the standard approach and to compare the results with matched control subjects, looking for a possible difference in BR ictally and interictally, and clarify whether migraineurs with aura differ from those without aura.

## Methods

This is case-control research that was conducted at the Neurophysiology Department of Al-Imamein Al-Kadhimein Medical City in Baghdad from October 2019 to September 2020. The Iraqi Board of Medical Specialization approved the study (order no. 931; date: 1/3/2020). All the participants were informed about the technique and the aim of the study and informed consent was obtained from them.

### Subjects

There were seventy-seven subjects investigated. According to the International Classification of Headache Disorders, forty individuals (28 females and 10 men) were diagnosed with a migraine, with or without aura [12]. The age range was 22 to 54 years (35.5±7.82 years).

Patients who had received prophylactic treatment within the previous 3 months, had a disease that could affect electrophysiological examination or involved the trigeminal or facial nerve, had a structural lesion detected on cranial images, had headaches other than migraine, or were younger than 18 or older than 60 years old were excluded from the study.

Another 39 age- and sex-matched healthy subjects comprised of 25 females and 15 males served as the control group.

### Methods

#### *History and clinical examination*

After a brief medical history was taken from each patient, data on gender, type of migraine (with or without aura), the time interval between examination and onset of attack (during, within, or 72 hours after onset of the attack), associated and relieving symptoms, frequency, and location of the attack were recorded by a senior neurologist.

#### *Electrophysiological assessments*

Keypoint (Medtronic functional diagnosis A/S - DK-2740 Skovlunde, Denmark) EMG machine was used throughout the study. The room temperature was monitored between (25-28°C) during the test procedure and skin temperature between (32-34°C) was ensured using a skin thermometer.

Preston and Shapiro's approved approach was used to examine the BR [13]. Surface recording electrodes were placed over the inferior orbicularis oculi muscles bilaterally for recording the compound muscle action potential from the orbicularis oculi muscle. The active recording electrode is best placed below the eyes just lateral and inferior to the pupil mid–position. The corresponding reference electrode is placed lateral to the outer canthus bilaterally. A ground electrode was placed at the forehead or chin using a special paste and fixed with plaster, to ensure good electrical conduction.

A bipolar surface stimulating electrode was used to stimulate the supraorbital nerve ipsilaterally.

An electrical pulse with a duration of 100 ms is used. The current was increased in modest increments (3-5 mA) from zero mA to supramaximal stimulation, resulting in a response with the shortest latency and largest amplitude potential. A modest current can easily excite the nerve. Supramaximal

stimulation requires no more than 15 to 25 mA.

Because the BR is a multisynaptic route, there is some fluctuation between subsequent supraorbital nerve activations (particularly the R2), and at least 8-10 responses should be induced. Following the completion of one side's investigation, the contralateral side is stimulated and responses are recorded. The shortest latency of the Rl, iR2, and cR2 components was measured.

The electromyography settings were 5 or 10 ms per division sweep speed, the bandwidth of 10 Hz - 10 kHz, and the sensitivity of 100 or 200 µV per division.

**Statistical analysis**

All statistical analyses were performed using statistical package for social sciences (SPSS), version 25 (IBM Corporation, USA). Quantitative variables were presented as mean ± standard deviation (SD) and analyzed with an independent student t-test. Categorical variables were expressed as counts and percentages and analyzed with a chi-square test. For all tests, a significant level of statistics was considered when $p<0.05$.

## Results

No significant difference was noticed in the age and gender between the migraine and control groups (Table 1).

**Table 1. Demographic characteristics of migraine and control groups**

| Variables | | Migraine group | Control group | P-value |
|---|---|---|---|---|
| Number of participants | | 38 | 39 | |
| Age (years) | Mean±SD | 35.5±7.82 | 39.31±9.77 | 0.065 |
| Gender | Males, n (%)<br>Females, n (%) | 10 (26.3%)<br>28 (73.7%) | 14 (35.8%)<br>25 (64.2%) | 0.364 |
| Disease duration (months) | Range | 2-82 | | |
| Location | Unilateral, n (%)<br>Bilateral, n (%) | 17 (44.7%)<br>21 (55.3%) | | |
| Type of migraine | With aura, n (%)<br>Without aura, n (%) | 17 (44.7%)<br>21 (55.3%) | | |

Table 2 illustrates the data of BR components in migraine and control groups. The right cR2 latency and the left iR2 were significantly prolonged in the patients versus the controls ($p = 0.024$; $p = 0.019$, respectively). Other BR components were not different between the two groups.

Dividing the patients with migraine into those with and without aura, none of the BR components were different between the two subgroups (Table 3).

Based on the time of the attack, patients were divided into two groups (Table 4). The right iR2 and cR2 latency values acquired from patients following 2 days of the headache attack were substantially longer than those obtained from patients during the headache attack ($p = 0.002$; $p < 0.001$, respectively).

**Table 2. Comparison of blink reflex latency values in migraine and control groups**

| Variables | | Migraine group N=38 Mean±SD | Control group N=39 Mean±SD | P-value |
|---|---|---|---|---|
| iR1 latency (ms) | Right | 10.48±1.66 | 9.71±2.76 | 0.144 |
| | Left | 10.43±2.84 | 9.55±1.93 | 0.116 |
| R2 latency (ms) | Right iR2 | 33.25±6.36 | 33.31±5.02 | 0.969 |
| | Right cR2 | 35.53±6.26 | 32.27±6.19 | 0.024 |
| | Left iR2 | 36.73±7.21 | 33.13±5.81 | 0.019 |
| | Left cR2 | 36.1±6.77 | 36.33±7.31 | 0.887 |

R1: Early component; iR2: Ipsilateral late component; cR2: Contralateral late component; SD: Standard deviation

**Table 3. Comparison of latency values obtained on eye blink reflex test in patient groups with migraine**

| Variables | | Migraine without aura N=17 Mean±SD | Migraine with aura N=21 Mean±SD | P-value |
|---|---|---|---|---|
| iR1 latency (ms) | Right | 10.75±1.31 | 10.14±2.0 | 0.269 |
| | Left | 11.14±3.14 | 9.54±2.18 | 0.085 |
| R2 latency (ms) | Right iR2 | 33.7±6.1 | 32.7±6.82 | 0.637 |
| | Right cR2 | 36.38±6.25 | 34.48±6.3 | 0.362 |
| | Left iR2 | 33.22±5.3 | 33.02±6.56 | 0.918 |
| | Left cR2 | 35.68±7.45 | 36.1±6.77 | 0.671 |

R1: Early component; iR2: Ipsilateral late component; cR2: Contralateral late component; SD: Standard deviation

**Table 4. Comparison of blink reflex latency values according to the time passed between the start of the examination and the last attack**

| Variables | | Ictal N=16 Mean±SD | The last 2 days N=22 Mean±SD | P-value |
|---|---|---|---|---|
| iR1 latency (ms) | Right | 9.98±1.98 | 10.8±1.66 | 0.137 |
| | Left | 9.64±2.23 | 10.94±3.11 | 0.17 |
| R2 latency (ms) | Right iR2 | 29.41±6.47 | 35.75±5.0 | 0.002 |
| | Right cR2 | 30.72±5.06 | 38.67±4.85 | <0.001 |
| | Left iR2 | 31.55±6.78 | 34.42±4.82 | 0.090 |
| | Left cR2 | 35.19±7.22 | 36.7±6.55 | 0.510 |

R1: Early component; iR2: Ipsilateral late component; cR2: Contralateral late component; SD: Standard deviation

## Discussion

In this study, significantly longer right cR2 and left iR2 latencies were recorded in the migraineurs independent of stimulation. These changes could be the result of aberrant synaptic transmission in the brainstem and interneuronal excitability. This idea is

supported by neural networks mediated by the R2 component of BR, which include the trigeminal caudal nucleus, excitatory interneurons of the bulbopontine lateral reticular formation, and pontine facial nuclei innervating orbicularis oculi. Furthermore, because R2 response is controlled by segmental and suprasegmental mechanisms, it assesses the excitability of brainstem reticular formation and corticoreticular circuits [9,11].

Imaging investigations on migraine sufferers revealed increased brainstem activity, implying that the brainstem may be a migraine generator [14]. During ictal and interictal migraine headache attacks, these imaging tests revealed anomalies in the ascending and descending nociceptive pathways [15,16].

Several investigations comparing the latencies of R1, iR2, and cR2 waves in migraine sufferers and controls (Table 5) yielded diverse results. Unal et al. [17] discovered significantly longer R1 latency values on both the right and left sides, as well as both the left iR2i and cR2, in the migraine group compared to the control group in their investigation.

Patients who experienced an episode at the time of the research showed longer R1 and R2 latencies [17]. Their findings corroborate the trigeminovascular theory of migraine by pointing to abnormalities in the brainstem and trigeminovascular connections of migraine patients.

**Table 5: Main published studies about the blink reflex in migraine**

| Authors | N | Diagnosis | Age Mean±SD | Timing of recording | Significant findings |
|---|---|---|---|---|---|
| Present study | 38 | 21 MO, 17 MA | 35.5±7.82 | 1-3 days after the attack | R2 latency prolongation |
| Avramidis et al. (2017) [21] | 55 | | | Ictal | Reduced R2 amplitude and area |
| Uygunoglu et al. (2017) [29] | 20 | 6 MO, 14 CM | 37.5±8.9 | Within 48 h after the attack | No difference |
| Unal et al. (2016) [17] | 40 | 31 MO, 9 MA | 37.36±9.67 | During the attack | R2 latency prolongation |
| Brooks and Fragoso (2013) [28] | 160 | CM | 50.8±18.2 | NA | No difference |
| Zduńska et al. (2013) [27] | 29 | | | NA | No difference |
| Yildirim et al. (2011) [18] | 40 | 25 MO, 15 MA | 30.85±8.03 | Interictal | R2 latency prolongation |
| De Marinis et al. (2007) [11] | 35 | CM | 37.0±6.0 | >72 hours after the attack; <3 hours before the next attack | No difference |
| Sand et al. (2006) [26] | 23 | 13 MO, 10 MA | 33.9±12.5 | NA | No difference |
| De Marinis et al. (2003) [25] | 30 | MO | 33.0±8.0 | >72 hours after the attack | No difference |
| de Tommaso et al. (2002) [5] | 70 | 50 MO, 20 MA | | during different cognitive situations | Altered cR2 amplitude and latency |
| Aktekin et al. (2001) [24] | 34 | 24 MO, 10 MA | 32.7±8.5 | Interictal | No difference |
| Avramidis et al. (1998) [20] | 19 | MO | 37.5 | Ictal | Reduced R2 amplitude |
| Sand and Zwarts (1994) [23] | 15 | 10 MO, 5 MA | 19.0±12.0 | NA | No difference |
| Bánk et al. (1992) [19] | 43 | 33 MO, 10 MA | 31.1±9.6 | > 14 days after the attack | Prolonged R2 latency |

MO = Migraine without aura; MA = Migraine with aura; CM = Chronic migraine

When a 40-migraineur group was compared to a control group, there was a statistically significant increase in bilateral R2 latencies [18]. They confirm that migraineurs have functionally defective brainstem and trigeminovascular connections, as evidenced by trigeminal system activation, sensitization of the brainstem trigeminal nucleus, aberrant synaptic transmission, and suppression of brainstem interneuron areas.

Furthermore, significantly prolonged R2 but similar R1 latencies in the migraine group were found in the patient group [19] harmonizing the results of this study. These data suggest that in migraine, the trigeminal afferent and/or polysynaptic pathways in the brainstem may be functionally changed.

Avramidis et al. [20,21] observed reduced R2 amplitude ictally and proposed that the brain stem interneuron part of the BR arc may be diffusely suppressed in migraine, with an abnormality in the R2 component only during the attack phase of migraine and that this dysfunction returned to normal thereafter. Other investigations have found that migraineurs have a lack of habituation to pain stimuli [11,22].

On the reverse, many studies found no difference between controls and migraineurs [11,23-29]. They believed BR to be a primitive reflex or relatively basic reflex that is not impaired unless there is considerable brainstem damage; others stated that this is proof that migraine-specific trigeminal dysfunction is a temporary disorder.This disparity in results was attributed to different methodologies utilized, differences in patient selection, latency recording time (ictal/interictal), a lower sample size, or incorrect designation of individuals as migraine sufferers. Furthermore, methodological discrepancies between research may cause disagreement when comparing the results. On the other hand, no single approach or gold standard is advised for BR research in migraine or other primary headaches.

When dividing patients according to the type of migraine, 55.3% were without aura and 44.7% with aura. Although the discrepancy is likely due to the limited sample size, studies show a decreased prevalence of migraine with aura compared to the most frequent migraine without aura [30].

In this study, no significant difference was noticed between migraineurs with and without aura regarding BR latencies. This is in harmony with the findings of other groups of researchers [17]. Meanwhile, the right R2 amplitude value was found significantly higher in the group with aura than without aura as noticed by another group [18].

While dividing the patients into two subgroups based on whether the testing is done ictally or intericately, the right iR2 and cR2 latencies were prolonged interictally than those obtained ictally. This shows that trigeminovascular dysfunction in migraine patients is not a transient phase. This finding was in harmony with the results of other researchers [18,19], but in contradiction to those of Unal et al. [17]. This situation may depend on the special selection of the patients.

This study, as well as other ictal and interictal investigations, looked into the relationship between the pain localization site and the recorded side of BR. There was no correlation discovered between the area of pain and the recorded side [11]. The current study's findings are most likely because BR measurements were taken while some of the patients were pain-free. However, another study showed a significant correlation between the symptomatic side and prolong R1 and R2i latency values during the ictal phase [17]. The latter authors interpret their findings as a result of trigeminal nucleus sensitization on the symptomatic side during an ictal phase. Another group of researchers found an increase in the area-under-the-curve of R2 on the affected side during migraine attacks [28].

In conclusions, patients suffering from episodic migraine may have altered interneuronal

brainstem circuits. BR data would not change whether migraine was with or without aura. Interictal changes in BR suggest trigeminovascular dysfunction in migraineurs patients is not a transient phenomenon.

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## Author contribution

Both authors contributed directly to the creation of this paper and approved the final version that was submitted. The electrodiagnostic tests were performed by both authors. Likewise for the manuscript. Also, the study was conceptualized, designed, and interpreted by both authors. Similarly, the final manuscript has been read and approved by both authors.

## Conflict of interest

The authors declare no conflict of interest.

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# Significance of HbA1c Test and Different Sociodemographic Factors in The Development of Complications in Type 1 Diabetes in Children

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## Abstract

**Background** Despite adequate treatment, about 50% of patients with type 1 diabetes mellitus (T1DM) usually develop a serious complication during their lifetime. The level of hemoglobin A1c (HbA1c) reflects glycemic control, the screening of which is particularly helpful in community-based care settings where tests requiring fasting are not that practical.

**Objective** To describe the difference in HbA1c level among a sample of children with T1DM and to identify the most common type of compilations and its association with HbA1c and different sociodemographic factors.

**Methods** A cross sectional study, included 96 children with T1DM, their age ranged between 8-18 years, that attended Medical City Complex in Baghdad in the Pediatrics outpatients Clinic and Endocrine Outpatient Clinic during period from 1st of July 2021 to 30th of October 2021.

**Results** Males formed 47.9%, 45.8% of them were in school age group, the mean HbA1c level was (10.74±2.974) mg/dl. Employed fathers formed 52.1%. Family history of T1DM was among 13.5% and that of T2DM was among 34.4%. Complication appeared among 71.9% of children, mainly neuropathy found among 50% of them. Children with retinopathy, positive family history have significantly poor controlled DM (last reading HbA1c ≥7 mg/dl).

**Conclusion** HbA1c level in children with T1DM is found to be associated with family history, parents' education and employment, in addition a significant association with development of retinopathy in those children was also found in this study.

**Keywords** Type 1 DM, HbA1c, retinopathy

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**List of abbreviations:** DM = Diabetes mellitus, T1DM = Type 1 diabetes mellitus, T2DM = Type 2 diabetes mellitus. ISPAD = International Society for Pediatric and Adolescent Diabetes

## Introduction

Type 1 diabetes mellitus (T1DM) is a metabolic disease causing many complications and pathological changes due to β-cell destruction. It is associated with high morbidity and mortality. Despite adequate treatment, about 50% of patients usually develop a serious complication during their lifetime [(1)]. Some will lose eyes vision, and others may develop nephropathy, cataracts, gastroparesis, hypertension, neuropathy, coronary disease increased susceptibility to infections, and peripheral vascular disease are common complications. For patients who pass the first 20 years safely, the prognosis is good [(2)].

Maintaining euglycemia is associated with severe anxiety and depression for both

patients and their parents; for many patients with T1DM, the quality of life is unsatisfying [3].
The level of hemoglobin A1c (HbA1c) reflects glycemic control. The screening of which is particularly helpful in community-based care settings where tests requiring fasting are not that practical. HbA1c screening should be used whenever possible to diagnose diabetes. HbA1c ≥6.5% is the most reliable indicator of the diagnosis and follow up of diabetes [4]. Patients with an HbA1c level between 6% and 6.4% in screening programs are considered to be at high risk for developing diabetes, while HbA1c levels of around 7% had the best outcomes especially regarding long-term complications. That's why most clinicians aim for HbA1c values of 7-9%. for all children. The International Society for Pediatric and Adolescent Diabetes (ISPAD) recommends and suggest a target level of 7.5% (58 mmol/mol) or less for good glycemic control [5,6].
This study aimed to describe the difference in HbA1c level among a sample of T1DM children and to identify the most common type of compilations and its association with HbA1c and other different socio-demographic factors.

## Methods

A cross sectional study, included T1DM patients (age range of 8-18 years) that attended Pediatrics Outpatients Clinic and Endocrine Outpatient Clinic in the Medical City Complex and during period from 1st of July 2021 to 30th of October 2021. Data collection was planned for follow up of their condition; for 2 consecutive months, 2 days/week, 5 hours/ day were included. A normal HbA1c level cutoff is below 5.7%
The complications were diagnosed by a specialist endocrinologist, nephrologist, neurologist, in addition to ophthalmologist and confirmed in the patient record.
The main complications were followed up were:
***Retinopathy*:** often refers to retinal vascular disease, or damage to the retina caused by abnormal blood flow [5].

***Nephropathy*:** which is the deterioration of kidney function. According to the CDC, diabetes is the most common cause of end stage renal disease [5].

***Hypoglycemia*:** A state low blood sugar level that cause symptoms [2].

***Poor weight gain and short stature*:** measured according to growth chart of appropriate age and gender [3].

The children who admitted to the wards or emergency units for other reasons were excluded from the study.
A questionnaire paper was filled by researcher; included the socio-demographic and clinical features (age, gender, parents' job, parents' education, family history, age at diagnosis, complications, in addition to laboratory test of HbA1c.

### Statistical analysis

Microsoft Excel 2010 and IBM SPSS (statistical package for social sciences) version 24 were used for data entry, management, and analysis. Descriptive analyses of the variables were expressed as frequencies and percentage for categorical data. While mean of standard deviation was used for quantitative data that is normally distributed, represented by figures and tables. To compare qualitative variables, we utilized the chi-square test, and we used P at level 0.05 to determine statistical significance.

### Ethical approval

This study was approved by Ministry of Health, Medical city Directorate, and Medical City Hospital ethics committee and the requirement for informed consent was waived by the Ethics Commission due to the observational nature of the study. Verbal consents were taken from parents.

## Results

Table 1 shows that males formed 47.9% of the 96 children with T1DM enrolled in this study, their mean age was 10.85±3.35 years and

mean HbA1c level at diagnosis 10.74±2.97 mg/dl; while females formed 52.1%, their mean age 10.8±3.03 years and mean HbA1c level at diagnosis 10.95±3.19 mg/dl.

**Table 1. Gender distribution of patients according to the mean age and mean level of HbA1c at diagnosis**

| Gender | N | % | Age (years) Mean±SD | HbA1c at diagnosis Mean±SD |
|---|---|---|---|---|
| Male | 46 | 47.9 | 10.85±3.35 | 10.74±2.97 |
| Female | 50 | 52.1 | 10.8±3.03 | 10.95±3.19 |
| Total | 96 | 100% | P=0.94 | P=0.74 |

The socio-demographic features of the patients are represented in table 2; which revealed that employed fathers formed 52.1%, and 79.2% of them with secondary school education or less. Employed mothers formed 11.5%, and 81.3% of them with secondary school education or less. Family history of T1DM was among 13.5% and that of T2DM was among 34.4%. Complication appeared among 71.9% of patients.

**Table 2. Socio-demographic features of type 1 diabetes mellitus patients**

| Socio-demographic features | | N | % |
|---|---|---|---|
| Age Group | <10 years | 14 | 14.6 |
| | School age (10 to 15 years) | 44 | 45.8 |
| | >15 years | 38 | 39.6 |
| Father Occupation | Employed | 50 | 52.1 |
| | Not employed | 46 | 47.9 |
| Father Education | > Secondary school | 20 | 20.8 |
| | ≤ Secondary school | 76 | 79.2 |
| Mother Occupation | Employed | 11 | 11.5 |
| | Not employed | 85 | 88.5 |
| Mother Education | > Secondary school | 18 | 18.7 |
| | ≤ Secondary school | 78 | 81.3 |
| Family History of DM | No | 50 | 52.1 |
| | T1DM | 13 | 13.5 |
| | T2DM | 33 | 34.4 |
| Complications | No | 27 | 28.1 |
| | Yes | 69 | 71.9 |
| Total | | 96 | 100 |

T1DM: Type 1 diabetes mellitus, T2DM: Type 2 diabetes mellitus

Figure 1 shows types of complications among children. The commonest complications found in children was neuropathy (50%) followed by nephropathy (40.6%). Other complications

were hypoglycemia, which found among 12.5% of children, retinopathy found among 9.4% of children, and poor weight gain found among 9.4% of children. While the least complication was short stature, which found among 8.3% of children.

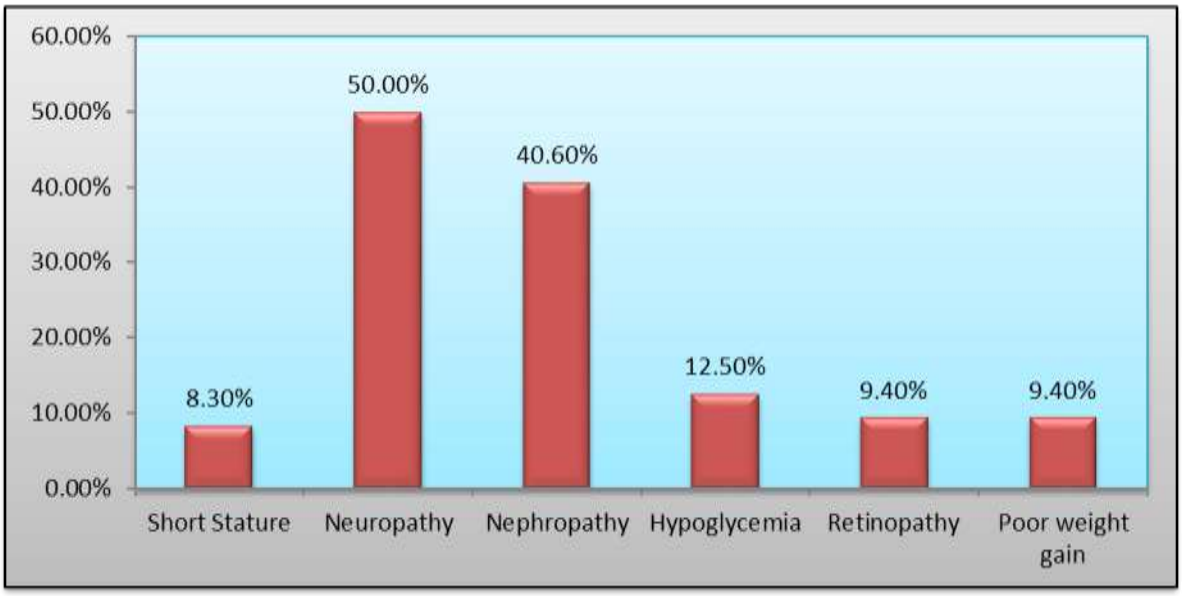


**Figure 1. Complications among children with type 1 diabetes mellitus**

According to the current HbA1c levels, there were 13 (76.5%) children with employed fathers have significantly good controlled DM (last reading HbA1c <7 mg/dl), 44 (88%) children with negative family history have significantly poor controlled DM (last reading HbA1c ≥7 mg/dl), and 74 (93.7%) children with retinopathy have significantly poor controlled DM (last reading HbA1c ≥7 mg/dl), with P=0.027, P=0.014, and P=0.02 respectively. (Table 3).

The association between having complications and other features of children was shown in table 4. There was a significant difference between family history of DM type 2 and having complications, P<0.001.

**Table 3. Sociodemographic distribution of patients according to current HbA1c level**

| Parameter | | Controlled DM (HbA1c.mg/dl) | | | | P value |
|---|---|---|---|---|---|---|
| | | Good (HbA1c <7) | | Poor (HbA1c ≥7) | | |
| | | N | % | N | % | |
| Age Group | <10 years | 1 | 5.9% | 13 | 16.5% | 0.17 |
| | 10 to 15 years | 6 | 35.3% | 38 | 48.1% | |
| | >15 years | 10 | 58.8% | 28 | 35.4% | |
| Gender | Male | 6 | 35.3% | 40 | 50.6% | 0.25 |
| | Female | 11 | 64.7% | 39 | 49.4% | |
| Father Occupation | Employed | 13 | 76.5% | 37 | 46.8% | 0.027 |
| | Not employed | 4 | 23.5% | 42 | 53.2% | |
| Father Education | > secondary school | 4 | 23.5% | 16 | 20.3% | 0.76 |
| | ≤ secondary school | 13 | 76.5% | 63 | 79.7% | |
| Mother Occupation | Employed | 3 | 17.6% | 8 | 10.1% | 0.37 |
| | Not employed | 14 | 82.4% | 71 | 89.9% | |
| Mother Education | > secondary school | 3 | 17.6% | 15 | 19.0% | 0.9 |
| | ≤ secondary school | 14 | 82.4% | 64 | 81.0% | |
| Family History of DM | No | 6 | 12% | 44 | 88% | 0.014 |
| | T1DM | 6 | 46.2% | 7 | 53.8% | |
| | T2DM | 5 | 15.2% | 28 | 84.3% | |
| Complications | No | 4 | 23.5% | 23 | 29.1% | 0.64 |
| | Yes | 13 | 76.5% | 56 | 70.9% | |
| Short Stature | No | 15 | 88.2% | 73 | 92.4% | 0.56 |
| | Yes | 2 | 11.8% | 6 | 7.6% | |
| Neuropathy | No | 9 | 52.9% | 39 | 49.4% | 0.78 |
| | Yes | 8 | 47.1% | 40 | 50.6% | |
| Nephropathy | No | 13 | 76.5% | 44 | 55.7% | 0.11 |
| | Yes | 4 | 23.5% | 35 | 44.3% | |
| Hypoglycemia | No | 16 | 94.1% | 68 | 86.1% | 0.36 |
| | Yes | 1 | 5.9% | 11 | 13.9% | |
| Retinopathy | No | 13 | 76.5% | 74 | 93.7% | 0.02 |
| | Yes | 4 | 23.5% | 5 | 6.3% | |
| Poor weight gain | No | 15 | 88.2% | 72 | 91.1% | 0.71 |
| | Yes | 2 | 11.8% | 7 | 8.9% | |

**Table 4. Association between having complications and sociodemographic features of children**

| Parameter | | Complications | | | | P value |
|---|---|---|---|---|---|---|
| | | No | | Yes | | |
| | | N | % | N | % | |
| Age Group | < 10 years | 7 | 25.9% | 7 | 10.1% | 0.13 |
| | (10to 15 years) | 10 | 37.0% | 34 | 49.3% | |
| | >15 years | 10 | 37.0% | 28 | 40.6% | |
| Gender | Male | 12 | 44.4% | 34 | 49.3% | 0.67 |
| | Female | 15 | 55.6% | 35 | 50.7% | |
| Father Occupation | Employed | 15 | 55.6% | 35 | 50.7% | 0.65 |
| | Not employed | 12 | 44.4% | 34 | 49.3% | |
| Father Education | > secondary school | 6 | 22.2% | 14 | 20.3% | 0.83 |
| | ≤ secondary school | 21 | 77.8% | 55 | 79.7% | |
| Mother Occupation | Employed | 2 | 7.4% | 9 | 13.0% | 0.43 |
| | Not employed | 25 | 92.6% | 60 | 87.0% | |
| Mother Education | > secondary school | 3 | 11.1% | 15 | 21.7% | 0.23 |
| | ≤ secondary school | 24 | 88.9% | 54 | 78.3% | |
| Family History of DM | No | 18 | 36% | 32 | 64% | <0.001 |
| | Type 1 DM | 9 | 69.2% | 4 | 30.8% | |
| | Type 2 DM | 0 | 0.0% | 33 | 100% | |

## Discussion

T1DM has a high morbidity and mortality in children. The HbA1c test is the routine index of an average blood glucose level over the past 2-3 months. The test results offer important feedback to health care providers as well as to the parents [2,7]. This study included 96 children with T1DM. Females formed more than half of the sample, the mean HbA1c level at diagnosis was high for both females and males (10.95±3.19 and 10.74±2.97) mg/dl respectively with no significant association between age and HbA1c level, which can be explained as the HbA1c level reflect the glycemic control in the last three months at any age.

This is slightly differing from a longitudinal cohort study of T1DM pediatric patients in Bart Hospital/England [8], in which 52.6% of patients were male, the mean diagnostic age was 9.0±4.1 years. HbA1c level was differed markedly across age groups, with older patients experiencing greater deterioration and higher levels than their younger counterparts ($p<0.001$) [9].

Glycemic control, duration of diabetes, and age, are important critical factors contributing toward development of complications. Other risk factors may include family history (genetic predisposition). In this study, complications appeared among 71.9% of children, they were mainly neuropathy found among 50% of children, nephropathy in 40.6%, hypoglycemia in 12.5%, retinopathy found among 9.4%, poor weight gain found among 9.4% and short stature among 8.3% of those children. The Diabetes Control and Complications Trial (DCCT) stated clearly the importance of glycemic control through controlled HbA1c level and emphasized the ability of improved this control to prevent and/or decrease complications such as retinopathy, neuropathy, and nephropathy using a multidisciplinary approach in addition to targeted glycemic and HbA1c values [10]. This is in agreement with a retrospective analysis by the Department of Metabolic Diseases in Krakow, in which the percentages of diabetic complications were: diabetic retinopathy in 41.5%, polyneuropathy in 29%, nephropathy in 17%, cardiovascular autonomic neuropathy 8.7%, and coronary artery disease 7.1% of patients. (71.4%) [11].

The current study found an association between poor control DM and the development of complications, it was significantly found between HbA1c level and

retinopathy, this is expected as HbA1c variability predicts the establishment of diabetes pathological changes like retinopathy, early nephropathy, and vascular problems, in addition to the presence of other risk factors, in adolescents with T1DM. That is why minimizing long term fluctuations in glycemic level could provide additional possible protection against the development and progression of microvascular complications.

These facts are agreed with a prospective cohort study from 1990 to 2014 done on 1706 adolescents of less than 20 years of age, in which HbA1c was associated with early retinopathy in those patients after adjusting other risk factors, including diabetes duration, environmental factors, blood pressure, and lipids [12].

Current study found that family history of T1DM was positive in 13.5% and that of T2DM was among 34.4% of parents. There was a significant difference between family history of DM and having complications. Employed parents formed 63.6%, and majority of them with secondary school education or less (76.5%) children of employed parents have significantly good controlled DM (last reading HbA1c <7 mg/dl)

On the other hand, Andreasson et al, on their study to evaluate the parental involvement in blood glucose monitoring through interviews with parents in 10- to 15-year-old patients with T1DM, found that that encouraging parental involvement in glycemic monitoring may help to prevent the well-documented deterioration in glycemic control and adherence to treatment that may occurs in later on in adolescence. There were significant differences in the mean HbA1c values between the groups who had more educated and oriented parents, parental knowledge and involvement was significantly related to adherence to glycemic control (number of blood sugar concentrations checked per day) in both groups of children and adolescent patients [13].

In conclusion, the most common complication associated with DM in this study was neuropathy and nephropathy. Significant association was detected between high HbA1c and both negative family history of DM in patients and patients having unemployed fathers. Also, there is significant association between poorly controlled DM and the occurrence of retinopathy. There is significant association between positive family history of type 2 DM and the occurrence of complications.

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## Author contribution

Dr. Qasim: Data collection and results analysis. Dr. Tawfeeq: Writing and editing of manuscript.

## Conflict of interest

None.

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# Brainstem Auditory Evoked Potential in Patients with Posterior Circulation Ischemic Stroke

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## Abstract

**Background** Approximately 20% of ischemic strokes affect the posterior circulation of brain structures. Mortality and risk of recurrent stroke are high with the possibility of misdiagnosis. As a result, an effective mode of examination is required for early diagnosis.

**Objective** To investigate the clinical diagnostic utility of brainstem auditory evoked potentials (BAEPs) in patients with posterior circulation ischemic stroke (PCIS).

**Methods** Twenty patients with PCIS aged 40 to 70 years were studied, along with 20 age-matched healthy controls. Medical history was taken, as well as a clinical neurological examination and BAEPs.

**Results** Waves IV and V latencies, and III-V inter-peak latencies were significantly prolonged and wave V amplitude was significantly reduced in patients with PCIS. Fifteen patients with PCIS show retrocochlear (central) dysfunction abnormality. Wave V latency and amplitude showed the highest estimated specificity and sensitivity with 80 % and 90%, respectively. Wave V latency negatively correlated with a duration from onset of stroke to neurophysiological examination.

**Conclusion** BAEPs are a useful tool as a biomarker for the clinical evaluation of PCIS. Wave V latency and amplitude is the most specific and sensitive among BAEPs parameter in the diagnosis of PCIS. Topographic distribution of the lost BAEP waves (according to diffusion-weighted MRI findings) may be closely linked to lost generators of individual waves, which suggests regional diagnostic validity of BAEPs. Finally, an early post-stroke BAEPs examination is better in detecting the abnormality and can provide reference values for further evaluation.

**Keywords** Posterior circulation ischemic stroke, brainstem auditory evoked potentials

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**List of abbreviations:** AUC = Area under the curve, BAEPs = Brainstem auditory evoked potentials, IPL = Inter-peak latencies, PCIS = Posterior circulation ischemic stroke, ROC = Receiver operating characteristic

## Introduction

The posterior cerebral circulation provides only about one-third of the total flow perfusing the brain [(1)]. This circulation supplies blood to the posterior portion of the brain that includes the occipital lobe, most of the anterior and posterior portions of the brainstem, thalamus, hippocampus, and all of the cerebellum [(2,3)].

Stroke is a clinical syndrome caused by vascular diseases with a high incidence rate and quite complicated etiological causes [(4)]. Approximately 20% of the strokes involve the posterior cerebral circulation [(5)].

The clinical course of posterior circulation ischemic stroke (PCIS) is difficult to predict and the clinical manifestations tend to disappear quickly making it difficult to detect positive

symptoms. There may be progressive stroke-induced brain damage during the subacute stage, which further aggravates the neurological outcome [6].

As a result of the lack of objective techniques for diagnosing the disease, the diagnosis is primarily dependent on the neurologist's clinical experience and is thus prone to misdiagnosis. Moreover, even imaging studies such as skull computerized tomography (CT) and magnetic resonance imaging (MRI) may not discover any responsible foci [7]. Therefore, precise monitoring and evaluation of brain injury are paramount for establishing effective treatment strategies and prognosis prediction.

Evoked potentials are commonly used to predict the outcome of individuals suffering from sudden severe strokes. These evoked potentials were examined at various points following the commencement of the stroke [8,9]. Physicians frequently seek early prognosis forecasts to improve treatment techniques.

Brainstem auditory evoked potentials (BAEPs) are a sensitive objective indicator of brainstem injury that can objectively reflect peripheral auditory sensitivity and brainstem conduction capability. Clinically, wave I-V is the most consistent and stable of the seven waves. The integrity and normal function of the auditory nerve and brainstem pathway is required for normal BAEPs [10].

Wave I represents the extracranial section of the auditory nerve; wave III represents the activity of the medial superior olive nucleus or cochlear nuclear power, and wave V principally represents the inferior pontine segment and the central nucleus of the inferior colliculus of the midbrain [11,12].

The origin of BAEP waves I-V corresponds to the blood supply area of the vertebral-basilar artery (posterior circulation) system [13]. Thus, detection of latencies and inter-peak latencies (IPL) can reflect brainstem ischemia and changes in blood flow perfusion in brainstem nuclei, as well as more early subclinical abnormalities in patients with PCIS [14]. Furthermore, publications imply that BAEPs have a unique diagnostic value and can provide objective proof for PCIS diagnosis [15,16].

The objectives of this work are to study BAEPs in PCIS, explore the clinical diagnostic value of BAEPs in the evaluation of patients with PCIS, look for the site of abnormality within the BAEPs pathway, and evaluate the sensitivity and specificity of different waves of BAEPs in PCIS.

## Methods

This is a prospective case-control study that was conducted at the Neurophysiology Unit of Al-Imamein Al-Kadhimein Medical City from May 2019 to November 2020. The Iraqi Board of Medical Specialization approved the study (order no. 931: date: 1/3/2020. All subjects provided written consent for participation. All participants were informed about the technique and aim of the study and written informed consent was obtained from them.

Twenty patients of either gender (12 males and 8 females) were studied and chosen from those attending the Department of Neurology with a diagnosis of PCIS according to clinical history, examination, and diffusion-weighted MRI. Their age ranges between 40 and 70 years (58.25±7.95 years). The duration from stroke onset to neurophysiological study was in the range of 2 to 30 days.

Another 20 healthy and symptoms-free normal persons (10 males and 10 females), aged 40 to 70 years (58.9±5.72 years) served as the control group.

A senior Neurologist performed a thorough medical and neurological history and assessment. All patients who met the following criteria were included in the study: in the case history, age ranges from 40 to 70 years, neurological deficits signs and symptoms must be able to be located in a specific posterior circulation distribution region, abrupt start, peaks within a few minutes, and usually subsides within 24 hours, at least 2-3 occurrences of signs and symptoms such as dizziness, vomiting, nystagmus, ataxia, perioral numbness, trouble swallowing, and sudden deafness, as well as indications of an ischemic

lesion in the territorial circulatory system detected by diffusion-weighted MRI.

The study excluded any patient with diabetes mellitus, a history of significant hearing loss, a history of a previous stroke, craniocerebral trauma or hemorrhage, or an intracranial tumor.

Keypoint electromyography (EMG) machine (Medtronic functional Diagnostic A/S -DK-2740 Skovlunde Denmark) was used in this study. Monoaural stimulation was done by conventional audiometric earphones to deliver an electric square wave "click with a rate of 10/sec". The preferred stimulus intensity for waveform recognition was 60 dB above the click hearing threshold. The non-stimulated ear was masked by white noise at 60 dB. Two replication was done for each ear and the averages of 2000 responses (number of sweeps per replication) were obtained from each ear after auditory stimulation. The filter band-pass used in this study was LF 100– HF 3000 Hz [17].

The recording was done by surface electrodes and all electrodes have an impedance of less than 5 kΩ that is placed at the vertex (CZ) (reference electrode) and on the nasal root (ground electrode) and each ear mastoid (A1 and A2) (active electrodes) to record the auditory waveforms. The channel derivations include ipsilateral ear to vertex and contralateral ear to vertex [18].

The peak latencies of waves I, III, and V, as well as the IPL of waves I-III, III-V, and I-V, and the amplitudes of waves I and V to determine the V/I amplitude ratio were investigated.

Statistical package for social sciences (SPSS) software, version 25, was used for all statistical analyses (IBM Corporation, USA). A normality test (Shapiro Wilk test) was performed on continuous data, and it was discovered that the data was normally distributed. An independent student t-test was used to evaluate quantitative data that were reported as mean standard deviation (SD). The chi-square test was used to assess categorical variables that were expressed as counts and percentages.

The receiver operating characteristic (ROC) curve was used to assess the diagnostic significance of wave IV, V, and ILP-IIIV delay, as well as wave V amplitude, in the context of patients with stroke and control discrimination. The two-tailed Pearson's correlation analysis was used to examine the relationships between age and disease duration and other conduction characteristics. A statistically significant level of statistics was accepted for all tests when $p<0.05$.

## Results

Table 1 demonstrates no significant difference between the patients and control groups regarding age and sex. Stroke onset ranges from 2 days to 30 days. According to the diffusion-weighted MRI, brain stem ischemia is present in 11 (55%), occipital lobe ischemia in 6 (30%), and cerebellar ischemia in 3 (15%) of patients.

No significant difference was noticed in the mean values of all BAEPs parameters between the right and left ear and between males and females of the control group. Also, no significant difference was noticed in the mean values of all BAEPs parameters between the right and left ear and between males and females of the patients. Accordingly, these data were pooled together and tabulated as one group for further comparison.

According to the cutoff values for the abnormality of wave IV, which is 4.5 msec, of wave V, which is 5.5 msec, and of III-V IPL, which is 1.8 msec, 10 (50%) patients showed a central abnormality, 5 (25%) showed central and peripheral abnormality at the same time, 1 (5%) showed peripheral abnormality and 4 (20%) showed no abnormality (Figure 1).

**Table 1. Age and sex difference in the study population**

| Variables | | Patients N=20 | Controls N=20 | P-value |
|---|---|---|---|---|
| Age (years) | Mean±SD | 58.0±8.1 | 55.2±7.31 | 0.267 |
| | Range | 40-70 | 42-63 | |
| Sex | Males | 12 (60%) | 10 (50%) | 0.408 |
| | Female | 8 (40%) | 10 (50%) | |
| The onset of stroke (days) | Mean±SD | 9.0±9.48 | | |
| | Range | 3-30 | | |
| DW-MRI | Brainstem ischemia | 11 (55%) | | |
| | Occipital lobe ischemia | 6 (30%) | | |
| | Cerebellar ischemia | 3 (15%) | | |

DW-MRI = diffusion-weight Magnetic resonance imaging

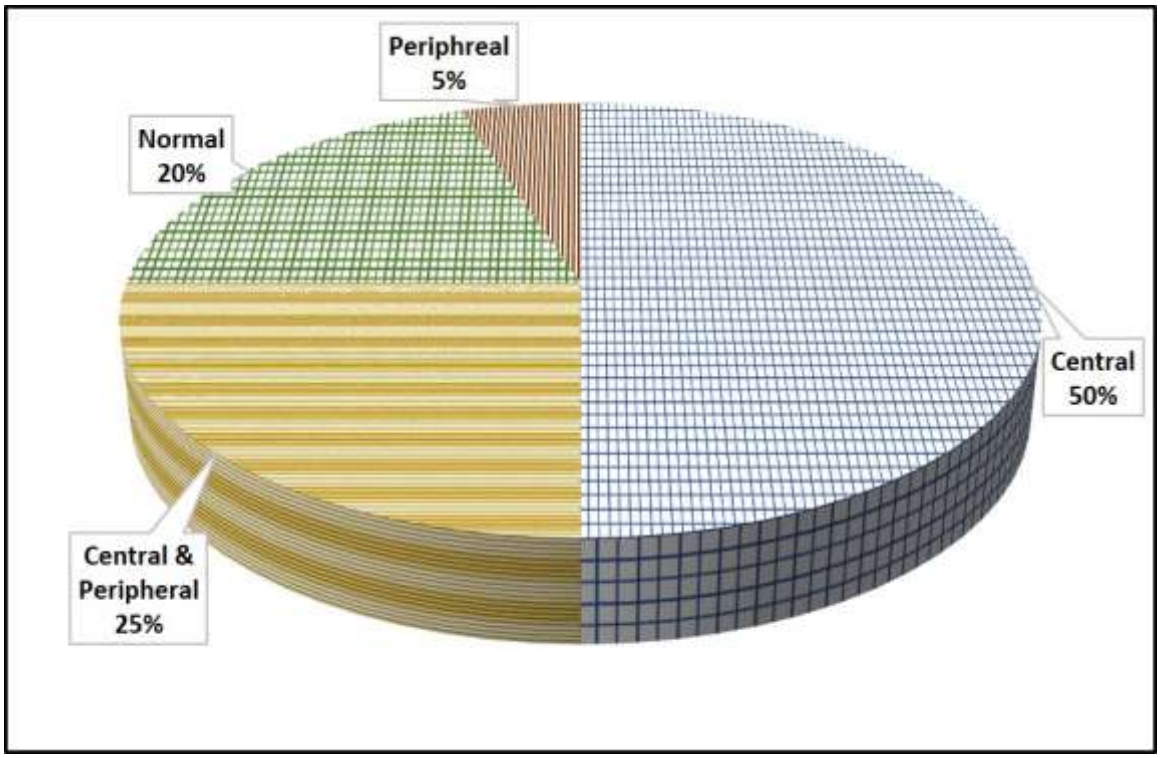


**Figure 1. Distribution of central and peripheral abnormalities of BAEPs**

Waves IV and V, and III-V IPL were significantly prolonged in patients with stroke as compared to the controls (p<0.001). On the reverse, wave V amplitude was significantly lower in patients with stroke as compared to the control group (p<0.001) as shown in table 2.

**Table 2. Comparison of brainstem auditory evoked potential parameters between the patients with stroke and controls**

| Variables | Patients N=20 Mean±SD | Controls N=20 Mean±SD | P-value |
|---|---|---|---|
| Wave I latency (ms) | 1.46±0.21 | 1.52±0.2 | 0.213 |
| Wave II latency (ms) | 2.48±0.25 | 2.47±0.22 | 0.825 |
| Wave III latency (ms) | 3.64±0.32 | 3.64±0.17 | 0.988 |
| Wave IV latency (ms) | 4.85±0.37 | 4.41±0.2 | <0.001 |
| Wave V latency (ms) | 5.78±0.34 | 5.41±0.19 | <0.001 |
| I-III IPL (ms) | 2.14±0.41 | 2.14±0.27 | 0.930 |
| III-V IPL (ms) | 2.12±0.41 | 1.75±0.24 | <0.001 |
| I-V IPL (ms) | 4.29±0.38 | 3.86±0.22 | 0.089 |
| Wave I amplitude (µV) | 0.35±0.11 | 0.4±0.12 | 0.089 |
| Wave V amplitude (µV) | 0.7±0.12 | 0.95±0.18 | <0.001 |
| V/I ratio | 2.2±0.9 | 2.57±0.83 | 0.077 |

IPL = Inter-peak latency

The distribution of BAEP abnormalities among 20 patients was as follows: abnormal wave IV latency in 9; abnormal wave V latency in 13; abnormal III-V IPL in 13; abnormal wave V amplitude in 7; unilateral absent response in 1; and bilateral absent response in 1 (Figure 2).

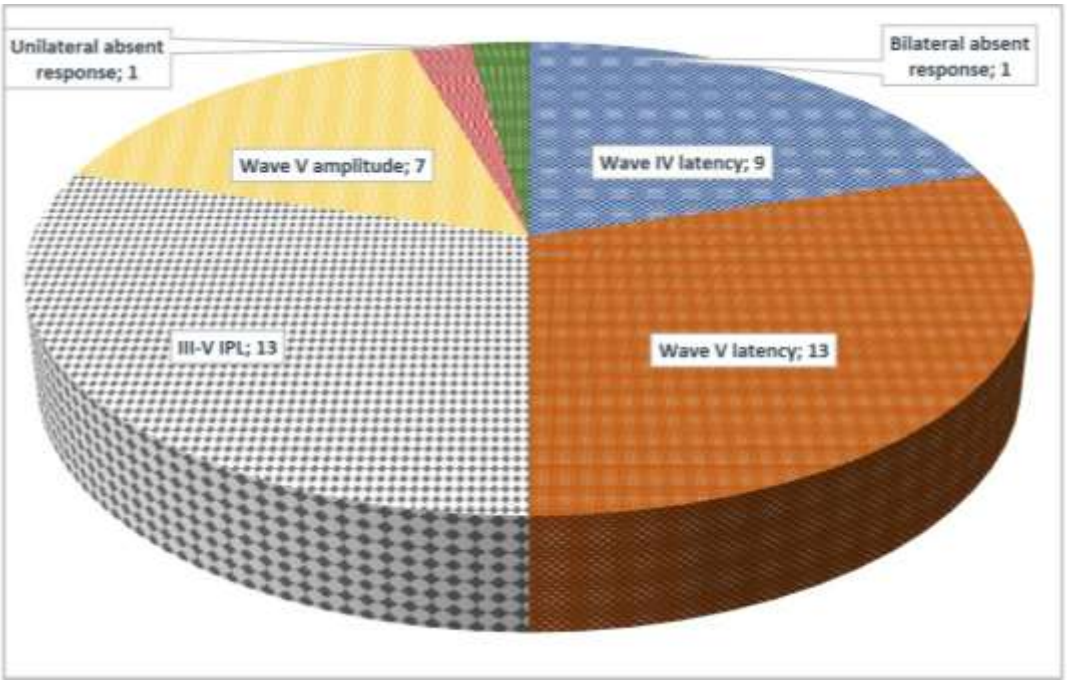


**Figure 2. The brainstem auditory evoked potentials abnormality in patients with stroke**

The ROC curve was used to assess the diagnostic significance of wave IV, wave V, III-V ILP, and wave V amplitude in distinguishing stroke patients from healthy volunteers.

The area under the curve (AUC) for wave IV was 0.84, 95% CI= 0.75-0.93, p<0.001. At a cut-off value of wave IV= 4.55 msec, the test's sensitivity and specificity were 0.75 and 0.70, respectively. The AUC for wave V was 0.792, 95% CI= 0.687-0.898, p<0.001. At a cut-off value of wave IV= 5.55 msec, the test's sensitivity and specificity were 0.63 and 0.80, respectively. The AUC for III-V ILP was 0.769, 95% CI= 0.651-0.887, p<0.001. The test's sensitivity and specificity at wave III-V ILP = 1.85 cut-off values were 0.78 and 0.65, respectively (Figure 3).

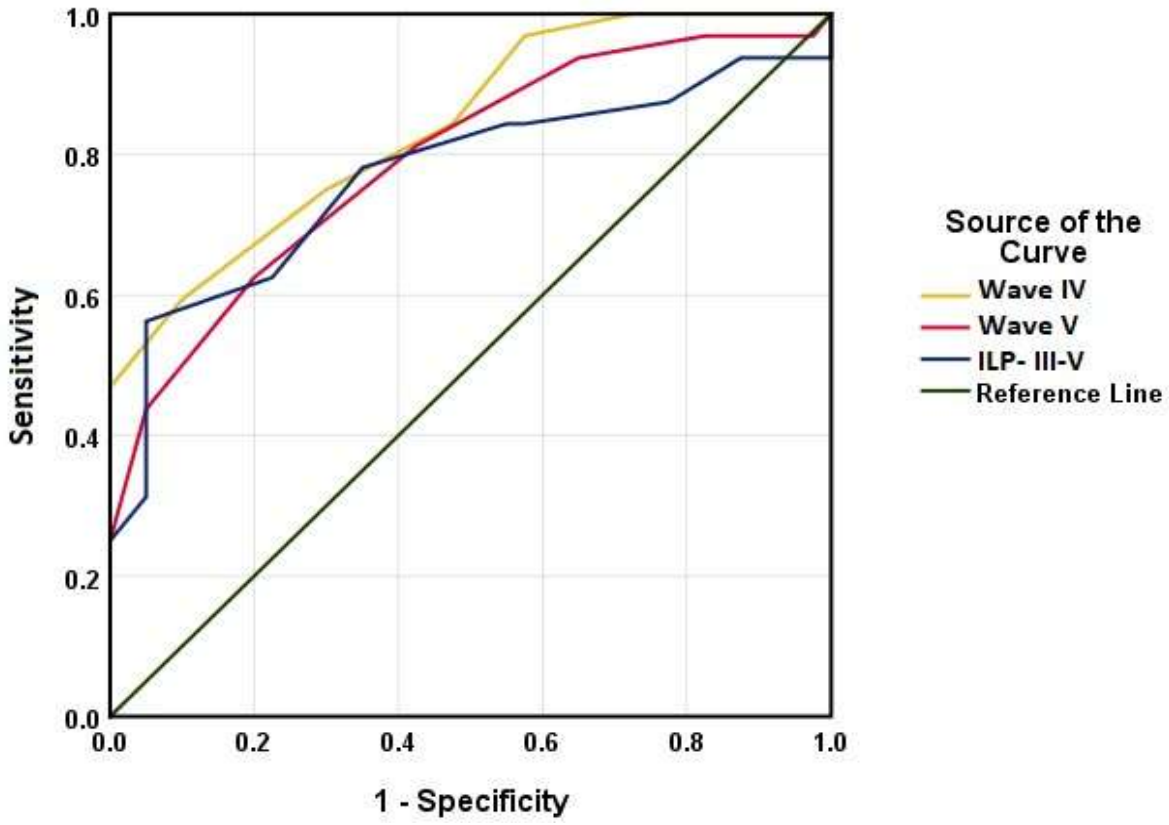


**Figure 3. ROC curve for wave IV, wave V and III-V IPL in the context of discrimination between patients with stroke and healthy controls**

The AUC for wave V amplitude was 0.888, 95% CI= 0.816-0.959, p<0.001. At a cut-off value of wave V amplitude = 0.75, the test's sensitivity and specificity were 0.90 and 0.70, respectively (Figure 4).

None of the BAEPs parameters was correlated with the age of the subjects. Nonetheless, wave V was significantly correlated (r = -0.332, p <0.045) with the duration (Figure 5).

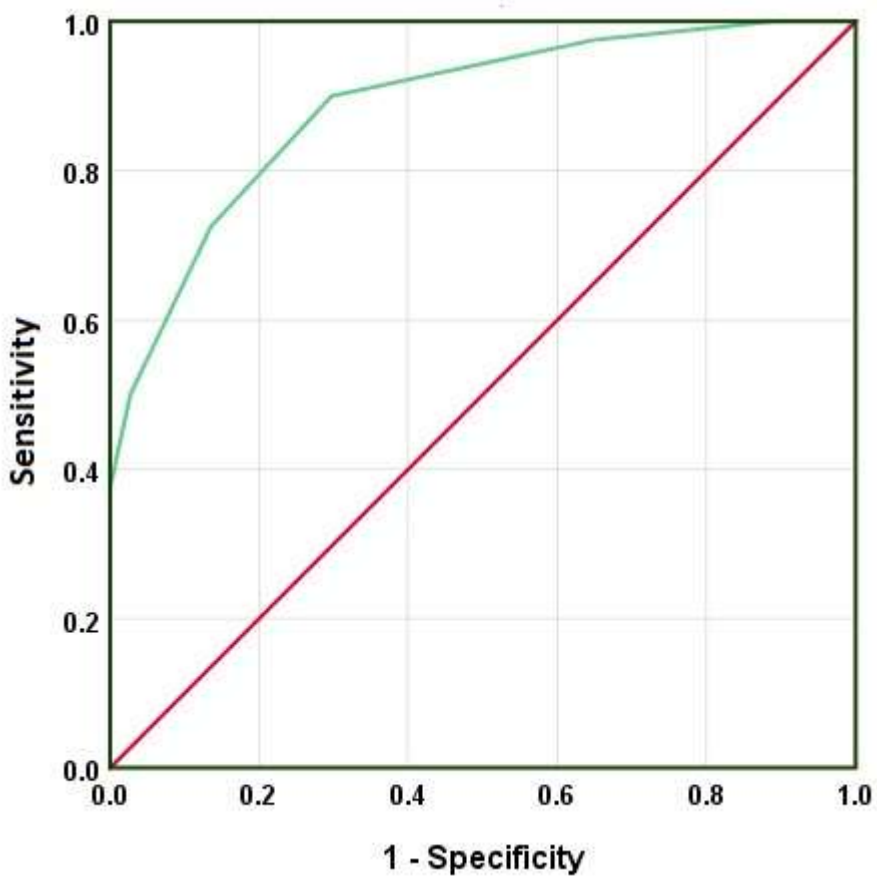


**Figure 4. ROC curve for wave V amplitude in the context of discrimination between patients with stroke and healthy controls**

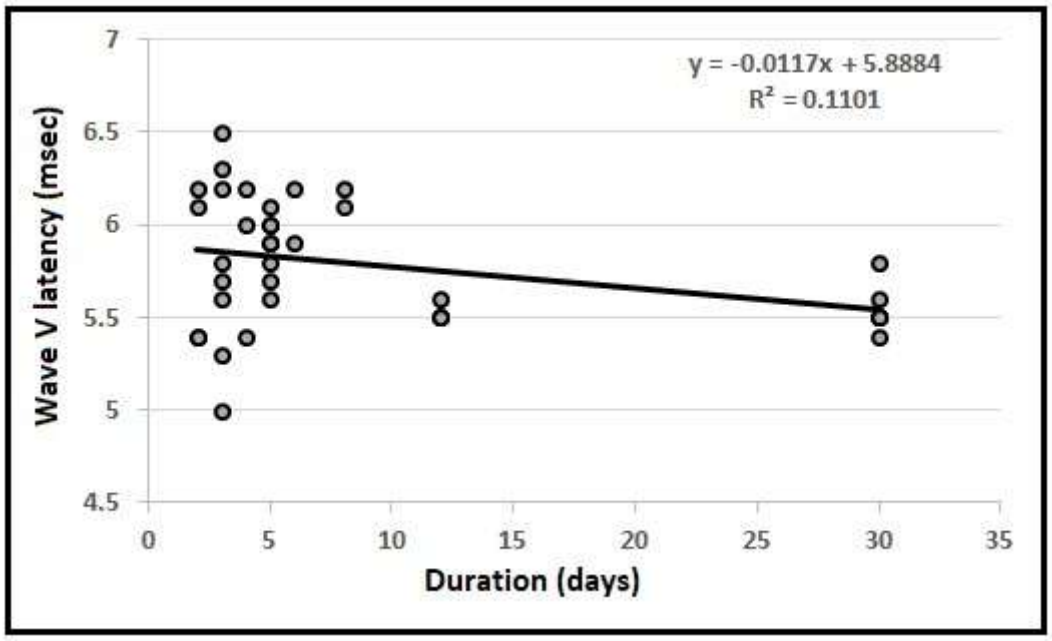


**Figure 5. Regression line between wave V latency and onset duration in patients with stroke**

## Discussion

In this study, 75% (15 cases) of patients with stroke present retrocochlear (central) dysfunction in form of significant prolongation in wave IV and V latencies, III-V IPL, and decreased wave V amplitude. The finding of the current study was in agreement with the results reported by other researchers [7,19-21].

Because the top region of the pons is primarily fed by the short circumflex branch of the pontine artery (a tiny branch from the basilar artery with a right angle), it is more susceptible to ischemia than the lower section, resulting in prolonged III-V IPL [22].

From a pathophysiological standpoint, the aberrant BAEPs came from a transitory lack of blood supply in the relevant functional area (ischemia of the posterior circulation), which causes decreased neuronal metabolism and a decrease in polarization. The large influx of calcium ions into these neurons damages them, slows conduction, and weakens electrical activity [7].

Because the BAEP diagnosis of PCIS can result in a greater abnormal rate, it is commonly employed clinically. In the present study, the BAEPs abnormal rate was as low as 35% (7 cases of low wave V amplitude) to as high as 65% (13 cases of prolonged wave V latency or III-V IPL). Similar findings were demonstrated by other groups [7,23].

The present study demonstrates that wave V latency and amplitude among other BAEPs parameters were the most sensitive and specific indicators of diagnostic utility for stroke. This finding was in agreement with the results reported by other researchers [8,22].

Because wave V of BAEPs as a reliable predictor of brainstem function, any primary or secondary disease that deteriorates and impairs brainstem function must first alter wave V, which originates in the inferior colliculus [9].

Moreover, when vertebrobasilar artery insufficiency leads to auditory pathway ischemia, wave V latency prolongs earlier than the others, or poor morphology is observed. Animal experiments have proved that, when the unilateral vertebral artery was clipped, wave V latency would prolong more obviously than waves I and III [24].

This study also showed a significant negative correlation between wave V latency and duration from onset of stroke to neurophysiological examination. The shorter the duration, the more abnormal wave V latency. This finding was similar to that reported by others [25,26] in which patients with ischemic stroke have delayed latencies of waveforms I, III, and V of BAEPs which are performed in the early phase of stroke.

Moreover, when an initial examination of evoked potentials is performed within the first week it provides valuable information for a prognostic purpose, however, serial examinations of BAEPs after the first weeks improve the prognostic information only slightly.

In contrast to the findings of other study [27], BAEPs can predict adverse outcomes of stroke patients more reliably when tested 4-7 days after stroke onset than when assessed 1-3 days after stroke onset. Perhaps this disagreement was because outcome evaluation of patients after 6 months by analysis of prognostic authenticity for possible predictors for unfavorable outcomes not included in the present study. Besides, only 6 patients out of the total number were examined within 1-3 days, also day one was not included in the present study.

Brain edema occurs 3-4 days after a stroke, and increased intracranial pressure occurs 4-7 days later [28]. During this time, patients frequently deteriorate. The predictive timing of acute stroke examinations at 4-7 days following start is thought to reflect brain function more accurately than assessments at 1-3 days.

The present study showed that lesions involving the pontine tegmentum and midbrain region of the brain stem often cause a bilateral absence of all BAEP waves. This is probably due to vascular territories (anterior inferior cerebellar and posterior inferior cerebellar arteries) that supply these regions of the brainstem which are considered physiological generators of BAEP waves, mainly waves IV and V, so it suggests regional diagnostic validity of BAEPs.

Lesions involving the pontine tegmentum always cause various abnormalities in the BAEPs or SSEPs, so it causes loss of the V wave in BAEPs or loss of N20 in SSEPs. A large lesion involving the bilateral pontine tegmentum causes the disappearance of more than one wave in the BAEPs [29].

In conclusions, BAEPs are a useful tool as a biomarker for the clinical evaluation of PCIS. Wave V latency and amplitude are the most specific and sensitive among other parameters in the diagnosis of PCIS. Finally, an early post-stroke BAEPs examination is better in detecting the abnormality and can provide reference values for further evaluation.

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## Author contribution

All of the authors contributed directly to the creation of this paper and approved the final version that was submitted. Dr. Al-Hamdani assessed and referred stroke patients clinically. The electrodiagnostic tests were performed by Dr. Tuaimeh and Dr. Hamdan likewise the writing of the document. All the three authors participated in the conceptualizing, designing, interpretation and final approval of the paper.

## Conflict of interest

The authors declare that they have no conflicts of interest.

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# A Retrospective Study Regarding Coronavirus Disease Epidemiological Features among People in Fallujah City, Iraq

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## Abstract

**Background:** A corona virus (COVID-19) pandemic in Iraq has extended all over the country, including Fallujah city in western of Iraq. Being aware of the epidemiological features of this novel disease can greatly assist in making the suitable decisions regarding the management and thus control of this epidemic.

**Objective:** To study the epidemiological characteristics of COVID-19 patients in Fallujah city, Iraq.

**Methods:** In this retrospective study, information in regard to the epidemiological features of COVID-19 suspected cases who underwent polymerase chain reaction (PCR) testing at Fallujah Teaching Hospital in Fallujah, Al-Anbar, Iraq, from September 12th, 2020 to January 5th, 2021. Inclusive, have been collected, analyzed and then reported. General features including age, sex, occupation and quarantine site were investigated. Whole data were retrieved from patients' official records.

**Results:** A total 3604 samples from COVID-19 suspected cases were collected, 575 cases of them were tested positive for COVID-19. Among the COVID-19 patients, 402 of them were males (69.9%) and 173 were females (30.1%). The age range of the patients was (15-86) years and the mean age ±SD was 40.98±14.3 years. Forty-one COVID-19 patients were healthcare workers (7.1%) and the rest 534 patients had non-healthcare related occupations (92.9%). Only 40 COVID-19 positive cases were admitted to the hospital (7%), and 535 patients were in home quarantine (93%). In case of the total 143 hospital admitted patients, the PCR test results were negative in 103 patients (72%) and positive in only 40 patients (28%).

**Conclusion:** This study found that COVID-19 was more prevalent among the young male individuals, whom represent the community active group. This study can serve in documenting the features of the patients with COVID-19 in Fallujah, Iraq, and also in helping the healthcare workers in detecting and managing the patients.

**Keywords:** COVID-19, Coronavirus, epidemiology, SARS-CoV-2, Iraq

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**List of abbreviations:** COVID-19 = Corona Virus Diseases - 19, MERS = Middle east respiratory syndrome, RT-PCR = Real-time polymerase chain reaction test, SARS–COV-2 = Severe acute respiratory syndrome coronavirus 2

## Introduction

Since emerging in Wuhan city, China, in December, 2019, the coronavirus disease 2019 epidemic has developed hastily into a pandemic [(1)]. On the 30th of January, 2020, the World Health Organization (WHO) officially announced that the coronavirus disease 2019

epidemic represents a serious threat to the world public health, and subsequently, in March 2020, the rapid spread of the disease internationally resulted in announcing the coronavirus disease 2019 pandemic [2]. Accordingly, the term COVID-19 (stands for coronavirus disease 2019) was decided as the official name for the disease, and SARS−COV-2 (stands for severe acute respiratory syndrome coronavirus 2) for the causative virus [3,4]. Similar to the Middle East Respiratory Syndrome (MERS) and the severe acute respiratory syndrome (SARS), the trend of COVID-19 has been seen in the epidemiology of this new emergent disease [5,6]. The nature of SARS-CoV-2 virus and the long incubation period indicates that COVID-19 is highly contagious disease, in which every infected person is able to infect at least 3 other persons [7,8].

An individual is said to be COVID-19 suspect when primarily presents with respiratory symptoms such as cough, shortness of breath and fever [9,10]. COVID-19 is associated with a wide range of clinical signs and symptoms including respiratory and non-respiratory manifestations. Even though the majority of the cases present with mild symptoms, some cases can be complicated into developing severe respiratory diseases and pneumonia as far as developing venous thrombosis, renal and heart failure [11].

There are various methods to diagnose COVID-19, such as nasopharyngeal or oropharyngeal swab reverse transcriptase polymerase chain reaction test (RT-PCR), viral detection from sputum sample or bronchoalveolar lavage, the rapid antigen detection test and chest radiograph [12]. The nasophaygeal swab is considered the standard method for screening or diagnosis of COVID-19 [13].

Iraq, along with other countries around the world attained COVID-19 mainly through individuals who have history of travel abroad. The first confirmed COVID-19 case in Iraq was reported on the 24th of February, 2020 in Al-Najaf city, south of Baghdad. While Baghdad, the capital of Iraq, reported the largest share (about 40%) of conformed cases at that time [14].

The rapid spreading of COVID-19 across Iraq could be explained by the fact that thousands of Iraqis had travelled outside Iraq visiting countries like Iran during the spring break which made them more liable to catch the COVID-19. In addition, upon their return home, they did not face any obligatory quarantines, which has led to the increase in the risk of spreading the infection to their families, neighbors and friends [15].

Since the emerging of COVID-19 pandemic last year, a large number of studies around the world, including Iraq, have been done to study the epidemiological characteristics of the patients with COVID-19. Studying the epidemiological features of this novel disease will help in making appropriate decisions and thus control the epidemic [14,16-18]. So, this study is aiming to investigate and report the basic epidemiological topographies of COVID-19 patients in Fallujah, west Iraq as a city with a population of more than 350 thousand people.

## Methods

### Data sources

Data for this study was obtained from the official records at the Central Laboratory of Fallujah Teaching Hospital after obtaining the relative approval. Data covered cases reported in the first five months of the second wave of epidemic (from September 12th, 2020 to January 5th, 2021 inclusive) in Fallujah City, West Iraq. Additional sources were also viewed to verify the accuracy of numbers reported.

### Definitions

The participants included in this study were all the COVID-19 suspected cases seeking PCR tests at Fallujah Teaching Hospital. A suspected case of COVID-19 is defined as any individual presented to any designated health institutions with symptoms suggestive of COVID-19 or with a history of a recent contact with a COVID-19 positive patient. The individual is said to be a

COVID-19 patient when it is confirmed by clinical picture and a positive nasopharyngeal swab (RT-PCR) [9,10].

**Data analysis**

General information regarding the age, sex, occupation and quarantine site from all the subjects were obtained, and the information were entered into a Microsoft Office (Excel) sheet. The data analysis was carried out using the Statistical Package for Social Sciences (SPSS) software, version 24. Descriptive analysis was carried out to estimate the frequencies and percentages of each variable in all the cases and in the PCR test positive cases.

**Ethical issue**

Ethical approval for this study was obtained from the Scientific Committee of University of Fallujah, College of Medicine. A research proposal was also approved by the Central Research Committee at the Directorate General for Health in Al-Anbar, Iraq.

## Results

Information from a total 3604 COVID-19 suspected cases were collected for this study. Fifty-three cases were excluded from the data analysis because of missed information. Of those 3551 remaining cases, 2467 were males (69.5%) and 1084 were females (30.5%). The age range of the participants was (3-86) years and the mean age ±SD was 37.75±13.93.

Out of the 3551 suspected cases, 3408 were in home quarantine (96%) and only 143 were admitted to the hospital (4%). In term of occupation, 3338 COVID-19 suspected subjects were non-healthcare workers (94%) and 213 subjects were healthcare workers (6%). Table 1 summarizes the general epidemiological features of all the participants in this study. The PCR tests results showed that 575 cases tested positive for COVID-19 (16.2%), and 2976 cases tested negative (83.8%).

**Table 1. Epidemiological characteristics of COVID-19 suspected cases in Fallujah, Iraq (from September 12th, 2020 to January 5th, 2021)**

| Variable | | Frequency | Percentage (%) |
|---|---|---|---|
| Gender | Male | 2467 | 69.5% |
| | Female | 1084 | 30.5% |
| Age group (years) | 1-18 | 161 | 4.5% |
| | 19-29 | 955 | 26.9% |
| | 30-39 | 1001 | 28.2% |
| | 40-49 | 694 | 19.5% |
| | 50-59 | 413 | 11.6% |
| | ≥60 | 327 | 9.3% |
| Occupation | Healthcare worker | 213 | 6.0% |
| | Non-healthcare worker | 3338 | 94.0% |
| Quarantine site | In hospital | 143 | 4.0% |
| | Home quarantine | 3408 | 96.0% |
| PCR result | Positive | 575 | 16.2% |
| | Negative | 2976 | 83.8% |

N=3551

Table 2 demonstrates the general epidemiological features of the 575 patients tested positive for COVID-19; 402 patients were males (69.9%) and 173 patients were

females (30.1%). The age range of the patients was (15-86) years and the mean age ±SD was 40.98±14.303 years. Forty-one COVID-19 patients were from healthcare workers (7.1%) and the rest 534 patients had non-healthcare related occupations (92.9%). Only 40 COVID-19 positive cases were admitted to the hospital (7%), and 535 patients were in home quarantine (93%). In case of the total 143 hospital admitted patients, the PCR test results were negative in 103 patients (72%) and positive in only 40 patients (28%).

**Table 2. Epidemiological characteristics of COVID-19 patients in Fallujah, Iraq (from September 12th, 2020 to January 5th, 2021)**

| Variable | | Frequency | Percentage (%) |
|---|---|---|---|
| Gender | Male | 402 | 69.9% |
| | Female | 173 | 30.1% |
| Age group (years) | 1-18 | 12 | 2.1% |
| | 19-29 | 119 | 20.7% |
| | 30-39 | 154 | 26.8% |
| | 40-49 | 129 | 22.4% |
| | 50-59 | 87 | 15.1% |
| | ≥60 | 74 | 12.9% |
| Occupation | Healthcare worker | 41 | 7.1% |
| | Non-healthcare worker | 534 | 92.9% |
| Quarantine site | In hospital | 40 | 7.0% |
| | Home quarantine | 535 | 93.0% |

N=575

## Discussion

The present study intended to investigate and document the basic epidemiological features of the patients with COVID-19 in Fallujah city, Iraq from the period of September, 12th, 2020 to January, 5th, 2021. During this period, only 3604 persons underwent nasopharyngeal swap RT-PCR test at Fallujah Teaching Hospital, and 575 patients infected with COVID-19 were diagnosed. One explanation to the small number of the PCR tests carried out could be due to the public fear from the pandemic and the hospitals, whom they mainly rely on the private medical cares. Furthermore, some patients could be diagnosed with COVID-19 by other means, such as the rapid antigen detection tests or chest radiology without undergoing the nasopharyngeal swap (PCR) test [19].

The general epidemiological characteristics of the COVID-19 patients showed that the majority of the patients were young age males and this finding agrees with the previous predominant in males worldwide like [20,21]. The male predominance finding can be explained by the differences between males and females regarding their immune responses to COVID-19 infection, as well as, women are usually less susceptible to viral infections in comparison to men, based on a different innate immunity, factors related to sex chromosomes and steroid hormones [22].

An interesting finding in the current study is that the majority of the patients admitted in the COVID-19 unit at Fallujah Teaching Hospital were PCR negative (72%). The high negative PCR results were also found in other studies [23,24] Arevalo-Rodriguez and colleagues (2020), found that up to 54% of COVID-19 patients had false-negative RT-PCR results initially (very low certainty of evidence), and that repeating the testing in patients with suspicion of SARS-Cov-2

infection is important to overcome any false-negative results [24]. Also, Lindner and colleagues [8] suggested that one of main reasons leading to false-negative RT-PCR results in COVID-19 patients is the technique of performing the nasopharyngeal swab in which the swab was generally rotated against the nasopharyngeal wall for less time than recommended by the manufacturer. And this may affect the sensitivity of the test with nasopharyngeal sampling, but also reflects the difficulty of collection of this sample type [25].

This study has a number of limitations; the information gained were accessed to from the official records which were lacking details about the presenting symptoms, the duration of the symptoms and any co-morbidities due to this study was performed in a single center. Also, authors did not have access to the clinical outcomes of the admitted cases in term of cure and mortality rates.

In conclusions, the results of current study found a higher incidence of COVID-19 among the young and male individuals, whom represent the active age group in the community, which has been mentioned earlier in other studies. Findings from this study will serve in documenting the features of the patients with COVID-19 in Fallujah city, Iraq, and also in helping the healthcare workers in detecting and managing the patients.

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## Author contribution
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## Conflict of interest
The authors declare that there is no conflict of interest.

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# Association of DVWA rs11718863 Gene Polymorphism with Knee Osteoarthritis in Iraqi Patients

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## Abstract

**Background** Osteoarthritis (OA) is a complex degenerative articular disease that has an ambiguous pathogeny because variant risk factors participate in the process of cartilage deterioration. Genetic factors may have a role in the onset and progression of OA.

**Objective** To investigate potential association between severity of knee OA (KOA) and Double von Willebrand factor A domains DVWA rs11718863 gene polymorphism.

**Methods** One hundred and twenty Iraqi patients diagnosed with KOA, aged (45 years and above) and sixty healthy people (control) at the same age range, with no family history of OA were evaluated at Al-Imamein Al-Kadhimein Medical City (Rheumatology Department). The degree of severity of KOA was assessed by clinical and radiographic assessment. DVWA rs11718863 genotyping was performed using DNA sequencing (Sanger's method).

**Results** Two different genotypes wild homozygote (TT) genotype and heterozygote (AT) appeared by genotyping of DVWArs:11718863. Highly significant difference ($p<0.001$) was found in distribution of the two genotypes in the three study groupsl the genotype (TT) was more frequent in control group (95.0%). Also, high significant difference ($p<0.001$) was noted in the two alleles (A, T) frequencies between control group and KOA patients group giving odd ratio 6.437 with 95% confidence intervals of (1.93-21.42).

**Conclusion** Iraqi subjects carrying AT genotype of DVWA rs11718863 gene are mostly susceptible for developing of KOA and the allele A of Tyr169Asn polymorphism are more frequent in those patients indicating that allele A may be a risk factor of onset of this disease. Age and body mass index are considered risk factors of onset and progression of KOA.

**Keywords** Double von Willebrand factor A domains (DVWA), single nucleotide polymorphism (SNP), body mass index, Knee osteoarthritis, KOA

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**List of abbreviations:** DVWA = Double von Willebrand factor A domains, GWAS = Genome-wide association studies, KOA = Knee osteoarthritis, OA = Osteoarthritis, PCR = Polymerase chain reaction, SNP = Single nucleotide polymorphism, VWA = von Willebrand A

## Introduction

Knee osteoarthritis (KOA) occurs as the cartilage in the knee wears away finally causing bone on bone contact between joint surfaces. Most common symptoms would comprise joint stiffness, joint swelling, and pain. KOA can be diagnosed by radiographs showing boney cysts, narrowing joint space, and sclerosis of the bone. This means that KOA comprises the deterioration of joints, including articular cartilage and subchondral bone. But also, ligaments, the capsule and the synovial

membrane disintegrate causing eventually pain and loss of function [1]. Various risk factors such as genetics, aging, obesity and joint deformation may be related with KOA onset and progression [2,3].

The genetic background of OA likely embraces numerous genes that encode proteins with considerable functions in the underlying disease process, suggesting that genetic factors are intensive stimulus of OA emergence [4]. Nineteen common variants associated with OA have been established by Genome-wide association studies (GWAS) reaching or approaching genome-wide significance [5,6].

According molecular genetic examinations have acquired more considerable role in the knowledge of OA etiology and have provided clue for a genetic component to OA [7-9].

Although OA is described as a heterogeneous disease, genetic factors have been found to be strongly affecting factor of this disease. Over the several last years, an increasing number of researches concentrated on the association between gene variants and OA, especially the double von Willebrand factor A domains (DVWA) gene [10-12]. The DVWA gene, that encodes for a protein with two von Willebrand A (VWA) domains, was found to have the rs11718863 single nucleotide polymorphism (SNP), showing a regular association with KOA in Asian OA cohorts (Japanese and Chinese) [10, 11]. Also, DVWA gene is known as collagen type VI alpha 4 pseudogenes 1 (COL6A4P1), particularly expressed in cartilage, encodes for a protein that have DVWA, which has a role in cellular adhesion and protein-to-protein interactions [13].

DVWA gene, on human chromosome 3p24.3, encodes short proteins (276 amino acid) with two regions corresponding to the VWA domain, which was presented in a variety of proteins [14] and it participates in cell adhesion, protein-protein interactions, and membrane transport [15,16]. OA emerges from VWA domain mutations of the matrilin 3 gene (MATN3) [17]. DVWA interacts with β-tubulin and this interaction may be considered a protective factor in OA pathogenesis. It is thought the strength of binding becomes weaker by alleles of two non-synonymous SNPs (rs11718863 and rs7639618) in VWA domain and that weaker binding between β-tubulin and the wild protein may increase the risk of developing OA [15].

Through a previous GWAS in Japanese, it was reported that a compelling association between two missense SNPs and OA risk in Japanese and Chinese KOA cohorts [15]. Then, UK cases study showed mild significant association between DVWA gene variants and OA development, but not in Netherlands, Spain and Greece [18]. Subsequently, separate European or Asian studies failed to replicate the association in Korean and UK samples [19, 20].

In a large GWA interaction study, DVWA genetic variants were tested by Miyamoto et colleagues. In particular, the DVWA rs11718863 SNP is reported to be strongly associated with OA knee injury and is able to affect β-tubulin binding in Asian populations [15].

This study aimed to figure out if the DVWA rs11718863 SNP will increase Iraqi individual`s susceptibility to developing KOA disease.

## Methods

This case-control study included 120 Iraqi patients affected by primary KOA with no family history of OA in addition to 60 healthy people (control) aged (45 years and above). Blood samples were obtained at Al-Imamein Al-Kadhimein Medical City (Rheumatology Department), Baghdad, Iraq during the period from March 2017 to May 2017 after submitting all the subjects (patients and control) to clinical (Western Ontario and McMaster Universities Arthritis Index WOMAC) scale [21] and radiographic (Kellgren and Lawrence) grading scale [22] examination and according to these two examinations, subjects were divided into the following 3 groups:

60 (35 females + 25 males) subjects with mild plus moderate KOA.

60 (35 females + 25 males) subjects with severe KOA.

60 (35 females + 25 males) healthy people as control.

All subjects with one of the following conditions were excluded from the study; any other pathological condition that may explain the symptoms (e.g., other rheumatic disease, previous knee joint replacement, intra-articular fracture, septic arthritis, ligament or meniscus damage), pregnant women and co-morbidity that prevents physical examination.

Renal functions test, complete blood picture and erythrocyte sedimentation rate (ESR) test were done for all subjects to make sure they don't have any renal dysfunction that could affect the level of serum sfrp3 protein and to exclude those having rheumatoid arthritis, diabetes. Physiological factors such as body mass index (BMI) and age were evaluated in this study.

Two ml of blood were obtained from each subject by vein puncture and put into Ethylene diamine tetra acetic acid (EDTA) tubes to store in – 70˚C (deep freeze) in order to be used later for genetic analysis. DNA extraction was done using Genomic DNA G-spin DNA extraction kit supplied by Intron Biotechnology, Cat. No. 17045. For analyzing the variation of DVWA gene, polymerase chain reaction (PCR) amplification had been done for all cases and control samples using specific primers pair. A fragment 924 bp of DVWA was amplified using a forward and reverse primers that supplied by IDT (Integrated DNA Technologies company, Canada) (Table 1).

**Table 1. The specific primer DVWA of gene [13]**

| Primer | Sequence | Tm (°C) | GC (%) | Product size |
|---|---|---|---|---|
| Forward | 5'- AGGCTGCCTGCCATTATTCTT- 3' | 57.1 | 47.6 | 924 |
| Reverse | 5'- CCCATGCTGTTTCCTTTGAACA- 3' | 56.1 | 45.5 | base pair |

PCR products (25 µl) (Figure 1) was prepared and sent for sequencing, to Macrogen, Korea. DNA sequence analysis have been done using BLAST program from National Center Biotechnology Information (NCBI) [23]. Patients DNA sequence had compared and alignment with DVWA gene sequence of both standard and control. Analysis of SNP also were done using the tools that provided by these sites. PCR product samples (120) were sent for sequence analysis represent patient and 60 sample represent healthy control; the samples were sequenced using DNA sequencer 3730XL, Applied Biosystem machine in national instrumentation center for environmental management NICM/USA company online at (http://nicem.snu.ac.kr/main/en_skin=index.html). The result of the sequence analysis was analyzed by blast in the NCBI online at (http://www.ncbi.nlm.nih.gov) and BioEdit program to detect gene mutation and polymorphism in DVWA gene.

**Statistical analysis**

The continuous data were presented as mean values ± standard error (SE); comparison of these data between two groups was performed by applying a Student's t test, and ANOVA (analysis of variance) and post hoc Tukey test was used for comparison of means of more than two groups. While for calculation of genotyping and allele frequency, Chi-square test was used to compare between percentages between groups. P value <0.05 was considered significant. The software used was statistical package for social sciences (SPSS), version 23.

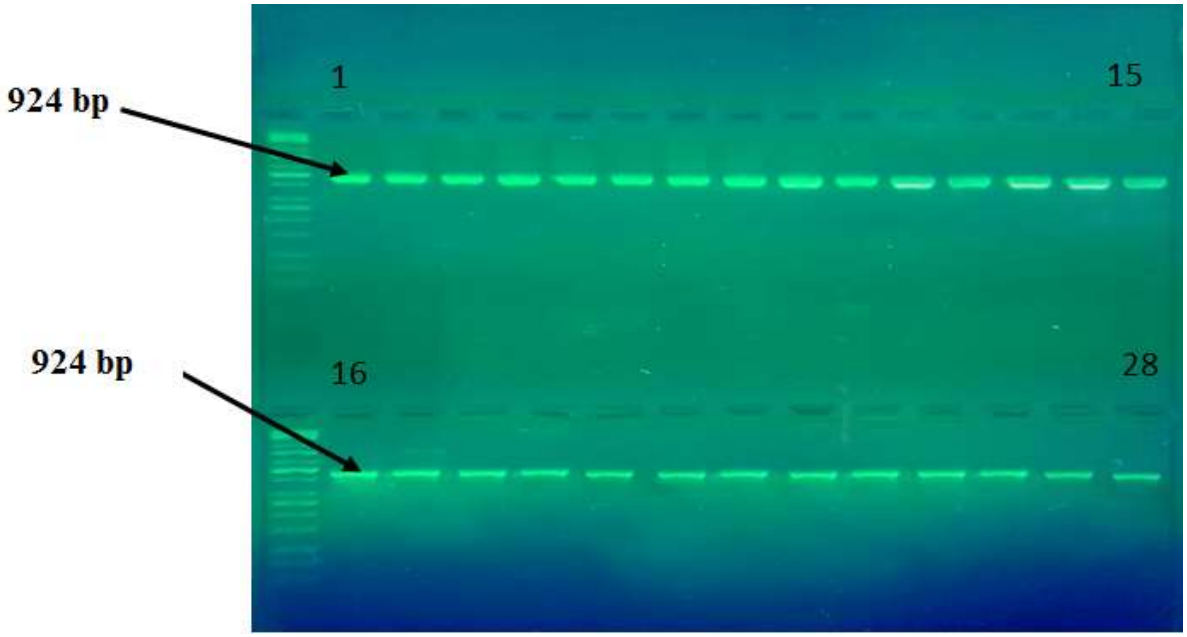


**Figure 1. PCR product of DVWA gene, the band size 924 bp. The product was electrophoresed on 1.5% agarose at 7 volt/cm$^2$. 1x TBE buffer for 1:30 hours. N: DNA ladder (100)**

## Results

There was high significant difference in the mean age and BMI of patients among the three study groups (p<0.001, p<0.001) respectively (Table 2).

Table (3) shows comparison between each two groups using post hoc Tukey test. Regarding age, there was significant difference between severe KOA group compared to control and mild plus moderate KOA groups (p<0.001, p<0.001) respectively, whereas, no significance in age between control and mild plus moderate KOA (p=0.967). BMI of both groups of KOA (mild plus moderate and severe) was significantly higher than control (p=0.028, p=0.004) respectively, while no significance was found between the two KOA groups (p=0.787).

**Table 2. Comparison of age and body mass index among the three study groups (according to severity) by ANOVA**

| Parameter | Control N=60 Mean±SE | Mild plus moderate KOA N=60 Mean±SE | Severe KOA N=60 Mean±SE | P value* |
|---|---|---|---|---|
| Age (yr) | 61.47±1.54 | 60.98±1.42 | 69.52±1.17 | <0.001 |
| BMI (kg/m$^2$) | 27.46±0.74 | 30.67±0.87 | 31.49±1.0 | ٠,٠٠٣ |

KOA = Knee osteoarthritis, BMI = Body mass index, *ANOVA

**Table 3. Comparison of age and body mass index between each pair of the three study groups (according to severity) by post hoc Tukey test**

| Dependent Variable | 1st Group | 2nd Group | P value |
|---|---|---|---|
| Age (yr) | Control | Mild plus moderate | 0.967 |
| | Control | Severe | <0.001 |
| | Mild plus moderate | Severe | <0.001 |
| BMI ($kg/m^2$) | Control | Mild plus moderate | 0.028 |
| | Control | Severe | 0.004 |
| | Mild plus moderate | Severe | 0.787 |

BMI = Body mass index

Two different genotypes were found by genotyping the DVWA rs11718863 gene; wild homozygote (TT) and heterozygote (AT). There was a highly significant difference in the distribution of them among the 3 study groups (p> 0.001); as the highest percentage of the wild type was found in control group 95.0% compared to 88.7% in mild plus moderate KOA group and to 51.7% in severe KOA group. While the highest percentage of the heterozygote type was found in severe KOA group 48.3% compared to 11.3% in mild plus moderate KOA group and to 5.0% in control group. The mutant genotype (AA) was not found in any group in this study (Table 4). The distribution of both genotypes of DVWA gene are shown in figures (2 and 3).

The frequencies of the two alleles (T and A) between control group and the KOA patients groups in general were demonstrated in the table (5). The percentage of T allele in control group was 97.5% compared to 85.8% in total KOA patients. While the percentage of allele A was 2.5% only in control group compared to 14.2% in total Knee OA patients. This difference was highly significant (p< 0.001) with a high odd ratio 6.437 and confidence interval of (1.93-21.42). shows high significant difference.

**Table 4. Genotyping distribution of DVWA gene polymorphisms in the three study groups**

| Group | DVWA | | | Total | P value |
|---|---|---|---|---|---|
| | TT | AT | AA | | |
| Control | 57 (95.0%) | 3 (5.0%) | 0 (0.0%) | 60 | < 0.001* |
| Mild plus moderate | 55 (88.7%) | 5 (11.3%) | 0 (0.0%) | 60 | |
| Severe | 31 (51.7%) | 29 (48.3%) | 0 (0.0%) | 60 | |
| Total | 143 (79.4%) | 37 (20.6%) | 0 (0.0%) | 180 | |

* Chi square test

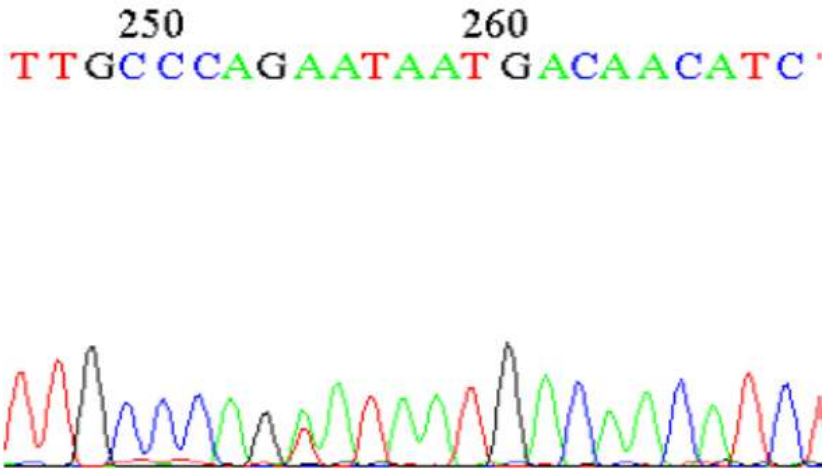


**Figure 2. Photographic of Macrogen appeared AT genotyping**

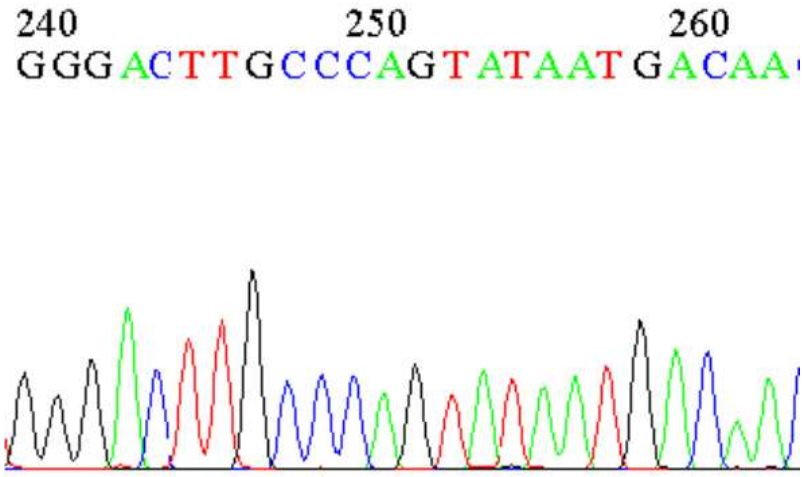


**Figure 3. Photographic of Macrogen appeared TT genotyping**

**Table 5. Allele frequencies and percentage, odd ratio (OR) and confidence interval (CI) of DWVA gene in control and knee OA patients' groups**

| Group | T | A | Total | P value | Odd ratio | 95% CI |
|---|---|---|---|---|---|---|
| Control | 117 (97.5%) | 3 (2.5%) | 120 | <0.001* | 6.437 | 1.93-21.42 |
| KOA | 206 (85.8%) | 34 (14.2%) | 240 | | | |
| Total | 323 | 37 | ٣٦٠ | | | |

KOA: Knee osteoarthritis, * Fisher exact test

When comparison done between control group and mild plus moderate KOA groups, there was no significant difference in the two alleles (T & A) percentage (p=0.772), the odd ratio was 1.696 with confidence interval (0.4-7.26) as illustrated in table (6).

**Table 6. Allele frequencies and percentage, odd ratio (OR) and confidence interval (CI) of DWVA gene in control and mild knee OA patients' groups**

| Group | T | A | Total | P value | Odd ratio | 95% CI |
|---|---|---|---|---|---|---|
| Control | 117 (97.5%) | 3 (2.5%) | 120 | 0.722 | 1.696 | 0.4-7.26 |
| Mild plus moderate KOA | 115 (95.8%) | 5 (4.2%) | ١٢٠ | | | |
| Total | 232 | 8 | ٢٤٠ | | | |

KOA: Knee osteoarthritis, * Fisher exact test

High significant difference (p<0.001) was noted in the two alleles percentage (T and A) between mild plus moderate KOA and severe KOA groups. For the allele T; high percentage 95.8% was found in mild plus moderate KOA group compared to 75.8% in severe KOA group, whereas the high percentage 24.2% of allele A was found in severe KOA group compared to only 4.2% in mild KOA group. This variation in frequencies gave high odd ratio 7.33 with confidence interval (2.728-19.69) (Table 7).

**Table 7. Allele frequencies and percentage, odd ratio (OR) and confidence interval (CI) of DWVA gene in control and mild knee OA patients' groups**

| Group | T | A | Total | P value | Odd ratio | 95% CI |
|---|---|---|---|---|---|---|
| Mild KOA | 115 (95.8%) | 5 (4.2%) | ١٢٠ | <0.001* | 7.33 | 2.728-19.69 |
| Severe KOA | 91 (75.8%) | 29 (24.2%) | ١٢٠ | | | |
| Total | 206 | 34 | ٢٤٠ | | | |

KOA: Knee osteoarthritis, * Fisher exact test

## Discussion

Considering OA as a degenerative disease, it always occurs in elderly populations indicating that aging is a major risk factor for primary OA, the most common form in humans [24]. Current results showed age of severe KOA patients was significantly higher than age of patients with mild plus moderate KOA patients and with controls. Regarding controls, it was hard to obtain complete healthy subjects at the same age group of severe KOA patients in our country with all its circumstances at time of the study. In concern to mild plus moderate KOA, it agreed with other study that found increasing age accompanied by increasing OA change [24]. Driban et al. [25] mentioned that during aging, senescence is caused by a continuous decrease in telomere due to repeated cell division or environmental stress factors, such as oxidative damage, chronic inflammation or ultraviolet radiation. Other studies showed that the role of reactive oxygen species (ROS) in many of the

age-related changes found in articular cartilage that alter cartilage homeostasis and contribute to the development of osteoarthritis. An increase in chondrocyte ROS levels occurs with aging [26-28]. Others factors suggested may reduce the "wear and tear risk" for developing OA [29,30].

BMI is one of the risk factors in developing of KOA; in current study, a significant association is found between the higher BMI and the severity of KOA as it is revealed in the results that show significant association.

Two previous meta-analyses studies found that increasing BMI associated with increased incidence of both knee and hip OA. They mentioned that for each 5-unit increase in BMI, the risk of KOA increased by 35%, and the risk of hip OA increased by 11% [31,32].

Concerning the mutational analysis; the current study revealed that there is a significant statistical association between DVWA rs11718863 genetic alterations and the susceptibility and progression of KOA in Iraqi patients as there was high percentage of A allele in KOA patient while T allele was higher in control group. The same significant difference was also shown between two groups of KOA patients where T allele was higher in severe group in comparison to mild plus moderate KOA group.

Current results agree with other studies, which showed that DVWA gene specifically expressed in normal and OA cartilage tissues, encodes a protein showing VWA domain, having a role in cell adhesion and protein-protein interactions [13,14].

Minafra et al. (2014) found the DVWA rs11718863 SNP is reported to be strongly associated with the risk of KOA (odds ratio = 1.43, P <0.001) and able to influence β-tubulin binding in Asian populations [13]. Nevertheless, Valdes and colleagues (2009) showed an association between this genetic alteration and KOA in the UK Nottingham total knee replacement OA group (P <0.046) [33].

Miyamoto et al. suggested that both rs11718863 DVWA and rs7639618 polymorphisms may be involved in the etiopathogenesis of OA in Japanese and Chinese people, in particular, DVWA rs11718863 SNP is reported to be strongly associated with KOA. The OA susceptibility rs11718863 polymorphism is found in the exonic region of the DWVA type and causes a change of missense by following the amino acidic Tyr169Asn. In addition, Tof169-Cys260 double-protein isoform modification, may have a weak binding effect of β-tubulin and appears to be overexpressed to OA, and suggests that this interaction may be a basis for protecting OA members [15].

Meulenbelt and colleagues also described a moderately important association in the UK sample (P = 0.046), which is not validated in other European countries. However, the high frequency of allele risk in European samples highlighted the unique penetration of OA susceptibility genes and the need to evaluate the distribution of alleles 'geographic [18].

The DWVA protein, which is involved in cell adhesion and protein-binding proteins, binds to β-tubulin microtubules and plays an important role in regulating chondrocyte separation, protecting articulate joints from the onset of OA. In particular, rs11718863 SNP attracts a diminished link between DVWA and β-tubulin and causes the development of OA [10,16].

Other study conducted on Finnish women found that the variants in many candidate genes including DVWA gene were associated with OA across multiple sites [34].

Furthermore, another study on 66 Sicilian individuals affected by primary KOA revealed a significant statistical association between severity of KOA disease and DVWA rs11718863 genetic alterations. This gene was associated with a more severe radiographic grade, displaying its predictive role as OA marker progression [13].

Bravata et al. (2015) proposed a hypothesis that DVWA gene rs11718863 and rs7639618 polymorphisms cause missense mutation with a consequent amino acidic substitution (Tyr169Asn and Cys260Tyr, respectively). The Tyr169-Cys260 isoform decreases the strength interaction between the DVWA protein and the β-tubulin, which affects regulation of chondrocyte differentiation, subsequently resulting in OA especially in Asians [20].

Hämäläinen et al. (2014) found in subgroup analysis, that DVWA gene rs7639618 and rs11718863 polymorphisms were closely associated with the risk of OA in Asians, but not in Caucasians [35].

In contrast, other studies did not find any significant association between the DVWA polymorphisms (rs7639618, rs9864422 and rs11718863) and susceptibility to Developmental dysplasia of the hip DDH in the Chinese Han population [36,37].

DVWA rs11718863 and rs7639618 polymorphisms have both been found in the exonic region (third) and cause genetic mutations by amino acidic substitution (Tyr169Asn and Cys260Tyr, respectively). These SNPs are involved in reducing the energy interaction between DVWA protein and β-tubulin, this protein-binding binding is important in regulating chondrocyte differentiation that protects the joints from the onset of OA [16,18].

In conclusion, Iraqi people carrying AT genotype of DVWA rs11718863 gene are mostly susceptible for developing of KOA and the allele A of Tyr169Asn polymorphism are more frequent in those patients indicating that allele A may be a risk factor of onset and progression of this disease.

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## Author contribution

Dr. Hasan: Design of the study, data collection, DNA sequencing, results interpretation. Dr. Alwasiti: Design of study, supervision of all steps of the study. Dr. Ahmed: Statistical analysis and final revision of the manuscript.

## Conflict of interest

The authors declare that they have no competing interests.

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# Hepatitis B Virus Genotypes and Pre-core and Core Genes Mutations in a Sample of Iraqi Patients with Chronic Hepatitis B Infection

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## Abstract

**Background** Genomic studies of hepatitis B virus (HBV) diversity are becoming increasingly significant to understand how HBV mutations interact with a wide spectrum of clinical and pathological disorders.

**Objective** This study focused on identifying HBV genotypes and determining the status of pre-core (PC) and core promoter (CP) mutants.

**Methods** Nested polymerase chain reaction was used to identify the viral genotypes of 100 patients with chronic HBV infection. Only 30 samples out of 100 were selected to determine the prevalence of mutations in the PC and CP by Sanger sequencing.

**Results** Over 95% of the samples had only D genotype and mixed genotypes 5% (B+C+D) in chronic hepatitis B (CHB) patients. G1898A, G1901A, G1910A and G1915A mutations in PC gene were found in total 15 out of 30 (50%) samples, which were distributed in the following proportions: 12 out of 25 (48%) in patients with hepatitis B e antigen (HBeAg) -ve, 8 samples with mutants at G1898A, G1901A, G1910A and G1915A (four mutations), 4 samples with mutants at G1898A, G 1901A and G1910A (three mutations). In addition, 3 out of 5 (60%) in patients with HBeAg +ve, while no type of any mutation was detected in the core gene in patients with chronic hepatitis type B.

**Conclusion** The genotype D was predominantly prevalent among HBV in CHB patients with 95% and with 5% in mixed genotypes (B+C+D), while genotype B and C were relatively less prevalent among CHB patients than genotype D. The mutations were found in the PC gene at nucleotides G1898A, G1901A, G1910A and G1915A, while no type of mutation was detected in the core gene.

**Keywords** Genotypes, pre-core mutation, core mutation, HBV

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**List of abbreviations:** CHB= Chronic hepatitis B patients, CP= Core promoter, HBeAg= Hepatitis B envelope antigen, HBV= Hepatitis B virus, PC= Pre-core

## Introduction

Hepatitis B virus (HBV) has infected two billion individuals worldwide. About 3.5% of the world's population, or 257 million people, have a chronic infection that increases their chance of developing severe liver disease, cirrhosis, and/or hepatocellular carcinoma [(1)]. HBV is a member of the family Hepadnaviridae, specifically the genus Orthohepadnavirus [(2)]. Blood or other bodily fluids from an infected individual can spread the virus, making it extremely infectious. For a

minimum of seven days, HBV can survive outside of the body [2,3].

HBV genomes have been classified into genotypes ranging from A to J based on their genetic variability (>8% for the entire genome) [4-6]. Each genotype was subdivided into multiple sub-genotypes [7]. Within specific populations and geographic locations, HBV genotypes are distributed differently [8,9]. There is a correlation between genotype/sub-genotype and severity of disease [9]. Numerous HBV genotypes and genome mutations have been identified as being associated with the progression of liver diseases. Their clinical significance, on the other hand, is still debatable [10].

Negative test for hepatitis B e antigen (HBeAg) in chronic hepatitis type B (CHB) patients is frequently associated with pre-core and core promoter mutants. The pre-core variation with the greatest prevalence is a G/A transition occurs at nucleotide (nt) 1898 (G1898A) and (G1899A), resulting in the formation of a premature stop codon that prevents the synthesis of the HBeAg [11]. Pre-core and core mutations down-regulate HBeAg synthesis, HBeAg can be detected in chronic hepatitis B patients as a marker of productive infection, the absence of HBeAg indicates a reduction in viral replication and infection due to the immune response of the host, however, the absence of HBeAg does not necessarily indicate a decline in HBeAg levels and viral replication, but in some cases, it is only a means of evading immune clearance caused by mutation at the pre-core and core, which results in the creation of a premature stop codon and HBeAg synthesis is no longer possible.

So, the aims of this study were to identify HBV genotypes, determine pre-core and core mutations in HBV and their impact on the subsequent course of liver disease and HBeAg expression.

## Methods

The study enrolled 100 CHB patients (68 males and 32 females) ranging in age from 4 to 70 years and attending Al-Imamein Al-Kadhimein Medical City in Baghdad, Iraq between July and November 2020. This study was approved by the Institutional Review Board (IRB) of the College of Medicine, Al-Nahrain University. Serum samples were collected from patients to determine the presence of hepatitis B surface antigen (HBsAg), HBeAg, anti-HBe, and anti-HBc IgG using enzyme-linked immunosorbent assay (ELISA) kits (Sure Bio-Tech, Hong Kong). Patients were classified into HBeAg +ve 13 (13%) and HBeAg -ve 87 (87%) CHB patients based on their HBeAg status. Then the serum was used to extract the viral DNA for genotyping using sets of primers to detect genotypes (A-F) by nested polymerase chain reaction (PCR). In addition, conventional PCR was used to amplify the pre-core and core region using specific primers, gel electrophoresis of the PCR products, and DNA sequencing to determine the presence of the mutations.

### DNA extraction and HBV quantification

Viral DNA extraction from serum sample was performed by using the Relia Prep™ Blood gDNA Miniprep System (Promega, USA), which provides a fast and simple technique for the preparation of purified DNA. The stages of the method are lysis of cells, binding of nucleic acid to column membrane, washing the bound nucleic acid and elution of the nucleic acid.

### HBV genotyping

To determine the genotypes of HBV, we used primers and applied PCR. Two rounds were made, the first round using a pair of outer primers, and the second round a pair of inner primers. The primers used in the study were designed based on the conserved nature of the nucleotide sequences in the pre-S1 to S gene regions, regardless of the genotype of the HBV [12]. The primers P1 (forward) and S1-2 (reveres) were used as outer primers (1,063 bases). To determine the genotypes, B2 was used as an inner primer (forward) in a combination known as mixture A, including A, B, C. We used (reveres) primers (BA1R) as (type A), BB1R as (type B), and BC1R as (type C); all these primers were in the mixture A. As for the determination of the genotypes (D, E, and F), B2R as (reveres)

primers were used in a combination known as mixture B, (forward) primers were used, BD1 (type D), BE1 (type E) and BF1 (type F) and all these primers were included in the B mixture. Variation in bands sizes specific to genotype types were determined on the basis of primers in the second-round combination using the polymerase chain reaction technique. Table 1 shows the strategy used to determine the types of genotypes of HBV.

The first round of the PCR was carried out in a tube containing the following components: a colorless ready to use master mixture 12.5 µl, 1 µl for each external primer, 1 µl of extracted DNA and 9.5 µl of nuclease-free water. The thermal cycler was set to run one cycle at 95°C for 10 minutes, followed by 40 cycles at 94°C for 20 seconds, 55°C for 20 seconds, and 72°C for 1 minute. The second round of PCR was carried out for each sample using B2 forward primer with mixture A that includes types A to C and reveres primer (B2R) with mixture B to determine the types D to F. As in the first reaction, 3 µl of the product of the PCR produced from the first round were added to two tubes, each tube containing the second group of pairs of internal primers with an amount of 0.5 µl for each B2, BA1R, BB1R and BC1R in addition to 7.5 µl of nuclease-free water and master mixture 12.5 µl to detect mix A genotypes, and in second tube the same composition but with another pairs of internal primers B2R, BD1, BE1 and BF1 to detect mix B genotypes, then it was amplified for 40 cycles as follows, preheating at 95°C for 10 minutes and 20 amplification cycles at 94°C for 20 seconds and 58°C for 20 seconds and 72°C for 30 seconds and additional cycles at 94°C for 20 seconds and 60°C for 20 second followed by 72°C for 30 seconds.

**Table 1. The primers used for detection of HBV genotypes and the size of PCR products [12]**

| | Primers | The size of PCR products |
|---|---|---|
| 1st round PCR | P1(F)/S1-2(R) | 900 bp |
| 2nd round PCR Mix A | B2+BA1R | 68 bp |
| | B2+BB1R | 281 bp |
| | B2+BC1R | 124 bp |
| 2nd round PCR Mix B | BD1+B2R | 120 bp |
| | BE1+B2R | 167 bp |
| | BF1+B2R | 97 bp |

**HBV mutations in the pre-core and core promoter regions**

The mutations in the pre-core and core genes of HBV were investigated using nested PCR with a set of primers (F and R) as given in Table 2. The first round of PCR was done using a ready to use 25 µl Taq colorless master mix, 1 µl of each external primers (P1 and P2), 5 µl of DNA extract and 18 µl of nuclease-free water. The reaction was carried out in a thermal cycler for 36 cycles of 1 minute at 94°C, 1 minute at 56°C, and 3 minutes at 72°C, followed by a 7-minute extension step at 72°C. For the second-round PCR, 2 µl of the first-round PCR product was added to 25 µl master mix, 1 µl of each internal primer (P3 and P4) and 21 µl of nuclease-free water. Five microliters of the second-round PCR products were electrophoresed in a 1% agarose gel stained with ethidium bromide (0.5 µg/ml). The gel electrophoresis apparatus was turned on and set to 72 volts for one hour before being photographed under ultraviolet light. All required precautions against cross-contamination were taken, and each test contained negative controls (the same composition of reaction mix omitted the DNA sample and replaced with nuclease-free water).

**Table 2. Primers sequences used for amplify pre-core and core regions [13]**

| Nested PCR | Primer | Sequences | PCR product size (bp) |
|---|---|---|---|
| External primers | P1(F) | 5´-TCGCATGGAGACCACCGTGA-3´ (Positions 1604-1623) | 450 bp |
| | P2 (R) | 5´-ATA GCTTGCCTG AGTGC-3´ (Positions 2076-2060) | |
| Internal primers | P3(F) | 5´-CATAAG AGGACTCTTGGACT-3´ (Positions 1653-1672) | 340 bp |
| | P4 (R) | 5´-GGAAAGAAGTCAGAAGGC-3´ (Positions1974-1957). | |

**Sanger sequencing analysis**

Purification of amplified PCR products using Qiaquick spin columns (Qiagen Inc.) was performed according to the manufacturer's instructions. The purified PCR products were then sent to Macrogen Corporation – Korea for Sanger sequencing using an ABI3730XL automated DNA sequences. The results were received by email and processed with the help of the genius prime software (version 2021).

**Statistical analysis**

The statistical package for social science (SPSS) software (version 25) was used to conduct the descriptive analysis. Frequency and mean± standard deviation (SD) of continuous data was calculated.

## Results

Results presented in this study showed the HBV genotypes distribution of the collected samples revealed that genotype D was the most common genotype among patients (95%) followed by mixed (B+C+D) genotypes (5%). However, genotypes A, E and F were not identified in any patient (Table 3 and Figures 1 and 2).

On comparing between 87 HBeAg -ve CHB and 13 HBeAg +ve CHB patients regarding HBV genotypes, revealed that genotype D was the most predominant genotype in HBeAg -ve patients (82%) followed by mixed infection with (D,B,C) genotypes (5%), while in HBeAg +ve patients, it was found that the genotype D percentage was (13%) and no any infected patient with mixed infection (B+C+D) genotypes (data not shown).

**Table 3. Hepatitis B virus genotypes of hundred chronic hepatitis B patients**

| HBV genotypes | CHB patients | |
|---|---|---|
| | No. | % |
| Genotype D alone | 95 | 95.0 |
| Mixed infection (B,C,D) | 5 | 5.0 |
| Mixed infection (A,E,F) | 0 | 0.0 |
| Total | 100 | 100 |

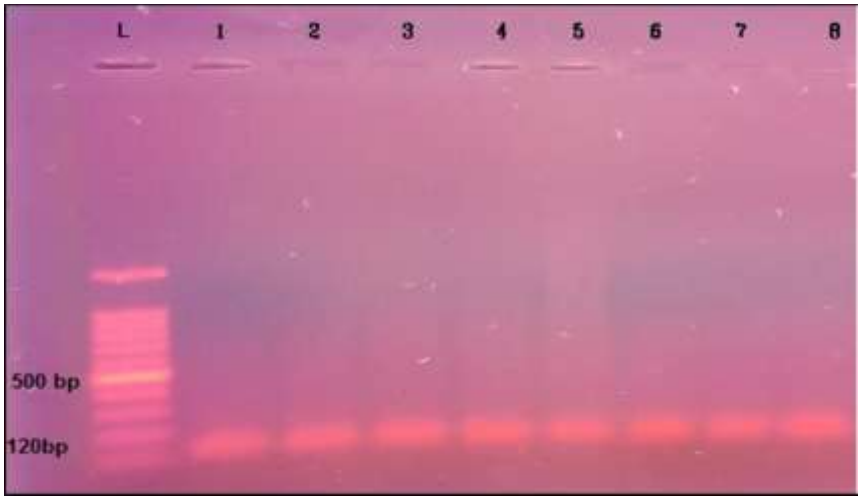


**Figure 1. Eight chronic hepatitis B samples using gel electrophoresis was turned on and set to 72 volts for one hour, show amplification of mix B (120 bp genotype D) (L: 100-1000 pb ladder)**

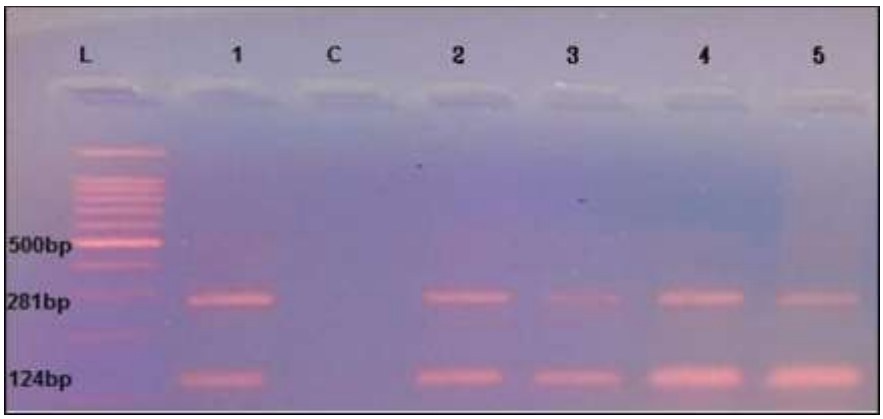


**Figure 2. Gel electrophoresis on Mix A genotypes (2nd round) shows mix infection (genotype C, 124 bp) and (genotype B, 281 bp). C is negative control (no template DNA sample), L (100-1000 pb ladder)**

**Pre-core and core promoter mutants**

G1898A, G1901A, G1910A and G1915A mutants were found in total 15 out of 30 (50%) samples. These mutations were detected in 12 of 25 (48%) of HBeAg -ve and 3 of 5 (60%) of HBeAg +ve samples. No mutations at core promoter positions were detected in this study, whether HBeAg -ve or HBeAg+ve. G1898A, G1901A, G1910A and G1915A mutants were distributed in the following proportions: 12 out of 25 patients (48%), 8 samples with mutants at G1898A, G1901A, G1910A and G1915A (four mutations), 4 samples with mutants at G1898A, G1901A and G1910A (three mutations) in HBeAg -ve patients, and 3 out of 5 (60%) in HBeAg +ve, as shown in table 4.

Patients with HBeAg -ve mutant viruses were older (30-65) years versus (20-48) years in HBeAg +ve. In comparison to HBeAg +ve patients, patients with HBeAg -ve had more mutations in the pre-core region at nucleotides G1898A, G1901A, G1910A and G1915A. While no mutations in the core region promoter at nucleotides A1762T and G1764A were detected in both groups, as shown in table 5.

**HBV mutations and genotypes**

In terms of genotypes, 25 of 30 patients (83.33%) were infected with HBV genotype D, whereas 5 of 30 patients (16.66%) were infected with HBV mixed genotype (B,C,D). In comparison to genotypes and mutations, this study found that genotype D-infected patients had more mutations at G1898A, G1901A, G1910A and G1915A than mixed genotype-infected patients. Additionally, demonstrated differences in age, sex, and the rate of HBeAg -ve and +ve between genotype D patients and mixed genotype (B+C+D) as shown in tables 4 and 5.

**Table 4. The percentage of mutations in the pre-core and core promoter genes in 30 individuals with chronic hepatitis B virus with regard to HBeAg status and HBV genotypes**

| | | Total | Mutation G1898A No. (%) | MutationG1901A No. (%) | MutationG1910A No. (%) | Mutation G1915A No. (%) | Mutation A1762T/ A1764G No. (%) |
|---|---|---|---|---|---|---|---|
| HBe Ag status | Positive | 5 | 3 of 5 (60%) | 3 of 5 (60%) | 3 of 5 (60%) | 3 of 5 (60%) | --- |
| | Negative | 25 | 12 of 25 (48%) | 12 of 25 (48%) | 12 of 25 (48%) | 12 of 25 (48%) | --- |
| Total | | 30 | 15 (50%) | 15 (50%) | 15 (50%) | 15 (50%) | 0 |
| HBV genotypes | Genotype D alone | 25 | 12 of 25 (48%) | 12 of 25 (48%) | 12 of 25 (48%) | 12 of 25 (48%) | 0 |
| | Mixed (B,C,D) | 5 | 3 of 5 (60%) | 3 of 5 (60%) | 3 of 5 (60%) | 3 of 5 (60%) | 0 |
| Total | | 30 | 15 (50%) | 15 (50%) | 15 (50%) | 15 (50%) | 0 |

**Table 5. The percentage of mutations in the pre-core and core promoter genes in HBeAg -ve and HBeAg +ve for 30 patients with chronic hepatitis B virus in relation to age and sex**

| | | HBeAg (-ve) No. = 25 (83.33%) | HBeAg (+ve) No. = 5 (16.66%) |
|---|---|---|---|
| Age(yr) | mean±SD | 33.92±1.44 | 24.6±1.22 |
| Sex | (male/female) (n=30) | 19M/6 F | 3 M/2 F |
| Core(CP) mutation | A1762T | --- | --- |
| | A1764G | --- | --- |
| Pre core mutation | G1898A | 12(48%) | 3(60%) |
| | G1901A | 12(48%) | 3(60%) |
| | G1910A | 12(48%) | 3(60%) |
| | G1915A | 12(48%) | 3(60%) |
| HBV genotypes | Genotype D alone | 20 (80%) | 5 (100%) |
| | Mixed (B,C,D) | 5 (20%) | 0 (0%) |

## Discussion

The HBV genotypes distribution of the collected samples revealed that genotype D was the most common genotype among patients (95%) followed by mixed (B,C,D) genotypes (5%), while genotypes A, E and F were not identified in any patient, as shown in table 3. The foregoing findings are consistent with Khaled et al. [14] who reported that HBV infections were significantly linked to virus genotype D, which accounted for 87% of all patients, a high prevalence compared to mixed infections (D and F), which accounted for 13 percent of all samples. These results are not matched with those in Sulaimania, Iraq, where they reported that 100% of samples had mixed genotypes (25% with B,C,D genotypes and 75% with A,B,C,D genotypes)[15]. In Baghdad, one study reported that genotype D contributes to 80% of the infection, which is the most prevalent in CHB patients, and mixed genotypes F and D contribute to 20% of the infection [16]. These findings disagreed with Ali et al. [17] in Wasit (Iraq) who reported that there was no single HBV genotype infection and that 77.7% of their patients had mixed infection with five genotypes out of six. Also, another study in Baghdad that involved eighty CHB patients reported six HBV genotypes (A, B, C, D, E, F). The most frequently found genotypes were B and F (72.5% each), whereas the least frequently occurring genotype was E (12.5%). Therefore, they concluded that HBV genotypes B and F were the most prevalent in Iraqi CHB patients from Baghdad [18]. So, the results in this study appeared in agreement with some other results of studies conducted in Iraq and the same time are contradicted with others which conducted in Iraq as well. The reason is due to the difference in the number of samples used in each study, the method used in the diagnosis, where some studies relied on traditional methods using primers and some relied on the use of ready-made kits for diagnosis, and this is one of the reasons for the mismatch of results between studies within the same country.

In comparison to genotypes and mutations, this study found that patients with genotype D-infected patients had more frequency mutations at G1898A, G1901A, G1910A, and G1915A than patients with mixed genotypes. This was in disagreement with a study in Nigeria by Mbamalu et al. [19] who revealed that 11 (12.4%) of the 89 patients had genotype B, and 78 (87.6%) had genotype C, in addition, G1896A (23.6%), G1764A (9%), and A1762T (6.7%) were the most frequently occurring alterations. Pre-core mutations were found to be substantially more prevalent in genotype B patients than in genotype C patients (54.5% vs. 19.2%, p=0.01).

In this study in Iraqi CHB patients, the presence of mutations in the pre-core gene at the nucleotides (G1889A, G1901A, G1910A and G1915A) was 15 out of 30 (50%) divided into: 3 out of 5 (60%) representing HBeAg +ve and 12 out of 25 (48%) for patients with HBeAg –ve, as shown in table 4. As for the core gene, the study did not show any mutations in core gene. These results are in disagreement with another study in Baghdad by Kadham [20] who reported presence of mutations in core gene of HBV at nucleotide position A1762T and G1764A. The inconsistency of the results in this study with the study of Kadham [20]is due to the difference in the number of samples included in the study and the difference in the technique used to diagnose mutations in the pre-core and core gene. But at the same time present results agree with Kadham[20] who found mutations at nucleotide G1896A and G1898A, and these mutations indicate severe form of chronic liver disease. The present results are disagreed with results of a study in China regarding the absence of mutations in the core gene at nucleotides position A1762T and G1764A in the core gene promoter region[21]. The results differ due to the difference in the number of samples included in the study, the difference in the environment, the geographical location, and the possibility of a different pathological phase of the virus. The results of this study agreed with another study in Taiwan regarding the presence of mutations in the pre-core region, and disagreed regarding the absence of mutations in the core promoter region [11]; the difference may be due to dissimilarity in the virulence of the virus in its local isolates differs from the virulence of the virus in global isolates, as well as the difference in the environment and geographical location. The current study was consistent with another study conducted on patients with CHB in Korea

and found the frequency of mutations in the pre-core region at the nucleotides G1896A and G1898A, and also, present results did not agree with this Korean study regarding the absence of mutations in the core region at nucleotides A1764T, G1768A, and C1766T [22].

In conclusion, the genotype D of HBV was predominantly prevalent among CHB patients with 95% and with 5% in mixed genotypes (B,C,D), while genotype B and C were relatively less prevalent among CHB patients than genotype D. The mutations were found in the pre-core promoter gene at nucleotides G1898A, G1901A, G1910A and G1915A, while no type of mutation was detected in the core gene. The clinical significance of these mutations G1898A, G1901A, G1910A and G1915A, shows us the stages of disease development and whether the patient is in the stage of carrying the disease. And when we do the serological examination of the HBeAg gives negative results because of the mutations that occurred in the genome of the virus that led to the absence of the formation of HBeAg.

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## Author contribution

As part of her PhD thesis, Dr. Hussain performed all laboratory work and wrote the draft of this paper. This work was designed and supervised by Dr. Al-Shuwaikh and Dr. Ahmed. The final version of this manuscript was read and approved by the authors.

## Conflict of interest

The authors declare that there is no conflict of interest.

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# Risk Factors for Relapses in Children with Steroid Sensitive Nephrotic Syndrome

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## Abstract

**Background** Nephrotic syndrome (NS) is the most common glomerular disease seen in the pediatric age group.

**Objective** To study demographic, socioeconomic, disease related characteristics and the physical examination including height, weight and blood pressure correlation with frequency of relapses in children with steroid sensitive NS (SSNS).

**Methods** This is a cross sectional study that was conducted at Nephrology Consultation Clinic, in Al-Imamein Al-kadhimein (peace on them) Medical City, Baghdad, Iraq starting from 1st of January, 2020 to 31 July, 2021.

**Results** Total number of patients was 60 children with SSNS, 24 (40%) patients were frequent relapsers and 36 patients (60%) were infrequent relapsers. Most of the children at onset of the disease were less than 5 yr (76.7%) and males were more than females (78.3%). Fifty-three patients were responder to steroid in less than 2 weeks (88.3%). Ten patients with low body weight (16.7%) and 6 patients with low body height (10%), 25 patients presented with elevated blood pressure (41.7%). A significant correlation was found between future relapses and following characteristics; response to steroid in less than 2 weeks, urinary tract infection (UTI) and gross hematuria, low body weight, short stature and elevated blood pressure.

**Conclusion** Comparing frequent with infrequent relapsers, the following factors found to be statically significant: response to steroid less than 2 weeks, UTI, gross hematuria, low body weight, short stature and elevated blood pressure.

**Keywords** Relapse, steroid, nephrotic syndrome, frequency

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**List of abbreviations:** NS = Nephrotic syndrome, SRNS = Steroid resistant nephrotic syndrome, SSNS = Steroid sensitive nephrotic syndrome, URTI = Upper respiratory tract, UTI = Urinary tract infection

## Introduction

Nephrotic syndrome (NS) is the most common glomerular disease seen in the pediatric age group. NS is defined as proteinuria >40 mg/h/m$^2$ or >50 mg/kg/day or protein/creatinine ratio >0.2 g/mmol (>2 g/g) and hypoalbuminemia <25 g/l with or without edema [1,2].

NS in children is generally classified into steroid sensitive nephrotic syndrome (SSNS) and steroid resistant nephrotic syndrome (SRNS) based on the initial response to corticosteroid therapy at presentation [3]. NS incidence and prevalence varies between different geographical regions of the world and ethnicities [4]. About 80-90% of SSNS children experience one or more subsequent relapses that can be infrequent or frequent relapses or

steroid dependence. The age of onset of the disease, time to respond to steroids and length of treatment, rapid steroid tapering and infections were reported to be the predictors of the relapses and their frequency. Two-thirds of childhood NS present before the age of 6 years. The ratio of boys to girls is 2:1. By late adolescence, both sexes are equally affected [5].

Most children with SSNS (89%) have repeated relapses. Although there is no proven way to predict an individual child's course, children who respond rapidly to steroids and those who have no relapses during the first 6 months after diagnosis are likely to follow an infrequently relapsing course. It is important to indicate to the family that the child with SSNS is unlikely to develop chronic kidney disease, that the disease is rarely hereditary, and that the child will remain fertile [6].

This study aimed to identify risk factors for relapses in children with SSNS.

## Methods

This is a cross sectional study that was conducted at Nephrology Consultation Clinic, in Al-Imamein Al-kadhimein (peace on them) Medical City, Baghdad, Iraq, starting from 1st of January, 2020 to 31st of July, 2021. In this study, 60 patients were included and diagnosed as cases of SSNS. Well-formed questionnaire was designed by researchers including demographic and socioeconomic: residency, gender, age and economic condition, which classified according to family income: poor if less than 500,000 Iraqi Dinars (ID), middle if 500,000 – 1000,000 ID, upper if more than 1000,000 ID). Disease related data including age of onset of NS, response to steroid, types of NS (frequent or infrequent), atopy (asthma), gross hematuria, asthma and infections (upper respiratory tract (URTI) (within last 2 weeks) and urinary tract infection (UTI) (within last 2 weeks) in the last year. Physical examination was conducted for each patient including blood pressure, height, and weight and plotted on reference charts.

Data collection was done by direct contact interview with patients and their parents, in addition to doctors involved in their management and the record files. A verbal consent from the parents was taken prior to enrollment in the study. Total no. of 60 children with NS were divided into 2 groups for comparison: frequent (24 patients - 40%) and infrequent (36 patients - 60%).

The following definitions were used: Relapsed NS is defined as proteinuria mg/h/$m^2$ or >50 mg/kg/day or Albustix +++ for 3 consecutive days after having been in remission. Frequent relapses (FR) are 2 or more relapses within 6 months of initial response or 4 or more relapses within a period of 1 year while infrequent relapses (IR) were less than 2 within 6 months or less than 4 for any year thereafter. SSNS is complete remission achieved with steroid therapy. SRNS defined as patients who fail to enter remission after 8 weeks of corticosteroid treatment [2,3,6].

Gross hematuria: is referred as blood in the urine is visible to the naked eye [3].

UTI: was considered positive when patient had symptoms and findings on urinalysis, confirmed by a urine culture [6].

Hypertension: was defined as BP ≥95th percentile for age, height, and sex [6].

Low height or length for age; if below 3rd centile or less than 2 standard deviations for that specific age and sex [6].

Low weight for age; if a child weight is below the 3th percentile or less than 2 standard deviations for that specific age [6].

The data analyzed by the statistical package for social sciences (SPSS version 20) and Microsoft office Excel programs (2013) and Graph Pad Prism (6) for mean, standard deviation and p-value.

P value was calculated, statistically significant if it is <0.05 and highly significant if <0.001.

## Results

Total number of 60 patients with SSNS were enrolled in this study; FR were documented in 24 patients (40%), while IR was documented in 36 patients (60%).

The demographic characteristics of the study group came out with the following data: The age of the patients was ranging between 2.5-16 yr at the time of the study; 45 patients (75%) of them were older than 5 yr, while 46 (76.7%) were less than 5 yr age at time of onset of the disease. Males were 47 (78.3%) while females were 13 (21.6%) with a male:female ratio 3.6:1. Forty patients (66.7%) were residing in urban area, while 20 patients (33.3%) came from rural areas. Thirty patients (50%) lie in the middle socioeconomic status (29 patients with poor status and 1 patient from upper status).

Considering the disease related characteristics, 53 patients (88.3%) responded to steroids in less than two weeks while 7 patients (11.7%) did not. Fifteen patients (25%) were reported to be asthmatics. Nineteen patients (31.7%) reported at least one episode of URTI in the last year, while 26 patients (43.3%) reported UTI in the last year. Episode(s) of gross hematuria was reported in 8 patients (13.3%).

Regarding the clinical characteristics of the disease, ten patients (16.7%) reported low body weight, six patients (10%) reported short stature, and hypertension was detected in 25 patients (41.7%).

Table (1) shows comparison of sociodemographic characteristics according to the frequency of relapse in the study group. Among this cohort group, the mean current age and age at onset were 8.0 yr and 4.5 yr for FR, while 7.9 yr and 4.2 yr for IR respectively. No statistical significance was noted among those parameters. Males were 22 (91.7%) in FR versus 25 (69.4%) in IR, while females represent 2 (8.3%) and 11 (30.6%) respectively, with no statistical significance. Fifteen patients (62.5%) of the FR were residing in urban area, while 25 patients (69.4%) of the IR do so. The comparison regarding the residency was not significant. Most of this cohort lie in the middle socioeconomic class 11 FR (45.8%) and 19 IR (52.8%) with a non-significant statistical comparison.

**Table 1. Comparison of sociodemographic characteristics according to the frequency of relapse in children with nephrotic syndrome**

| Characteristic | | Frequent relapse N=24 Mean±SD | Infrequent relapse N=36 Mean±SD | P value |
|---|---|---|---|---|
| Age at time of study (yr) | | 8.06±3.58 | 7.98±3.11 | 0.924* |
| Age at onset of NS (yr) | | 4.55±2.98 | 4.26±1.63 | 0.659* |
| | | N (%) | N (%) | |
| Gender | Female | 2 (8.3) | 11 (30.6) | 0.056** |
| | Male | 22 (91.7) | 25 (69.4) | |
| Residency | Rural | 9 (37.5) | 11 (30.6) | 0.590** |
| | Urban | 15 (62.5) | 25 (69.4) | |
| Socioeconomic status | Poor | 12 (50) | 17 (47.2) | 0.962*** |
| | Middle | 11 (45.8) | 19 (52.8) | |
| | Upper | 1 (4.2) | 0 (0.0) | |

NS: Nephrotic syndrome, * p value by unpaired ttest, ** p value by Fisher exact test, *** p value by Yates chi square test

Table (2) shows comparison of disease-related characteristics according to the frequency of relapse in the study group. Out of the FR, 18 patients (75%) responded to steroids in less than two weeks while 6 in more than two weeks (25%), 15 patients (62.5%) reported history of episode(s) of UTI in the past year, and 7 patients (29.2%) reported episode(s) of

gross hematuria. Out of the infrequent relapses, 35 patients (97.2%) responded to steroids in less than two weeks while 1 in more than two weeks (2.8%), 11 patients (30.6%) reported episode(s) of UTI in the past year, and 1 patient (2.8%) reported episode(s) of gross hematuria. The difference was significant in response to steroid among type of relapsers (frequent versus infrequent) with a p value of 0.013, significant in regard to UTI among both groups (P value 0.018, and also significant in regard to gross hematuria among both groups, p value 0.005). It is not Significant among both groups in regard to asthma history and URTI among both groups (p values 0.3 and 0.7 respectively).

**Table 2. Comparison of disease-related characteristics according to the frequency of relapse in children with nephrotic syndrome**

| Characteristic | | Frequent relapse N=24 N (%) | Infrequent relapse N=36 N (%) | P value* |
|---|---|---|---|---|
| Response to steroids | ≤2 wk | 18 (75.0) | 35 (97.2) | 0.013 |
| | >2 wk | 6 (25.0) | 1 (2.8) | |
| Asthma | Negative | 20 (83.3) | 25 (69.4) | 0.362 |
| | Positive | 4 (16.7) | 11 (30.6) | |
| URTI | Negative | 17 (70.8) | 24 (66.7) | 0.784 |
| | Positive | 7 (29.2) | 12 (33.3) | |
| UTI | Negative | 9 (37.5) | 25 (69.4) | 0.018 |
| | Positive | 15 (62.5) | 11 (30.6) | |
| Gross hematuria | Negative | 17 (70.8) | 35 (97.2) | 0.005 |
| | Positive | 7 (29.2) | 1 (2.8) | |

URTI: Upper respiratory tract infection, UTI: Urinary tract infection, * p value by Fisher exact test

Table (3) shows comparison of clinical characteristics according to the frequency of relapse in the study group. Among the FR cases, 16 patients (66.7%) had normal body weight while 8 patients (33.3%) showed low body weight, 18 patients (75%) showed normal height range while 6 patients (25%) were short in stature, and 15 patients (62.5%) reported high blood pressure reading. Among the IR cases, 2 patients (5.6%) showed low body weight, no patients were reported to have short stature, and lastly 10 patients (27.8%) showed high blood pressure. All comparisons among both groups were statistically significant (p values 0.01 for body weight, 0.003 for height, and 0.015 for blood pressure reading.

**Table 3. Comparison of clinical characteristics according to the frequency of relapse in children with nephrotic syndrome**

| Characteristic | | Frequent relapse N=24 N (%) | Infrequent relapse N=36 N (%) | P value* |
|---|---|---|---|---|
| Body weight | Low | 8 (33.3) | 2 (5.6) | 0.010 |
| | Normal | 16 (66.7) | 34 (94.4) | |
| Body height | Low | 6 (25.0) | 0 (0.0) | 0.003 |
| | Normal | 18 (75.0) | 36 (100) | |
| Blood pressure | High | 15 (62.5) | 10 (27.8) | 0.015 |
| | Normal | 9 (37.5) | 26 (72.2) | |

* p value by Fisher exact test

## Discussion

Nephrotic syndrome is a chronic relapsing kidney disease with higher incidence compared to other kidney diseases. Frequency of relapses is highly variable and there are different risk factors associated with frequency of relapses. This study was done to compare the risk factors for relapses.

In a study for sociodemographic characteristics by Moorani et al. [7], the mean age at time of the study and onset of the disease was 7.99±3.1 and 5.4±2.7 yr respectively, which is nearly similar to current results, demonstrating a gap between the age of study and age of onset of NS in both FR and IR. Also, these results are in agreement with previous studies performed by Constantinescu et al. [8], Fujinaga et al. [9] and Takeda et al. [10], we did not find any correlation between age at time of study, nor age at onset of disease with future relapses among patients with FR and IR NS, but this was inconsistent with 2 other studies by Sarker MN. et al. and Situmorang et al. [11,12].

This study showed predominance of male patients over female patients and the result was similar to elsewhere [6,13,14]. Like this study, statically nonsignificant correlation between gender and frequency of relapse was reported in Rahi et al. study [15].

Most of the patients in this study came from urban area surrounding the hospital, however non-significant correlation between residency and frequency of relapse and was detected. The same result was noticed previously by previous Iraqi study [13]. Yet, this finding is in contrast to Sarker et al. [11] who reported a significantly higher incidence of frequent relapse in rural children than in urban children and their explanation for this observation was the delay in the initiation of specific treatment in rural areas.

No statistical significance regarding socioeconomic status and relapse was present in both study groups and this because of the percentages in both frequent and infrequent relapsers were roughly the same. Sarker et al. [11] and Ali et al. [16] detected low socioeconomic status was another risk factor for frequent relapses and their explanation was that such children are vulnerable to infection and hence more likely to relapse.

Regarding disease related characteristics; apropos of response to steroid, interesting finding in this study was a statistically significant association between early response to steroid therapy and increased incidence of IR. The same was noticed by Fujinaga et al. [9] and Ali et al. [13]; who reported that patients who responded within early days of initial steroid therapy showed a favorable clinical course and less frequency of future relapses.

For asthma and URTI; asthma is common problem and mostly asthmatic attack is aggravated with respiratory infections, which

might underestimate the asthma itself as a trigger factor. The correlation between relapse and asthma in this study was statically nonsignificant and this was consistent with study done by Riar et al. [17] and Ali et al. [18].

Whilst URTI is also statistically nonsignificant in this study (p value = 0.784), which is similar with Rahi et al. [15], but URTI was important significant factor with relapses in Mantan et al. study [19]. This may be explained that different viruses implicated in exacerbations suggest that a host response to viral infection, rather than specific viral antibodies, may be the triggering factor for relapse following URTI [20].

UTI is another significant factor of frequent relapse in this study and same result was present with Sarker et al. [11] and Balaji et al. [21].

Regarding gross hematuria; there was highly significant difference between both groups and was evident by previous Iraqi study [13].

In regard to clinical characteristics; low body weight and height in both groups was shown to have statistical significance. This result may be interpreted by poorly nourished patients, more liable to infections with more steroids using, which affect height due to more frequency of relapses in these patients. Relating to hypertension, the positive statistical significance was similar to what was mentioned by Balaji et al. [21], and disagrees with Noer et al. [14] and these variable results may be explained by different pathophysiological contributing factors including renal factors (like albuminuria, Sodium retention, or decreased glomerular filtration rate) and extra-renal factors (like medication side effects, genetic predisposition, life style and diet or cardiovascular risk factors [22].

In conclusions, comparing frequent with infrequent relapsers, the following factors found to be statically significant: response to steroid less than 2 weeks, UTI, gross hematuria, low body weight, short stature and elevated blood pressure.

The authors recommend involvement of a larger sample is mandatory to confirm the results. Urine examination routinely with each relapse to detect UTI. Involvement of dieticians for proper nutritional education of children with NS. The blood pressure is important to be measure in each patient.

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## Author contribution

All authors have participated sufficiently in the intellectual content, conception and design of this work or the analysis and interpretation of the data, as well as the writing of the manuscript.

## Conflict of interest

The authors declare there is no conflict of interest.

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# The Role of Elastography in Predicting the Grade of Mammary Ductal Carcinoma

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## Abstract

**Background** Elastography is an imaging technique which has been used in the last decades and its role in breast masses characterization is well established. However, its value for predicting breast cancer grading is yet to be studied.

**Objective** To determine the role of strain elastography in the prediction of the grade of mammary ductal carcinoma.

**Methods** A cross sectional study enrolled 44 female patients with Breast Imaging Reporting and Data System (BI-RADS) Category 5 breast mass. Complete B-mode and elastographic ultrasound examination of the breast was performed with evaluation of the elastoscore and strain ratio. Correlation with the detailed histopathological report was done for cases that were proved to be ductal type breast carcinoma.

**Results** Significant correlation was found between the speculated outline of the tumor and grade III tumor (p=0.03). Breast tumors with higher Elastography/B mode size ratio (>1) and with high elasticity score (score 5) were significantly associated with grade III (p=0.02). The mean strain ratio of masses was significantly higher among grade III breast tumors (p<0.001).

**Conclusion** Elastography is a helpful non-invasive tool that has the potential for predicting breast ductal carcinoma grade and by such predicting the prognosis.

**Keywords** Breast ductal carcinoma, strain elastography, ductal carcinoma grades

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**List of abbreviations:** BI-RADS = Breast imaging reporting and data system, ER = Estrogen receptor, ROI = Region of interest, SR = strain ration, SWE = Shear wave elastography

## Introduction

Breast cancer is the most commonly diagnosed cancer and is the leading cause of cancer death among women, it represents 23% of the all cases of cancer and about 14% of the cancer related deaths, particularly in low- and middle-income countries, as a results of a combination of a late stage of presentation, diagnosis and limited access to proper treatment [(1,2)].

Owing to the lack of proper recording system in the Iraqi hospitals, there is no accurate reporting regarding tumor size, nodal state, hormonal receptor status, stage distribution at the time of initial diagnosis, proportion of patients with distant metastasis and of those treated with radical mastectomy or breast conservation surgery [(3,4)]. However, some previous recent studies had reported that invasive ductal carcinoma represents 88% of

breast cancer cases in Iraq with the tendency to occur at early age with moderately progressive grade and stage at time of presentation [5].

Elasticity is "the characteristic of a tissue or substance that makes it to be deformed when it is exposed to an external power and return to its original figure or size when the force is removed"[6]. The technique, which is now most widely used in clinical settings is real-time elastography, produce “strain imaging” by compression. Electrography can be performed using conventional ultrasound (US) device with dedicated software, by this examination we evaluate the relative elasticity of the tissues in a specific region of interest making an elastogram that is superimposed to the US image [7,8].

This study aimed to determine if quantitative and qualitative strain elastography have a role in the prediction of the grade of mammary ductal carcinoma.

## Methods

### Study design

The present study is a cross-sectional study conducted in Breast Clinic at Al-Imamein Al-Kadhimein Medical City for the period from October 2016 to August 2017.

### Study population

The study enrolled 44 female patients who were discovered to have Breast Imaging Reporting and Data System (BI-RADS) Category 5 breast mass for whom complete B-mode and elastographic ultrasound examination of the breast was performed, and a detailed histopathological report was obtained after excisional biopsy or mastectomy that proved the presence of ductal type breast carcinoma.

### Exclusion criteria

1. Previous surgical intervention and/or palliative therapy.
2. Histopathology finding of non-ductal carcinoma.
3. Women with previous breast or chest wall irradiation.
4. Women with breast masses with other BI-RADS Categories.

### Methods

#### *Data collection*

After full history and clinical examination, the eligible women were referred to B-mode US and elastography. The data were collected and filling a prepared questionnaire designed by the supervisor.

#### *US examination*

**Machine:** GE Voluson E6 ultrasound equipped with elastography software using high frequency linear array transducer (11L-D).

**Procedure:** B-mode US and elastography were performed at the same session. The patient lied in supine position with arm raised and palm placed beneath the head. The breast under assessment was exposed. Initially B-mode US was conducted, the mass was first localized and its maximum dimension measured, it was assessed for the presence of the following suspicious US features (microlobulation, spiculation, posterior acoustic shadowing, echogenic halo. After which, the elastographic examination was performed and the patient was instructed to maintain shallow respiration to prevent the counter strain caused by significant motion of the anterior chest wall. The field of view (FOV) box was set to include all the borders of the breast mass under examination as well as a portion of adjacent subcutaneous fat and some surrounding normal breast tissue. This procedure is the standard procedure applied in this setting according to BI-RADS [9].

The elastographic examination was performed by very gentle initial pressure with the transducer perpendicular to the skin surface. After obtaining adequate images according to green task bar, the largest lesion size was estimated, the elasticity score of the mass was estimated and the strain ration (SR) was obtained. For the size and SR, three measurements were taken and the average value reported. The SR was measured through

placing two small region of interest (ROI) circles of approximately comparable sizes: The first circle (Ref) placed within the fatty tissue at the same depth as the tumor if possible or placed within the adjacent subcutaneous fat if no fatty tissue was present at the same depth of the mass in the elastography FOV and the second circle (ROI) was put over the stiffest portion of the lesion as determined by color encoding. The SR from those two ROI circles was calculated automatically and displayed at the left lower corner of the monitor.

***Follow up***

After performing the B-mode US and elastography, the patients were followed up to receive their histopathology reports to correlate the sonographic and elastographic findings with the tumor grade the presence of the in-situ component and the lymphovascular invasion (LVI).

**Ethical considerations**

1. Approval was obtained from the Institutional Review Board, College of Medicine, Al- Nahrain University provided to the Journal Committee.
2. Verbal consent was taken from the patients to participate in the study.

**Statistical analysis**

The data of patients were analyzed by application of Microsoft excel program and Statistical Package for Social Sciences (SPSS) version 23. Outcomes of analysis were arranged in scales variables (means and standard deviation) and in categorical variables. Fishers' exact test was used for comparison between categorical data. One way ANOVA analysis was used to compare between more than two means. The level of significance (p value) was set as ≤ 0.05.

## Results

This study included 44 women with ductal breast carcinoma. Histopathology revealed that 6.8% (N=3) of women had grade I, 81.8% (N=36) of women had grade II and 11.4% (N=5) of women had grade III breast carcinoma. Mean age of studied women was 49.6±13.4 years, 6.8% (N=3) of them were <30 years age, and 20.5% (N=9) of them were above 60 years as shown in table 1.

**Table 1. Age groups and breast cancer grades in the study population**

| Variable | | No. | % |
|---|---|---|---|
| Age groups | <30 years | 3 | 6.8 |
| | 30-59 years | 32 | 72.5 |
| | ≥60 years | 9 | 20.5 |
| Grades of tumor | Grade I | 3 | 6.8 |
| | Grade II | 36 | 81.8 |
| | Grade III | 5 | 11.4 |
| Total | | 44 | 100.0 |

The breast tumor size of women with ductal breast carcinoma as measured by B-mode US was 25.3±8.9 mm; ranged between 8-52 mm. All the tumors exhibited a hypoechoic texture. The other most common gray scale US findings were irregular outlines 90.9% (N=40) followed by speculated margins (84.1%, N=37), post shadowing (70.5%, N=31), microlobulated (50%, N=22) and echogenic halo (34.1%, N=15). 2.3% (N=1) of women had size ratio of less than 1, 25% (N=11) had size ratio 1 and 72.7% (N=32) of them had size ratio of more than 1. The mean elasticity SR of women with breast carcinoma was 5.5±2.2; ranged between 3.5-

16.5. The elasticity SR mean±SD (5.5±2.2) and range (3.5-16.5), elasticity score of women with ductal breast carcinoma was score 3 among 2.3% (N=1) of women, score 4 among 25% (N=11) of them and score 5 among 72.7% (N=32) of women (Tables 2 and 3).

**Table 2. Elastography characteristics of breast tumor**

| Elasticity characteristics | No. | % |
|---|---|---|
| <1 | 1 | 2.3 |
| 1 | 11 | 25.0 |
| >1 | 32 | 72.7 |
| Total | 44 | 100.0 |

**Table 3. Elastography score of breast tumor**

| Elasticity score | No. | % |
|---|---|---|
| 3 | 1 | 2.3 |
| 4 | 11 | 25.0 |
| 5 | 32 | 72.7 |
| Total | 44 | 100.0 |

A significant association was observed between increased elasticity score and breast tumor grade III (p=0.02). No significant relationships were observed between each of LVI variable. Significantly higher mean of elastography SR was found among women with breast tumor grade III (p<0.001) (Table 4).

**Table 4. Elastography strain ratio according to grading of breast tumor**

| Variable | Grade I Mean±SD | Grade II Mean±SD | Grade III Mean±SD | P value* |
|---|---|---|---|---|
| Strain ratio | 3.8±0.2 | 5.0±0.8 | 10.2±4.1 | <0.001 |

*One way ANOVA test

## Discussion

During the last decade, the role of strain elastography in differentiating benign form malignant breast masses has been studied extensively, however; little has been said about its potential value in prediction of breast tumor grade [9]. In this study, the correlation of various strain elastographic parameters as well as some B-mode features with the grade of breast ductal carcinoma has been assessed.

The most frequently recorded B-mode US findings in breast ductal carcinomas in this study were spiculation, posterior acoustic shadowing, microlobulation and echogenic halo. These findings are in agreement with the results of a previous study conducted in Iraq, which stated that the main US findings of ductal breast carcinoma among a sample of women in Hilla city were irregularity, posterior acoustic shadowing and spiculated margins [10]. The spiculated margins detected by B-mode US

in the current study was significantly correlated with grade III breast carcinoma. A study done by Wojcinski et al. [11] in Germany reported that US criteria of breast tumors are dependent on biological and clinical profile of women (like presence of other risk factors, comorbidities, family history, etc.. and this might be helpful in grading breast cancer, however, they showed that grade III tumors were more likely to exhibit microlobulation and posterior acoustic enhancement. Likewise, a previous Canadian study [12] documented that grade III invasive ductal carcinomas of breast are more likely to show microlobulation and posterior acoustic enhancement, nevertheless, the findings in the current study might be attributed to the small number of grade III carcinomas (n=5).

Regarding elastography findings; a significant correlation of elasticity score and size ratio of ductal carcinoma with tumor grade (p=0.02) has been observed in this study. This finding is in agreement with results of Grajo et al. [4] study in USA, which studied the relation between tumor histological grade and E/B size ratio as an elastographic marker of tumor stiffness and found significant correlation between the two.

Current study found that the mean SR was significantly associated with the grade of ductal breast carcinoma (p<0.001). A study by Kim et al. [13] reviewed the records of 284 women found that the mean SR was significantly higher among women with positive LN status, unlike the current results, they did not report significant correlation between SR and histological grading of breast carcinoma. The larger sample size and the larger tumor size range (2-90 mm) in Kim et al. study could also have influenced the difference between the two results.

Several studies in the recent literature have evaluated the role of shear wave elastography in predicting breast tumor prognostic factors including histological grading. A study done by Evans et al. [14] in UK showed significant correlation between the mean stiffness and the histological grade of breast carcinoma.

Although the latter two studies used shear wave elastography (SWE) rather than strain elastography, the results showed that the diagnostic performance of strain elastography is not significantly different from that of SWE.

In regard to histopathological findings; the results of histopathology in this study showed that most (81.8%) of ductal breast carcinomas were grade II. This proportion of grade II ductal carcinoma is slightly higher than the results of a previous study in northern Iraq [15] in which, grade II carcinomas represented 55.5%. This difference might be attributed to the fact that in the aforementioned study all histological types of breast carcinomas were included unlike in this study which enrolled only ductal type of breast carcinoma.

Regarding LVI, several previous studies showed that it is correlated with the prognosis. A study in China by Wang et al [16] revealed that LVI among women with ER positive ductal breast carcinoma is an independent predictor for poor prognosis, and stiffness of elastography is great predictor for LVI in invasive ductal breast carcinoma. Another study in South Korea [17] revealed that lymphangiogenesis among women with ductal breast carcinoma is significantly correlated to breast tumor stiffness and histological grade.

The findings of the present study have many clinical impacts as it showed that elastography can be used not only for diagnosing breast carcinomas but also in predicting its grading and by such predicting the prognosis and biological behavior. Furthermore, implementing both elastography and B-mode US routinely for breast tumor radiological evaluation will be helpful in guiding the clinical work-up and will provide additional support in diagnosing and predicting grading of breast carcinomas preoperatively.

In conclusions, all three elastographic parameters implicated in the study have strong association with the grade of the tumor. Elastography is a helpful non-invasive tool that has the potential for predicting breast ductal carcinoma grade and by such predicting the prognosis.

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## Author contribution

Dr. Said: study design and data collection. Dr. Sheet: writing and editing. Dr. Nuaman: data analysis.

## Conflict of interest

None.

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# Serum Lipoprotein Ratios as Markers for Insulin Resistance Among Non-Diabetic Acute Coronary Syndrome Patients with Impaired Fasting Glucose

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## Abstract

**Background** Insulin resistance is a major risk factor in the development of cardiovascular diseases and type 2 diabetes mellitus. Some studies concluded that serum lipoproteins levels and hence lipoprotein ratios were altered in patients with insulin resistance.

**Objective** To identify the possibility of using lipoprotein ratios as markers for insulin resistance.

**Methods** A cross sectional study conducted in Baghdad (Medical City) and in Maysan (Al Shaheed Al Sadir Teaching Hospital) from February to December 2020. Eighty-three male and 51 female in Coronary Care Unit and Internal Medicine Wards patients were selected in the study group.

**Results** Lipoprotein ratios were significantly higher in individuals with homeostatic model assessment for insulin resistance (HOMA-IR) ≥2.5 as compared to subjects with HOMA-IR <2.5. There was a statistically significant association between lipoprotein ratios and insulin resistance when HOMA-IR ≥2.5 (P less than 0.05). Fasting insulin correlated significantly with lipoprotein ratios.

**Conclusion** Serum lipoprotein ratios and the best one is triglyceride/high density lipoprotein cholesterol could be used as markers for insulin resistance.

**Keywords** Lipoprotein ratios, insulin resistance, diabetes

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**List of abbreviations:** HDL-C = High-density lipoprotein cholesterol, HOMA-IR = Homeostatic model assessment for insulin resistance, LDL-C = Low-density lipoprotein cholesterol, TC = Total cholesterol, TG = Triglyceride

## Introduction

Insulin resistance is a decrease in the biological response to the stimulation of insulin hormone in target tissues, which are mainly liver, muscle, and adipose tissue [(1)]. This pathological process usually impairs the glucose disposal (Insulin-Stimulated Glucose Disposal), which results in a compensatory elevation in the production of beta cell of insulin and increase in serum insulin [(2)]. Metabolic syndrome is a group of disorders that occurs at the same time, increasing the risk of heart diseases, stroke and type 2 diabetes mellitus [(3)].

### Insulin resistance and dyslipidemia

Diabetic dyslipidemia is characterized by the lipid triad: high plasma triglycerides (TG), low high-density lipoprotein cholesterol (HDL), and high small dense low-density lipoproteins (sd LDL), in addition to excessive postprandial lipidemia [(4)]. Obesity is a global widespread epidemic and closely associated with the progression of coronary artery diseases and

type 2 diabetes mellitus. Obesity has a great effect on lipoprotein profile and many systemic, endothelial disorders and vascular inflammation. Abnormal level of lipids and Apo lipoproteins may cause disruption in the production, catabolism and conversion, of lipoprotein particles [5,6].

The objective of this study is to identify the possibility of using lipoprotein ratios as markers for insulin resistance.

## Methods

A cross sectional study conducted in Baghdad (Medical City) and in Maysan (Al Shaheed Al Sadir Teaching Hospital) from February to December 2020. All of the subjects were non-diabetic, impaired fasting glucose with acute coronary syndrome and the patients selected by convenient sampling (all patients presented during study visit and met the inclusion criteria). Lipid profile, fasting blood glucose (FBS), fasting insulin and HbA1c were measured for each participant. Immediate measurements of FBG, TG, HDL-C, Total cholesterol (TC), and LDL-C. The assay applied by automated method by using Abbott Architect C4000. Lipoprotein ratios: TG/HDL-C, TC/HDL-C, and LDL-C/HDL-C. Homeostatic model assessment for insulin resistance (HOMA-IR) was calculated by multiplying fasting insulin (µIU/mL) by FBG (mg/dL) divided by 405 [7].

## Results

Eighty-three male and 51 female in Coronary Care Unit and Internal Medicine Wards patients were selected in the study group, from them, 17 patients less than 40 years, 45 patients between 40 to 60 years and the remaining above age of 60 with high HOMA-IR among males, and age group more than 60 years (Table 1).

**Table 1. Distribution of the study group by socio-demographic characteristics (age and gender)**

| Characteristics | | HOMA-IR ≥2.5 (n=53) n (%) | HOMA-IR <2.5 (n=81) n (%) | Total | P value |
|---|---|---|---|---|---|
| Age groups (yr) | <40 | 6 (11) | 11 (14) | 17 | <0.001 |
| | 40-60 years | 18 (34) | 27 (33) | 45 | |
| | >60 | 29 (55) | 43 (53) | 72 | |
| Gender | Male | 33 (62) | 50 (62) | 83 | 0.950 |
| | Female | 20 (38) | 31 (38) | 51 | |

* P ≤0.05 is significant

Regarding biochemical parameters; the mean±SD of fasting glucose of study group was 107.7±6.05 mg/dl. But fasting insulin level in insulin resistance group (HOMA-IR ≥2.5) was significantly high (12.0±1.80) µIU/mL. All types of cholesterol and HbA1c were significantly high except the HDL-C, which it was significantly low (Table 2).

There was a statistically significant association between lipoprotein ratios and insulin resistance. The ratios were higher among insulin resistant group than non-insulin resistant patients. P value for all was <0.05. (Table 3).

**Table 2. Distribution of study group by biochemical parameters**

| Biochemical parameters | HOMA-IR ≥2.5 (n= 53) (Mean ± SD) | HOMA <2.5 (n= 53) (Mean ± SD) | Total (Mean ± SD) | P value |
|---|---|---|---|---|
| Fasting glucose (mg/dL) | 107.6 ± 6.03 | 107.7 ± 6.09 | 107.7 ±6.05 | 0.885 |
| Fasting insulin (µIU/mL) | 12.0 ± 1.80 | 7.7 ± 1.14 | 9.4 ±2.56 | <0.001 |
| HbA1c % | 6.1 ± 0.26 | 5.7 ± 0.35 | 5.8 ± 0.35 | <0.001 |
| Total cholesterol (mg/dL) | 192.6 ± 26.74 | 166.9 ± 20.44 | 177.1 ±26.28 | <0.001 |
| LDL-C (mg/dL) | 119.6 ± 25.13 | 109.1 ± 20.75 | 113.3 ±23.07 | 0.010 |
| HDL-C (mg/dL) | 46.0 ± 6.38 | 51.8 ± 3.43 | 49.5 ±5.58 | <0.001 |
| Non-HDL-C (mg/dL) | 146.6 ± 28.02 | 115.0 ± 19.65 | 127.5 ±27.92 | 0.001 |
| TG (mg/dL) | 158.5 ± 19.25 | 119.8 ± 26.59 | 135.1 ±30.51 | <0.001 |

* P ≤0.05 is significant

**Table 3. Distribution of study group by lipoprotein ratios and HOMA-IR**

| Parameter | HOMA ≥2.5 (n=53) Mean±SD | HOMA <2.5 (n=81) Mean±SD | P value |
|---|---|---|---|
| T. Cholesterol/ HDL-C | 4.2±0.86 | 3.2±0.39 | <0.001 |
| TG/HDL-C | 2.5±0.67 | 2.1±0.45 | <0.001 |
| LDL/HDL-C | 3.5±0.64 | 2.3±0.53 | <0.001 |
| Non-HDL-C/ HDL-C | 3.2±0.86 | 2.2±0.39 | <0.001 |

* P ≤0.05 is significant

To determine the efficiency of TC/HDL-C, LDL/HDL-C, TG/HDL-C and Non-HDL-C/HDL-C as markers of insulin resistance in non-Diabetic acute coronary syndrome patients. ROC curve was calculated as showed in figure 1.

The TC/HDL-C is sensitive and specific (77.4%, 81.5%), the cut-off value was ≥3.58, and area under curve (AUC) 0.888, while LDL/HDL-C has AUC 0.695 with cutoff value ≥2.41 and lower sensitivity and specificity 64.2%, 70.4% respectively. The TG/HDL-C is more sensitive and specific (90.6%, 80.2%), the cut-off value was ≥2.69 and AUC 0.910 as showed in table 4.

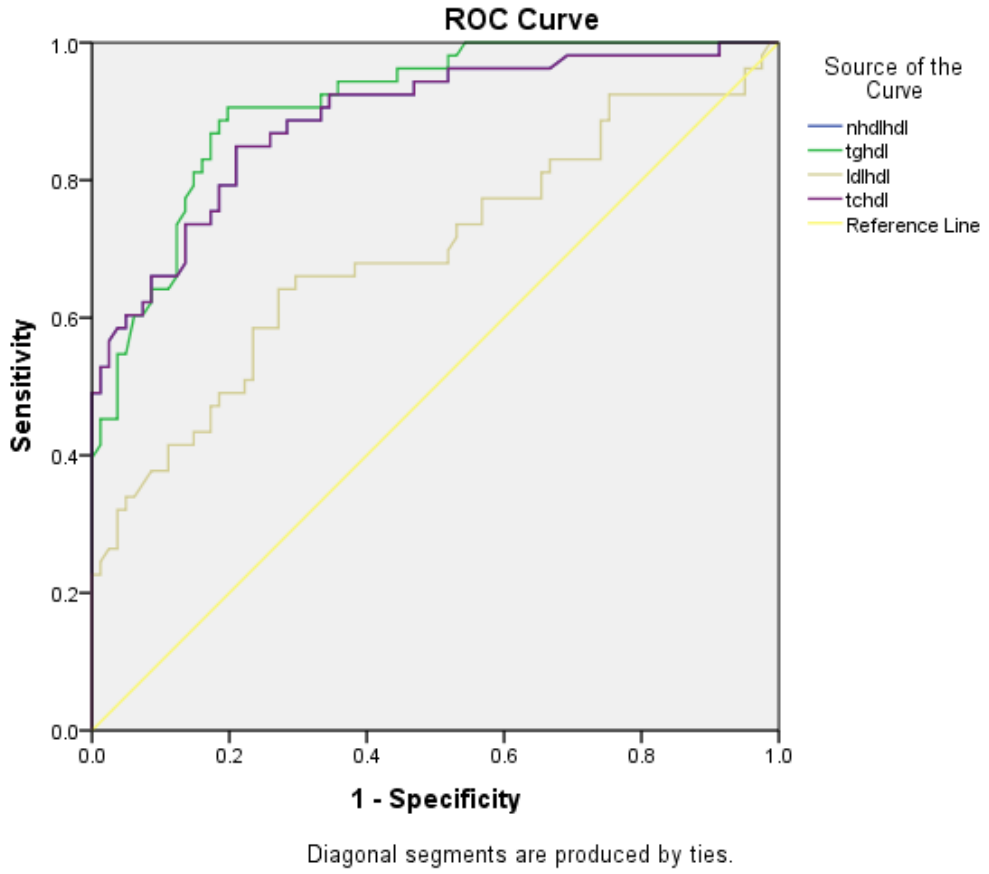


**Figure 1. ROC Curves for the detection of insulin resistance in non-diabetic acute coronary syndrome patients**

**Table 4. Cut-off points corresponding to the highest percentage of sensitivity and specificity calculated from roc curves for the detection of insulin resistance in non-diabetic acute coronary syndrome patients**

| Lipid indices | Cut-off point | Sensitivity | Specificity | P value | AUC | 95% CI |
|---|---|---|---|---|---|---|
| TC/HDL-C | 3.57 | 77.4 | 81.5 | <0.001 | 0.888 | 0.83-0.94 |
| LDL/HDL-C | 2.41 | 64.2 | 70.4 | <0.001 | 0.695 | 0.59-0.79 |
| TG/HDL-C | 2.69 | 90.6 | 80.2 | <0.001 | 0.910 | 0.86-0.95 |
| Non-HDL-C/ HDL-C | 2.57 | 77.4 | 81.5 | <0.001 | 0.888 | 0.83-0.94 |

* P ≤0.05 is significant

## Discussion

This study was conducted to determine the association between serum lipoprotein ratios and resistance to insulin in acute coronary syndrome patients with impaired fasting glucose.

In this study, there is significant association between age and insulin resistance, that defined as HOMA-IR ≥2.5 (p ≤0.05), this is different from results of another study from Rajappa et al. in 2014; who found that there is no significant association between them, while

the same study found that BMI, blood pressure and elevated waist:hip ratio were significantly associated with insulin resistance (p ≤0.01), the same as what found in this study [8].

In this study, about 62% of those with HOMA-IR ≥2.5 and 61.7% from other group were males, according to another study (Ray et al., 2015), 76.9% of insulin resistant group and 78.4% from other group were males [9].

In comparison with another study (Ormazabal et al., 2018) [1] who found that there was significant association between insulin resistance and CAD, this may be due to future development of CAD in follow up. The study of (Rajappa et al., 2014) who found that family and personal history of CAD are significantly associated with insulin resistance (p <0.01) [8].

The HOMA-IR is a good marker for insulin resistance in both patients and healthy individuals as it not required more than single measurement of fasting serum glucose and fasting serum insulin levels. Insulin resistance, in this study was identified as HOMA-IR ≥2.5, if less they considered sensitive to insulin without regard to the participant's gender. HOMA-IR is a commonly used measure to estimate insulin resistance. However, there were few studies focusing on the use of it in predicting the Framingham risk score (FRS). In one study, (Lu et al., 2020), participants who have elevated HOMA-IR levels were with higher capability to develop high FRS if compared with those had low levels of HOMA-IR. With adjustment for risk factors, such as sex, age class, BMI, tobacco smoking, the fasting sugar level, and blood pressure through multiple logistic regression statistical models, HOMA-IR also significantly associated with high FRS. These results may indicate that HOMA-IR is risk factor for high levels of FRS. They concluded that the power of HOMA-IR in identification of individuals with an elevated FRS. But, the lower specificity and low area under the curve, HOMA-IR using alone for prediction of high FRS was restricted [11].

In this study, there was significant difference in mean Total cholesterol, TG, HDL-C and LDL-C between the insulin resistant group and non-insulin resistant group, this is exactly similar to the results of International Scholarly Research Notices study (Rajappa et al, 2014) [8]. Fasting insulin level was significantly different between the two groups by the use of independent sample t test, this is similar to another study, (Johnson et al., 2010), who found that from individuals had fasting insulin level of >9 µIU/mL. In spite of presence of several methods for evaluation of insulin resistance, they are not cost effective and consume more time, the serum lipoprotein ratios (especially TG/HDL-C) can be used by physicians to classify patients into insulin resistant or sensitive [12]. The study of (Giannini et al., 2011) found that the relationship was statistically significant in white overweighted children but not significant in Hispanic individuals and African-American, the study found that TG/HDL-C ratio was significantly associated with the resistance to insulin in Korean individuals and the obese South-East Asian Immigrants [13].

In this study, serum lipoprotein ratios (TG/HDL-C, TC/HDL-C, LDL/HDL-C and Non-HDL/HDL-C) were higher among patients with insulin resistance than other group, and the difference is statistically significant. This is similar to the results of another study from India (Ray et al, 2015), in which, ninety participant who were known non-diabetic patients, with impaired fasting glucose level admitted to the hospital with ACS, lipoprotein ratios were higher in patients with HOMA-IR index ≥2.5 significantly, if compared to participants with lower index <2.5. The study of (Ray et al., 2015) also found that TG/HDL-C and TC/ HDL-C were significantly correlated with fasting insulin [9], this is similar to what found in this study, but In this study, fasting glucose and LDL-C were not correlated significantly with fasting insulin, this may be due to selection of impaired fasting glucose patients. According to another study (Kheirollahi et al., 2020) from Iran, on 305 individuals, the both groups, the insulin-resistant and the insulin-sensitive, diagnosed by the HOMA-IR were different in the levels of TG/HDL-C, TC/HDL-C ratios [14].

There is significant association between increased serum lipoprotein ratios and insulin resistance among the study group and are correlated significantly with fasting insulin. Serum lipoprotein ratios are cost effective and

not time-consuming method for prediction of insulin resistance.

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## Author contribution

Dr. Issa: Data collection, writing, and analysis. Dr. Rasheed: study deign, editing and revision.

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# Effect of TNF-Gold Nanoparticles Combination on Kidney and Liver Parameters of Female Mice

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## Abstract

**Background** Nanomedicine has emerged as a powerful platform for applying nanotechnology in the prevention and treating many diseases.

**Objective** To investigate the toxicity of designated anti-cancer drugs composed of gold nanoparticles (GNPs), tumor necrosis factor-alpha (TNF-α), and cysteine-Alanine-Leucine-Asparagine-Asparagine (CALNN) peptide on kidney and liver parameters, using animals' model.

**Methods** A Combination of TNF-α and CALNN peptide on the surface of GNPs were achieved depending on formation of dative covalent bond between molecules and pH of solutions, which leads to react with active groups of different molecules of nanoparticles, which characterized using ultraviolet visible spectroscopy. Moreover, their effects on kidney and liver blood variables had been examined. These blood values included measurement of the glutamic oxaloacetic transaminase, glutamic pyruvic transaminase and alkaline phosphatase, creatinine, and urea levels after intraperitoneal injection of an anti-cancer drug in female albino mice.

**Results** Overall, the results indicated that there was no significant change in the concentration of different biochemical values between treated and control groups.

**Conclusion** The results suggest that GNPs-TNF-α-CALNN combination have no harmful impact on kidney and liver organs of tested mice.

**Keywords** Nanomedicine, GNPs, TNF-α, CALNN peptide, drug delivery, cytotoxicity

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**List of abbreviations:** CALNN = Cysteine-Alanine-Leucine-Asparagine-Asparagine, GNP = Gold nanoparticles, GOT = Glutamic oxaloacetic transaminase, GPT = Glutamic pyruvic transaminase, TNF-α = Tumor necrosis factor-alpha, UV-VIS = Ultraviolet-visible

## Introduction

Nanomedicine is the usage of nanotechnology in different areas of medicine, present-day nanomedicine utilizes structured nanoparticles such as dendrimers, carbon fullerenes (Buckyball's), and nanoshells to target specific tissues and organs [(1)]. Nanoparticles present particular physical and chemical features that cannot be accomplished by other materials [(2)]. One interesting example of nanoparticles is colloidal gold that exhibits many applications in biology and medicine field [(1)]. Concerning other nanoparticle materials, gold nanoparticles (GNPs) have been used broadly in a different area of nanomedicine [(3)]. In the last two decades, highly advances in the field of nanoparticles-based therapeutically agents and in diagnostic tools for varying diseases like cancer, asthma, allergy, diabetes, infections, and a lot more were achieved [(4,5)]. These therapeutic agents could be more effective

when injected in an appropriate route of administration, lowering the toxicity of drugs, and increase the lifetime of the product, as a result, this will reduce health care costs [6]. Development of cancer nanotechnology enables and motivates the growing of securer yet more effective therapeutic agents and diagnostic methods for cancer therapy [7,8]. One of the important issues in nanoparticles-based studies is to achieve an efficient drug delivery system targeting to tumors as well monitoring the drug bioavailability throughout the body and accumulation within tumors [9,10]. GNPs have special characteristics over other types of nanoparticles that made them dedicated to using in different aspect of medicine and cancer research due to their size, shape and surface area, which can be easily customized, besides several lines of evidence have investigated the biocompatibility of GNPs and the minimal effect of toxicity [3,11].

Recently, CALNN peptide had been used in the treatment of different types of diseases, it is a pentapeptide composed of 5 amino acids, include Cys-Ala-Leu-Asn-Asn (CALNN) [12]. The use of therapeutic peptides over proteins relying on many characteristics including the size of a peptide, and the ability to penetrate the cell membranes. Besides peptides have increased activity, specificity and affinity; and biological and chemical diversity of therapeutic the agents [13]. Several lines of evidence emphasized the use of tumor necrosis factor-alpha (TNF-α) as anti-cancer therapy since it can affect both the cells and vasculature of the tumor and result in a reduction of the tumor volume [14,15]. However, dose-limiting toxicity is considered as the main problem for the systemic administration of TNF-α [16,17]. Therefore, there is a need for selective tumor delivery of TNF-α to reduce systemic toxicity.

The objective of this research is to determine whether the designated nanoparticles delivering system have any side effect or toxicity on the function of the kidney and liver of female albino mice after repeated intraperitoneal injections.

## Methods

Characterization of the combination of GNPs, TNF-α (obtained from Sigma Aldrich, USA), and CALNN peptide (obtained from the University of Ioannina, Greece) by using ultraviolet-visible (UV-VIS) spectrum analysis.

### Animals

Female healthy mice (8-10) weeks old, (15-25 g) weight housed that has been brought from Iraqi Center for Drug Monitoring, Baghdad, and kept in Iraqi Center for Cancer and Medical Genetic Research (ICCMGR) animal house, with controlled conditions of temperature (23±5°C) and relevant humidity. Animals were kept in partitioned cages and supplied with wood dust and tested for any accidental infection before starting the experiment [18].

The animals were distributed randomly into 3 treatment groups besides one control group, each group consisting of 3 mice. The concentration of compounds used in the treatment as followed 500 µg/Kg for GNPs and CALNN and 0.5 µg/Kg for TNF-α, the compounds were injected intraperitoneally every 3 days for 1, 2, 3 and 4 weeks and animals were sacrificed at the end of the experimental period as follows first, second, third and fourth weeks, the weight of mice was measured by using digit balance before each drug administration, the activity of mice also monitored during the experimental period. The first group was administrated with GNPs only, whereas GNPs-TNF-α was delivered intraperitoneally, as for the third group the animals were injected with a combination of GNPs-TNF-α-CALNN. For biochemical testing, the blood samples were collected using heart puncture, and then blood samples were centrifuged for about 10 minutes at 4000 rpm, serum aspirated in clean tubes, and kept at -20°C for later use.

### Statistical analysis

Graph Pad Prism 7.0 software was done to analyses the results by using the unpaired T-test method, P-value of $< 0.05$ considered to be significant [19].

## Results

### Characterization

The combination of GNPs-TNF-α and GNPs-TNF-α-CALNN was confirmed by UV-VIS spectroscopy as showed in figure (1). The top peak of GNPs could be noticed at 520 nm, whereas for TNF-α that attached to GNPs, the top band was shifted and appear very clear bands. However, when GNPs-TNF-α binding to CALNN peptide the top band would be shifted more to be at 680 nm.

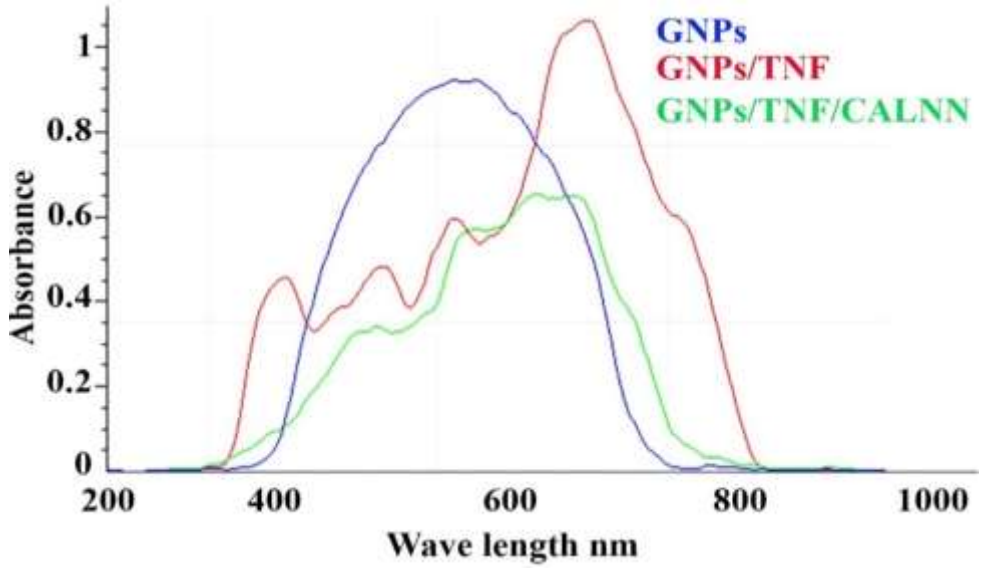


**Figure 1. Ultraviolet-visible spectroscopy of GNPs, TNF-α loaded on GNPs and GNPs-TNF-α-CALNN**

### Effect of drug delivery system on the weight of experimental animals

Evaluation of the body weight of experimental animals throughout the experimental period after intraperitoneal injection with GNPs, GNPs-TNF-α, GNPs-TNF-α-CALNN compounds was achieved by using electric balance, the body weight value was recorded for each group and results of body weight appeared to be unaffected by different types of treatment (Figure 2).

### Effect of drug delivery system on biochemical parameters of kidney and liver of experimental animals

To investigate the effect of these compounds on kidney and liver function, urea, creatinine, glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT) and alkaline phosphatase concentration were measured and compared the data with a normal value of control groups [20,21]. The results exhibited no significant differences between treated and control groups as set out in figures 3 and 4.

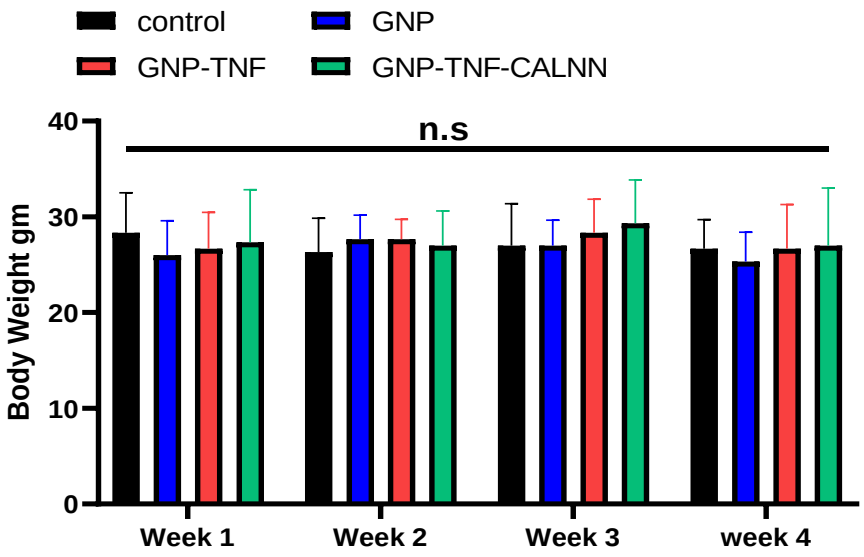


**Figure 2. Effect of GNPs, GNPs-TNF-α and GNPs-TNF-α-CALNN in animal body weight**

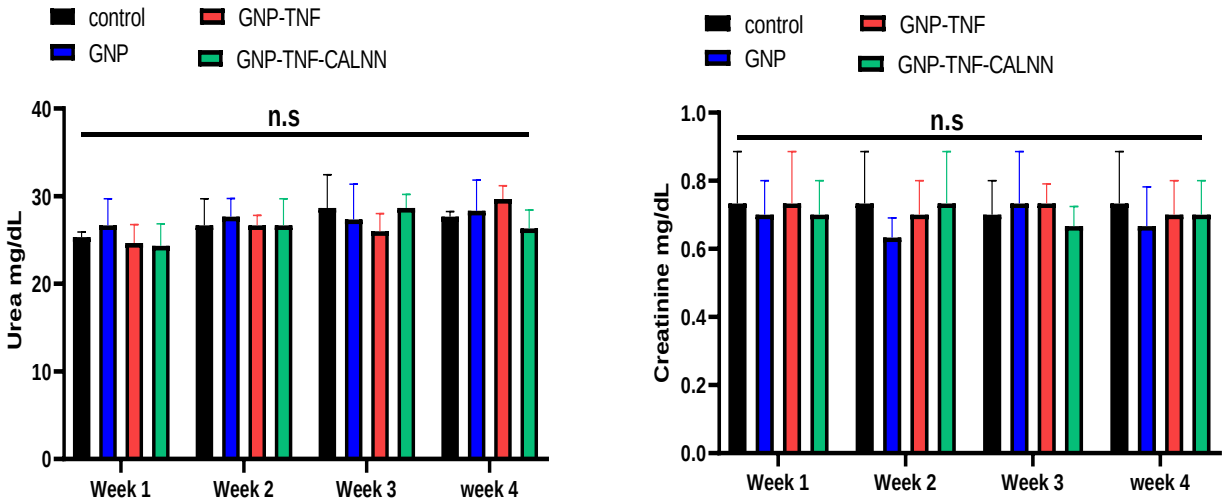


**Figure 3. Effect of GNPs, GNPs-TNF-α, and GNPs-TNF-α-CALNN treatment group in urea and creatinine concentration**

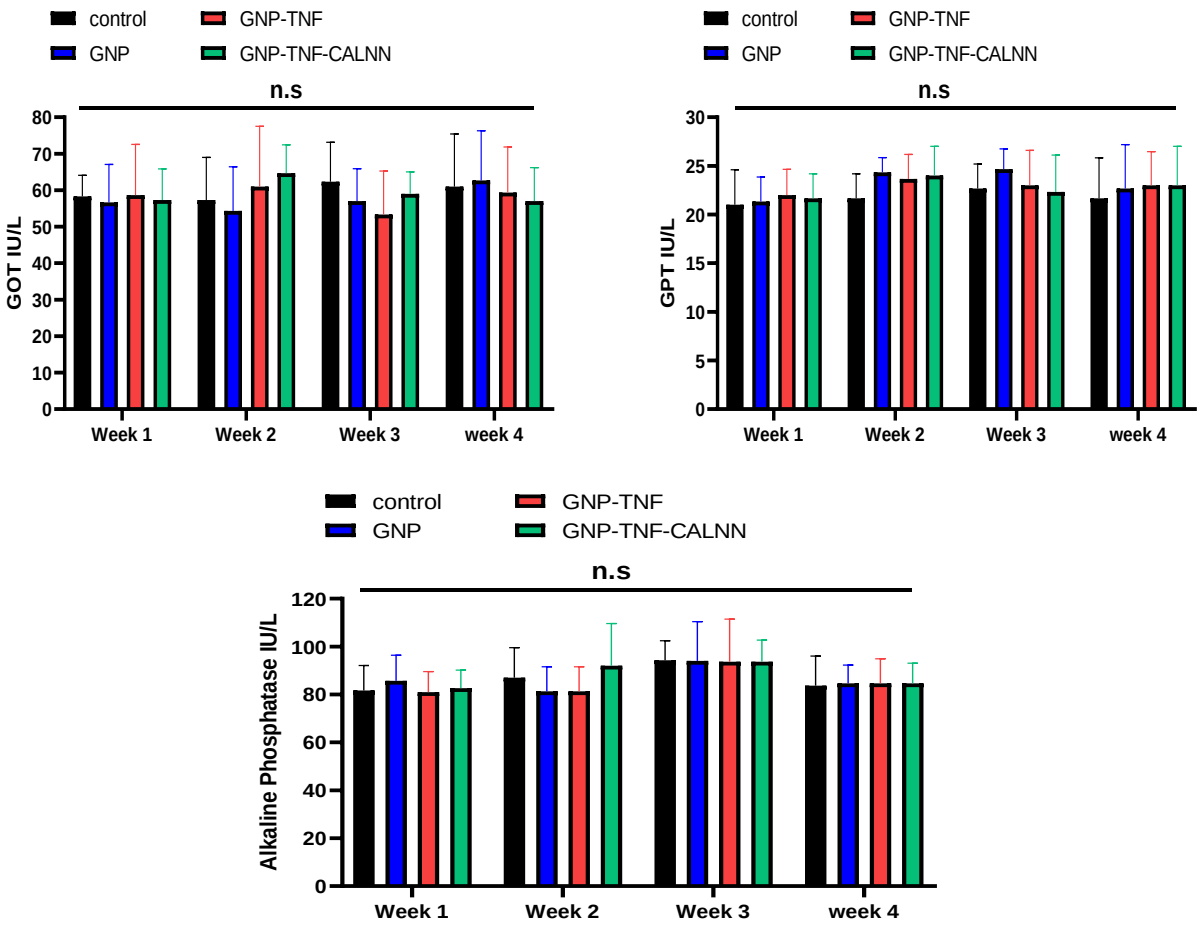


**Figure 4. Effect of GNPs, GNPs-TNF-α, and GNPs-TNF-α-CALNN treatment group in GOT, GPT, and Alkaline Phosphatase**

## Discussion

Data suggested that healthy animal does not exhibit any cytotoxic effect after received the treatment drugs intraperitoneally, also their biological parameters were normal. The body weight for three different groups showed non-significant differences in compared to control group. Also, the present results corroborate the idea of Zang et al. [22], who suggested that particles used in his experiment were absorbed by both hepatocytes and Kupffer cells and secreted in two different periods, as for hepatocytes that engulf particles within first 2-6 hours and secreted through hepatobiliary pathway during the first 24 hours, while Kupffer cells secreted the ingested particles lately (after 24 hours) by an unknown mechanism. These results seem to be consistence with Renaud et al. [23] who suggested two phases explained as an early phase and late phase of gold particles excreted from the liver, the clearance mechanism lay on the earlier circulation of gold nanoparticles with parenchymal and nonparenchymal cells and does not exhibit a cytotoxic effect on the liver. A comparison of the findings with those of other studies confirms that GNPs loaded with TNF-α and CALNN peptide do not have any cytotoxic effect on the liver and kidney.

In conclusions, this study has argued the effect of designated drug delivery compounds on some of the essential biochemical parameters of the kidney and liver. The following conclusions can be drawn from the present study was injected GNP, GNP-TNF-α, and the combination of GNP- TNF-α-CALNN don't show a toxic effect on female albino mice after 1, 2, 3 and 4 weeks of intraperitoneal injections.

Together these results provide important insights into the fact that using these compounds together as a drug-delivering system does not affect kidney tissues and function and could be used as an anti-cancer drug system safely, without affecting normal tissues.

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## Author contribution
Dr. Abood: Performed the experiments and writing the manuscript. Dr. Kadhim: Supervised the study and made the final revision of the article. Dr. Jabir: Designed the study and carried out the data analysis.

## Conflict of interest
No potential conflict of interest was reported by the authors.

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# Investigation of The Prevalence of Secondary Bacterial Infection Associated with COVID-19 In Baghdad and Diyala Province

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## Abstract

**Background** Coronavirus disease 2019 (COVID-19) is an epidemic disease produced via the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) beta coronavirus, which affects the lower respiratory tract. Secondary bacterial infection (SBI) is a serious and public problem in patients hospitalized with COVID-19 and caused 50% of deaths. Type A and O blood groups are more susceptible to infection with SBIs.

**Objective** To examine the relationship between SBI and ABO blood group with COVID-19 hospitalized patients in Baghdad and Diyala province.

**Methods** Three hundred and forty-two patients with COVID-19 were collected from several sources (nasal swab, pharyngeal swab, sputum, blood, and urine) of patients of different ages for the period between September to November 2021. Real-time reverse-transcription polymerase chain reaction technique was used to diagnose COVID-19 in the patients as well as selective and differential media, biochemical tests BACT/ALERT system, and VITEK 2 compact system were used to diagnose the isolates of SBIs. The disk diffusion technique was used to assess the susceptibility test of all isolates. ABO group analysis was done for totally patients with COVID-19 under the study.

**Results** Antimicrobial sensitivity test showed all SBIs were highly strong resistant to antibiotics. Fifty-seven isolates of bacteria were diagnosed including *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Klebsiella pneumonia*e, and *Escherichia coli*. Group A and O showed a higher rate of acquired SBIs. Duration of infection with COVID-19 showed 61% for 10 days and 30% for one month while 9% in the patients infected with SBI for more than one month. The result appeared that SBIs infection at were very high rate in COVID-19 patients who had untreated antibiotics compared with the patient treated with antibiotics through the duration of infection.

**Conclusion** The study revealed that many COVID-19 patients were more susceptible to infection with SBIs, especially in the early days of infection as well as there was a correlation between ABO blood groups and SBIs of COVID-19 patients.

**Keywords** COVID-19; SBIs, AST, ABO blood group

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**List of abbreviations:** AST = Antibiotics susceptibility test, COVID-19 = Coronavirus disease 2019, MDR = Multiple drug resistance, RT-PCR = Real-time reverse-transcription polymerase chain reaction, SARS-CoV-2 = severe acute respiratory syndrome coronavirus 2, SBI = Secondary bacterial infection

## Introduction

A new strain of coronavirus triggered a pneumonia outbreak in Wuhan, Hubei Province, China, in December 2019. The new virus, now known as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has quickly spread in China and additional

parts of the world owing to its ability to successfully transmit among humans [1]. Corona virus disease 2019 (COVID-19) had been confirmed in over 12 million people worldwide as of July 15th, 2020, with over 570 thousand deaths [2]. Bacterial co-pathogens are frequently seen in viral respiratory tract illnesses like influenza, and they stay a major source of morbidity and mortality, needing prompt identification and antibacterial treatment [3]. Secondary bacterial infection (SBI) is a serious and common complication in patients hospitalized with COVID-19. It occurs at an estimated rate of 10-15% [4].

According to prior investigations, SBIs caused 50% of COVID-19 deaths; hence, patients with SBIs had a higher risk of death [5]. Because there are no randomized clinical trials on the use of empiric antibiotic medicines in COVID-19 patients, the present guidelines are based on the extrapolation of data from other viral pneumonia patients [6].

The ABO blood group might have a role in the immune pathogenesis of COVID-19 infection, according to increasing data [7]; A and B antigens are originating in the external the red blood cells and their presence or absence determines the ABO and Rh blood grouping of an individual.

The blood group antigens, glycolipids, and glycoproteins are hereditarily controlled and inherited in variable occurrences across human peoples [8]. However, studies are presently ongoing to identify biological signs that can forecast an individual's susceptibility to SARS-CoV-2; severity and COVID-19 clinical result have been related to serum levels of some laboratory factors [9]. The ABO blood group has been connected to diseases such as Influenza and Norovirus [10]. Now there is rising evidence that indicated the susceptibility to SARS-CoV-2 is linked to the ABO blood group of an individual. Also, conflicting evidence occurs on the relationship between the ABO blood group and the severity and the clinical result of COVID-19 disease [11].

The present study aimed to examine the relationship between SBI and ABO blood group with COVID-19 hospitalized patients in Baghdad and Diyala province.

## Methods

To investigate the prevalence of infectious bacteria associated with COVID-19 patients, 342 specimens were collected from patients of various ages and sources, including nasal swabs, pharyngeal swabs, sputum, blood, and urine, after confirming that they were infected with COVID-19 using real-time reverse-transcription polymerase chain reaction (RT-PCR) technique; these samples were collected during the period from September 2021 to November 2021 at Medical City, Al-Shifa Hospital, Imam Ali Hospital and Al-Imamein Al-Kadhimein Medical city – Baghdad as well as from the hospitals in Baquba City. Officials from the hospitals gave their consent. All participants and healthcare professionals were informed about the study's goal. The advantages are explored, and the patients have been informed. There are no hazards to their health as a result of the study.

Patient information was including age, gender, use of artificial respiration, duration of infection, and receiving antibiotics or not. All the specimens (324 were cultured on differential and selective media (MacConkey agar, Blood agar, Mannitol salt agar, Pseudomonas agar, and Eosin methylene blue), also for the isolation; used BACT/ALERT system to detect an early bacterial growth in a special blood culture bottle. Colony characteristics, microscopic examination, and biochemical tests (Catalase test, Oxidase test and IMViC) were used to diagnose the isolates, VITEK 2 compact system was used to confirm the identification of the isolates. The disk diffusion method on Mueller-Hinton agar was used to assess the susceptibility of all isolates to different types of antibiotics, as recommended by the Clinical and Laboratory Standards Institute [12]. Blood group (ABO) analysis was performed for all patients.

## Results

### Bacterial isolation and identification

This study uses traditional methods for identification of the bacteria, which have relied on determining the phenotype of the causative organism using bacteriological methods including selective media cultivation, colonial morphology, and microscopic characteristics. From the total 342 samples; 57 isolates were obtained from patients with COVID-19 and distributed between Gram-positive and Gram-negative bacteria as shown in table 1.

**Table 1. Isolation of Secondary bacterial infections according to sample sources**

| Types of Bacteria | Sources | | | | |
|---|---|---|---|---|---|
| | Nasal Swab N (%) | Pharyngeal swab N (%) | Sputum N (%) | Blood N (%) | Urine N (%) |
| *Staphylococcus aureus* | 11 (100) | 3 (15) | 0 (0.0) | 0 (0.0) | 2 (25) |
| *Pseudomonas aeruginosa* | 0 (0.0) | 0 (0.0) | 5 (33.3) | 1 (33.3) | 0 (0.0) |
| *Acinetobacter baumannii* | 0 (0.0) | 6 (30) | 3 (20) | 0 (0.0) | 0 (0.0) |
| *Klebsiella pneumoniae* | 0 (0.0) | 11 (55) | 7 (46.6) | 2 (66.6) | 0 (0.0) |
| *Escherichia coli* | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 6 (75) |
| Total | 11 | 20 | 15 | 3 | 8 |

### Antimicrobial susceptibility test

All bacterial species were evaluated against 12 antimicrobial compounds of each bacteria using the disk diffusion method. The results in the figures (1,2,3) were evaluated according to (12) standards and revealed that there were numerous bacterium isolates with multiple drug resistance (MDR) from distinct types of bacteria.

### Blood group associated with Secondary bacterial infection

Three hundred and forty-two patients with COVID-19, 57 patients were identified with SBIs. The current study showed that blood groups of COVID-19 patients associated with SBIs were graduated the risk of the infection at 42%, 35%, 20% and 3% for the O and A, B and AB respectively as appeared in figure 4.

### Relationship between SBIs in COVID-19 patients and antibiotics usage

The current results established that COVID-19 patients who were not given or treated with classical antibiotics were more likely to develop a secondary bacterial infection (SBIs), as the percentage of patients who were untreated with antibiotics was (70%) and the percentage of those treated was (30%) as showed in figure 5.

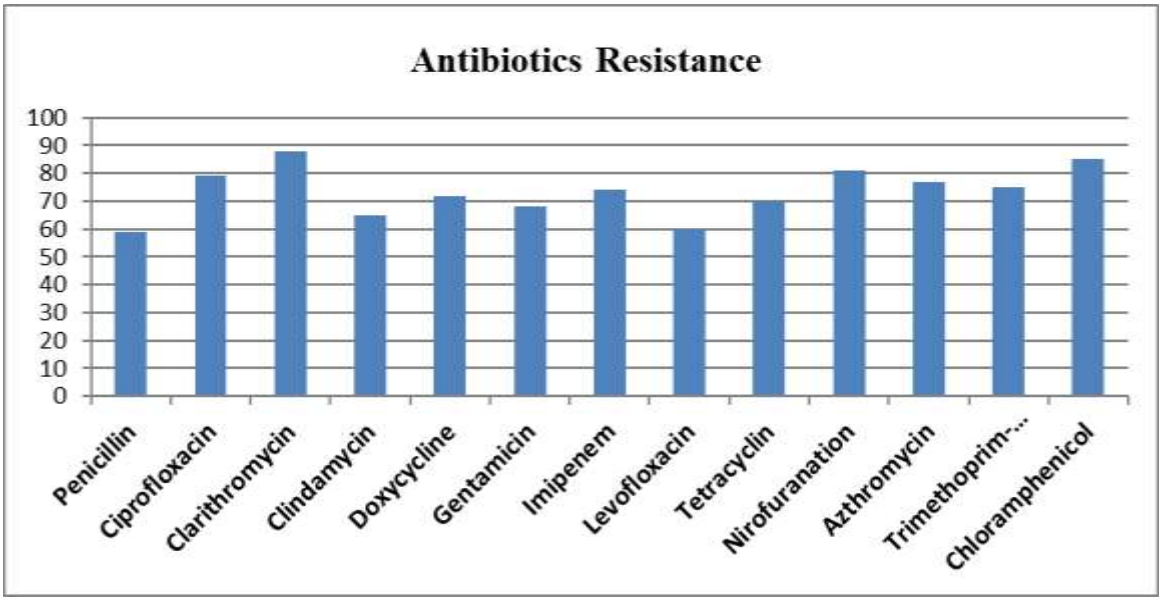


**Figure 1.** ***Staphylococcus aureus*** **resistance of antibiotics**

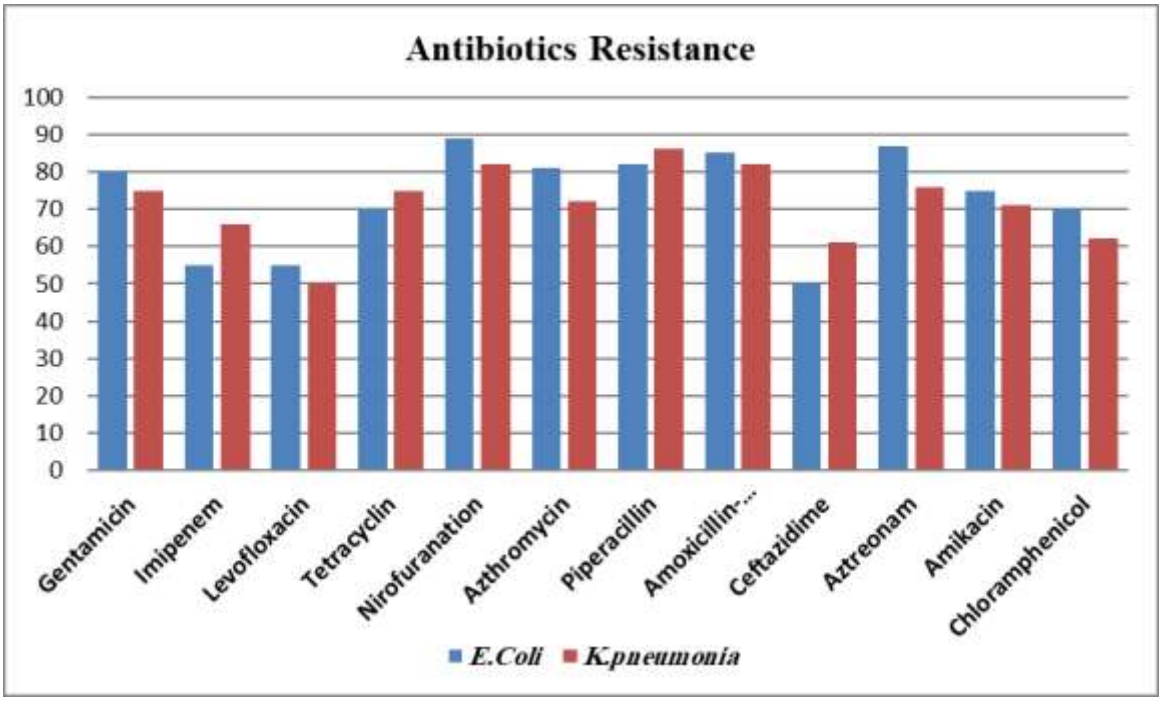


**Figure 2.** ***Escherichia coli*** **and** ***Klebsiella pneumoniae*** **resistance to antibiotics**

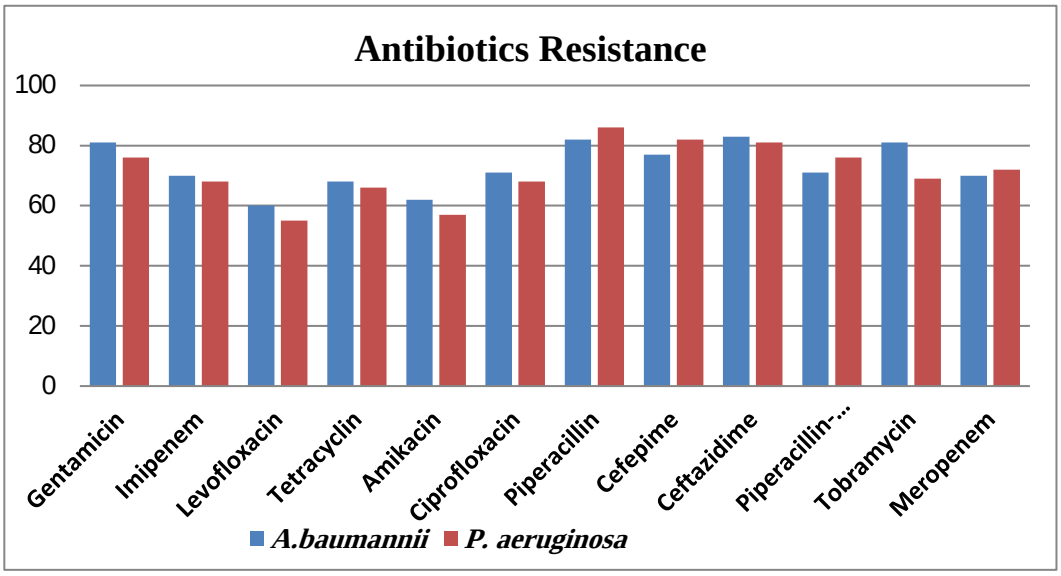


**Figure 3.** ***Acinetobacter baumannii and Pseudomonas aeruginosa*** **resistance to antibiotics**

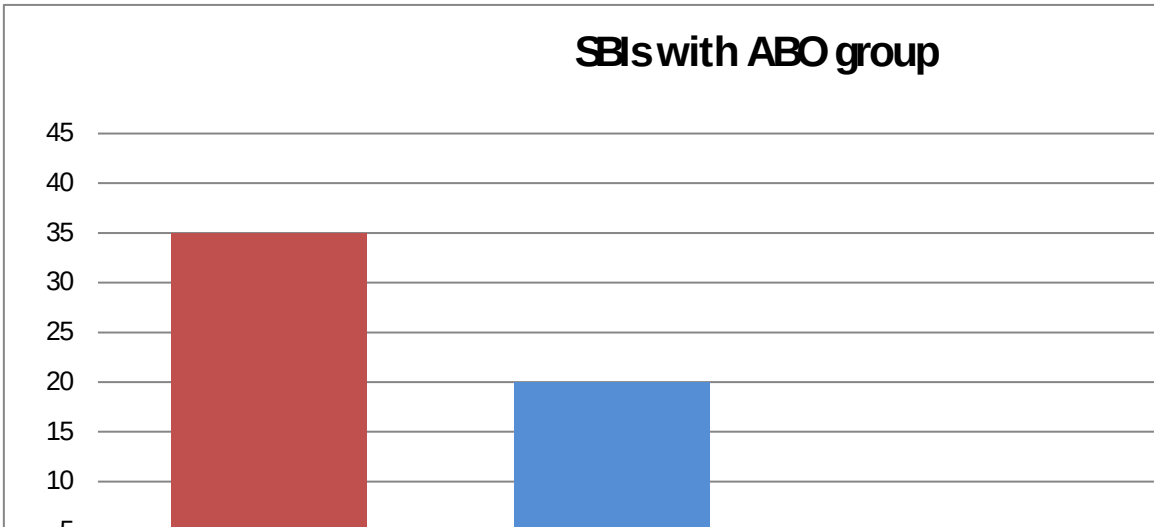


**Figure 4. The relationship between the secondary bacterial infection and ABO group**

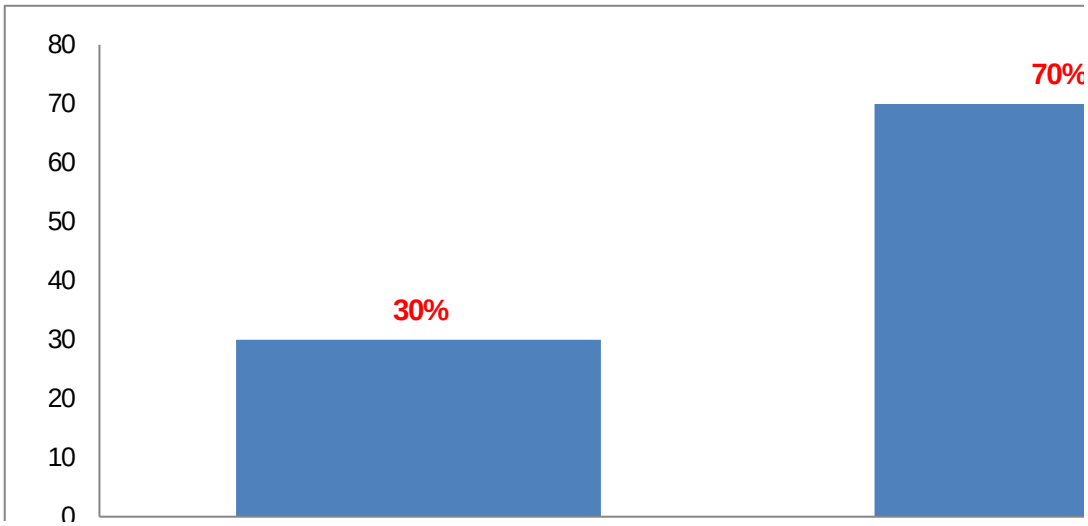


**Figure 5. The percentage of treated and untreated antibiotics in COVID-19 patients associated with secondary bacterial infections**

**Relationship of SBIs with a duration of COVID-19 patients**

The duration of infection with COVID-19 and SBIs included three parts, which included (<10 days, ١١-٣٠ days, and more than 1 month); the results shown in figure 6 showed that the high level of infection with SBIs was 61% during 10 days, followed by 30% in the period ١١-٣٠ days and 9% in more than 1-month, high levels of microorganisms will invade patients during the first month of infection with COVID-19 because of low immune system.

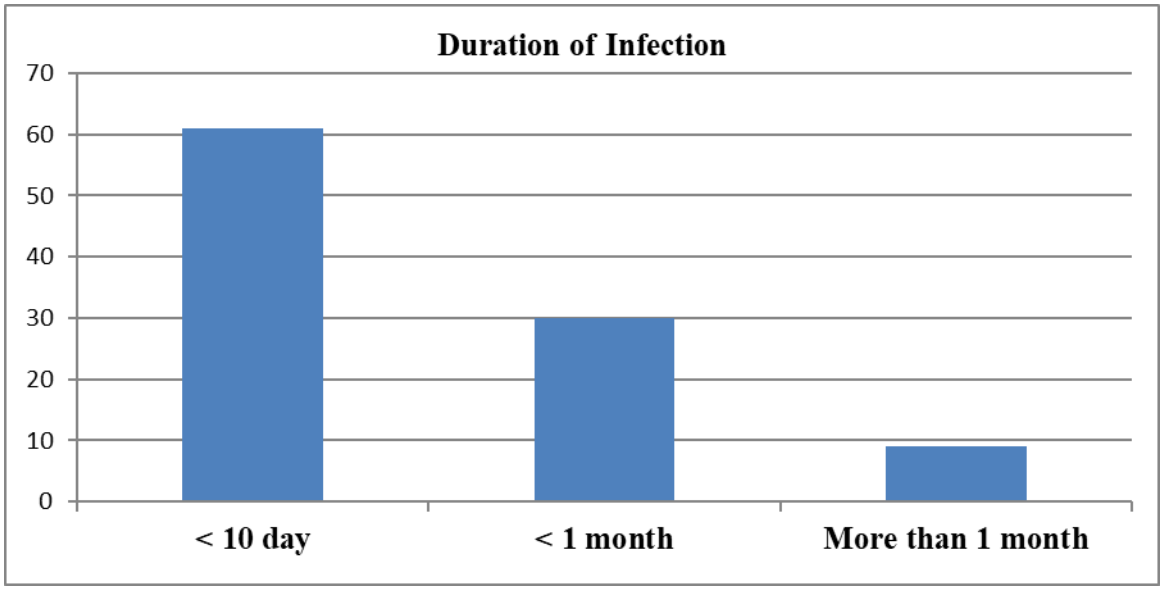


**Figure 6. The percentage of treated and untreated antibiotics in COVID-19 patients associated with secondary bacterial infections**

## Discussion

Gram-positive bacteria *Staphylococcus aureus*, (16 isolates) were isolated from nasal swabs, pharyngeal swabs, and urine whereas the percentage was 100%, 15% and 25% respectively. Instead, four types of Gram-negative bacteria; *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, and *Escherichia coli* were isolated and distributed as follows: 6 (33.3%,) isolates of *Pseudomonas aeruginosa* from sputum and blood, 9 isolates of *Acinetobacter baumannii* from pharyngeal swab and sputum were 6 (30%) and 3 ( 20%) respectively, as well as 20 isolates of *Klebsiella pneumoniae* isolated from the pharyngeal swab (55%), sputum (46.6%) and blood (66.6%) and 6 (75%) of *Escherichia coli* were isolated from urine samples only (Table 1).

The current results are in agreement with several studies as; Fu et al. [13] who showed most patients with COVID-19 developed a SBI that included *Acinetobacter baumannii*, *Klebsiella pneumonia*e, and *Staphylococcus aureus*, which isolated from blood, pharyngeal, and sputum. Multiple organisms were found in patients with COVID-19, according to Chen et al [1]. Drug-resistant microorganisms, such as *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli*, *Enterococcus*, *Mycoplasma pneumoniae* and *Chlamydia pneumoniae* have been linked to infections in COVID-19 patients [14-16]. SBIs can develop in COVID-19 patients, resulting in a significant fatality rate. The severity of the illness at the time of admission was linked to the occurrence of SBIs and the resistance levels of the principal isolated bacteria were usually high.

Fifty-seven isolates have been tested for sensitivity to antibiotics [12] and the current results shown in the figures (1,2,3) showed that isolates (SBIs) of *Staphylococcus aureus*, *Acinetobacter baumannii*, *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*, and were highly resistant to antibiotics, the current results agree with [17] when mentioned in his paper the high occurrence of carbapenem-resistant *Klebsiella pneumonia*e and *Acinetobacter baumannii* long-term nosocomial infection at the main campus hospital, particularly among Covid-19 patients. The bacteria *Acinetobacter baumannii* and *Klebsiella pneumoniae* were shown to be the most public causes of bacterial infection, accounting for 90.6% of all cases, and were closely linked to death [17]. The contaminated environment was proven to be a substantial cause of nosocomial infection [1]. The antimicrobial resistance rates of the principal identified bacteria are commonly high, implying that more precise antibacterial agent administration for SBIs in COVID-19 patients is required [18].

Behind COVID-19, SBIs had evolved into a secret threat. One of the most important factors in the successful treatment of COVID-19 is the use of an efficient antibiotic regimen. In severe COVID-19 patients with SBIs, a brief guide recommends empiric antibiotic treatment for all potential microorganisms [19].

Antibacterial drugs may be used more widely, which could lead to changes in etiology and antimicrobial resistance. SBIs in COVID-19 patients should be treated based on additional microbiological information [20]. In studies on the clinical features of COVID-19, certain occurrences of bacterial infections were recorded; however, there were no methodical studies on the origin of SBIs, and the total of positive cultures was minimal [21].

From figure (4), blood types, A and O were crucial parameters to consider when assessing the prognosis of COVID-19 patients with SBIs, these results agree with Muñiz-Diaz et al. [22] when showing the rate of blood groups, A, O, B and AB were (47%, 41%, 7% and 3%) respectively. The current results were similar to another results study done by Zheng et al. [23] looked at the clinical features of 134 COVID-19 cases in China and reported that the males were more prevalent than females, and the percentage of older patients with the underlying disease was comparatively high. Importantly, the researcher discovered in their study that the ABO group related to COVID-19 patients were A: 43.82%, B: 26.91%, O: 19.21%, and AB: 10.1 %.

According to the study carried out by Ngassaki-Yoka et al. [24], SBI has been linked to the ABO

polymorphism; the presence or lack of A/B antigens, as well as the presence or absence of anti-A/B antibodies, provide strong or weak defense barriers against infection. The ABO gene is found in many vertebrate species, and it has so benefited them. However, possessing both functional A and B genes in a species may not be necessary because anti-A/B antibodies may be lost over time. However, frequent A/B specificity gene conversions that result in amino acid changes or recombination with nonfunctional incomplete genes may have conferred resistance to microbial attacks [25].

ABO blood group antigens can be discovered in the human respiratory, digestive, and reproductive systems [26]. As well as ABO blood groups have been connected to the development and transmission of numerous diseases in the past, probably because blood group antigens act as virus receptors [27].

In Figure 5, as shown above, the high rate of untreated antibiotics for patients with COVID-19 who acquired SBI was (70%) while the patients who were treated with antibiotics were (30%), our results were close to a study carried out by Langford et al. [25] when studying the effect of antibiotics on COVID-19 patients and those who acquired SBI, noted most of the patients who treated antibiotics were a lower rate to infect SBIs. The number of COVID-19 patients with SBIs varies substantially, from 0 to 100% in those who died, as does antimicrobial use, which ranges from 20% to 100 % depending on the severity of the illness [28].

Antibiotics are useless in the treatment of COVID-19, but they are administered for a variety of reasons in patients with suspected or confirmed COVID-19. This includes the difficulty in excluding bacterial co-infection at the time of presentation, as well as the risk of bacterial secondary infection later in the disease [2]. Several strategies suggest the use of experiential antibiotics for severe COVID-19 patients [6] based on concerns about an increase in mortality in patients with bacterial superinfection during influenza pandemics. This premise, however, raises concerns about antibiotic misuse and the ensuing harm caused by bacterial resistance.

The current study compared the period of infection from the first days to more than ten days, and more than a month of patients with COVID-19 and SBI, the results shown in figure 6 revealed that the early days (<10 days) from infection with COVID-19 which were more susceptible to SBIs. The early stages of COVID-19 infection have severe consequences for individuals, especially those with weak immune systems, the elderly and those with chronic diseases [29]. Since it is a new virus, the correct medication protocol for it was not known at the beginning of the infection, so the patient is at risk of contracting it SBI. Respiratory failure or failure of many organs in the early days is the direct cause of death in COVID-19 patients and SBIs it has an important role in the infection [18].

Worry can also be influenced by financial means with fewer financial means, getting quality medical treatment and COVID-19 preventative tools like face masks and cleaning equipment can be problematic. Age and ethnicity are two demographic factors that may be linked to increased anxiety as a result of inequities in job insecurity [30].

In conclusion, SBI are a common source of morbidity and mortality in viral respiratory tract infections like COVID-19. Antimicrobial resistance rates of the most often discovered bacteria are generally high, emphasizing that SBIs in COVID-19 patients hospitalized require more specific antibacterial drug treatment. Individuals in groups A and O had a higher risk of SBIs, but those in groups AB and B had a lower susceptibility. SBIs were more common in COVID-19 individuals who did not get antibiotics and were in the early stages of infection (less than 10 days).

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## Author contribution

Both authors participated in concept and design, acquisition, analysis, interpretation of

data, statistical analysis, administrative, technical, and material support, and critical revision of the manuscript.

## Conflict of interest

None.

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# Adoption of Critical View of Safety versus Infundibular Technique in Laparoscopic Cholecystectomy: A comparative study

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## Abstract

**Background** Laparoscopic cholecystectomy (LC) is the most common elective surgery performed by a general surgeon. Although being a routine procedure, classical pitfalls as misperception of intraoperative anatomy is one of the leading causes of bile duct injuries (BDI). The critical view of safety (CVS) in LC can be a new safe technique for identification of anatomy to reduce such a risk.

**Objective** To assess the efficacy of CVS in LC compared with the traditional infundibular technique.

**Methods** This comparative study included 344 patients who suffered from symptomatic gall stones. Patients have been grouped into two groups: group A (172 patients) operated utilizing the traditional infundibular technique and group B (172 patients) by CVS technique. Preoperative patient assessment, operating time and intraoperative and postoperative events including hospital stay, were recorded. Those patients who were unfit and or with bleeding disorders were excluded from the study. Postoperatively, patients were assessed using clinical examination for the possible complications.

**Results** The main perioperative complications bleeding and bile leak were significantly reported in infundibular group 6.98% and 9.88% respectively compared to 0.58% and 1.74% in CVS group respectively. Postoperatively, both intra-abdominal collection and bile leak were significantly lower in CVS group (1.16% and 1.74%, respectively) than in infundibular group (7.58% and 6.98%, respectively). Furthermore, hospital stay was significantly less in CVS group (1.8±2.7 days) compared to infundibular group (3.14±2.8 days).

**Conclusion** Using the CVS is associated with shorter operative time, less frequent peri- and postoperative complications and shorter hospital stay compared with infundibular technique.

**Keywords** Laparoscopic cholecystectomy, critical view of safety (CVS)

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**List of abbreviations:** BDI = Bile duct injury, CVS = Critical view of safety, HC = hepatocytic, LC = Laparoscopic cholecystectomy

## Introduction

Laparoscopic cholecystectomy (LC) is one of the most commonly executed general surgical procedures worldwide. It is associated with an overall complication rate of nearly 10% with a higher risk of biliary injury (0.1-1.5%), when matched to the open method (0.1-0.2%) [(1)]. This complication, if persist, largely equipoises the benefit of the minimally invasive approach. Recent data suggest a decreasing trend in the bile duct injury (BDI), vascular-biliary injury (VBI) rate (0.32-0.52%)

without any significant change in the morbidity or mortality after LC [2].

A common source of biliary injury during LC is misidentification of structures in the hepatocytic (HC) triangle. Several procedures have been used to improve the identification of these structures. In the infundibular view error, cystic duct identification is established according to the appearance of the infundibulum-cystic duct junction as a funnel [3]. In certain conditions, this procedure can be misleading. When the cystic duct is merged with common hepatic duct (CHD) due to acute or chronic inflammation, when the cystic duct is too short or obliterated by a large stone impacted in the infundibulum, or when there is difficultly in uncovering the HC triangle due to insufficient retraction (e.g., due to fibrosis), the CBD may be misidentified as the cystic duct [4].

Many authors worldwide explored other techniques to overcome the problem of misidentification. Flum et al. used intraoperative cholangiography (IOC) and reported a significant decline in BDI [5]. Huang et al. adapted fundus-down LC and found it to be associated with lower complication rate and shorter postoperative hospital stay [6]. Al-Helfy et al. used intraoperative methylene blue in 98 Iraqi patients with symptomatic cholelithiasis and recorded a significant success in bile duct anatomical identification [7]. However, some cases of BDI/VBI (even accounting for very small percent) do occur in all these techniques.

The concept of the critical view of safety (CVS) was introduced in an attempt to decrease the misidentification injury [8]. The aim of the CVS is conclusive identification of the cystic duct and cystic artery (two targets) to avoid misidentification injury [9]. CVS is the final view that is achieved after a thorough dissection of the HC triangle to demarcate the cystic duct and the cystic artery before they are clipped and divided [3].

This study aimed to evaluate the efficiency of CVS in reducing the intraoperative complications compared with the traditional infundibular technique.

## Methods

This was a comparative study conducted on patients who attended Surgical Department at Al-Imamein Al-Kadhimein Medical City during the period from January 2019 to December 2021. Those patients were suffering from symptomatic gall stones confirmed by clinical examination and ultrasonography. Patients unfit for pneumoperitoneum due to cardiac or pulmonary causes, suspicion of gall bladder tumor or having bleeding disorders, combined gall bladder and common bile duct stones, patients who have suspicion of gall bladder mass and patients in whom both techniques of dissection had failed to applied intra operatively due to difficult anatomy secondary to inflammatory adhesion and fibrosis were excluded from the study.

Eligible patients provided written informed consent acknowledging all possible complications, and the study was approved by the Institutional Review Board, College of Medicine, Al-Nahrain University.

A total of 344 patients were qualified for the study. Patients given admission number. Group A (172 patients) who were operated by routine LC using infundibular technique and group B (172 patients) were operated by CVS technique.

For group A the older common method found in texts for ductal identification in laparoscopy has been used; the “infundibular” or “infundibular-cystic” technique. This method entails dissecting the gallbladder from its neck upward, the cystic duct is isolated by dissection on the front and the back of the triangle of Calot (cystic artery forms cephalad boundary instead of the liver surface) once isolated it is traced on to the gallbladder. Conclusive identification, i.e., the anatomic rationale for identification, occurs as a result of seeing the characteristic flare (funnel shape) as the cystic duct widens to become the gallbladder infundibulum.

In group B, the CVS was achieved by clearance of the HC triangle (CHD on the left, cystic duct on the right, and liver under surface superiorly). The triangle has been cleared of all the soft areolar tissue. Then exposure of the

lower cystic plate and the gallbladder been separated from its liver bed with the exposure of the lower third of the cystic plate. When two and only two tubular structures have be seen entering the gallbladder, the cystic duct and the cystic artery, clips are applied as shown in figure 1. Sub hepatic drain in both groups inserted when needed.

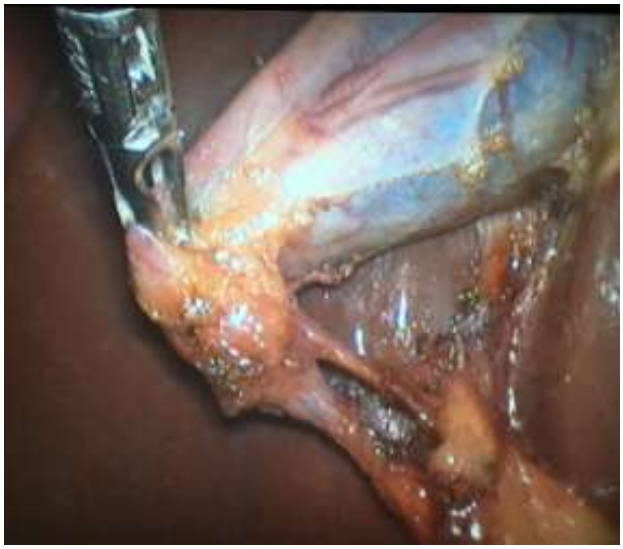

**Figure 1. Dissection of the hepatocytic triangle exposing the critical view of safety**

Preoperative patient characteristics, operating time and intraoperative and postoperative factors including hospital stay, were recorded. Postoperatively, patients were assessed using clinical examination for signs and symptoms of biliary leakage such as drainage bag content, abdominal distention, fever, jaundice, while sometimes, liver function test and abdominal US were needed to detect any bile collection. All the patients received adequate analgesia and were discharged once stable. The patients were called for follow up after 10 days for stitches removal and to look for any jaundice due to biliary strictures.

Results were statistically analyzed. Comparisons between the two groups were done by using T test and Chi square test. A p value of less than 0.05 was considered to represent a significant.

## Results

The mean age of the patients in group A and B was (36.9±6.21), (37.84±4.11) years subsequently. Stratifying of age in to classes revealed the age class 30-39 years was the most prevalent among patient both groups (109 patients, 63.37%, group A) and (101 patients, 58.72%, group B). Female preponderance was obvious in both groups. In group A the male:female ratio was 1:1.57 and 1:1.32 in group B with no significant difference.

The mean operative time for infundibular group was 67.8±20.9 minutes (range 45-112 min), which is significantly higher than that reported for CVS group (46.71±12.6 minutes, range 30-78 minutes) (p= 0.016).

In regard to perioperative complications, bleeding was encountered in 12 patients (6.98%) of infundibular group while only 2 patients (0.58%) of CVS group. Bile leak was reported in 17 patients (9.88%) in infundibular group versus 3 patients (1.74%) in CVS group. Subhepatic drain insertion was required in 25% and 2.33% of patients in infundibular and CVS group respectively, the details and P values are shown in table 1.

**Table 1. Perioperative complications in infundibular and critical view of safety groups**

| Complications | Infundibular (N=172) N (%) | CVS (N=172) N (%) | P value |
|---|---|---|---|
| Bleeding | 12 (6.98) | 1 (0.58) | <0.001 |
| Bile leak | 17 (9.88) | 3 (1.74) | 0.001 |
| Drain insertion | 43 (25) | 4 (2.33) | <0.001 |

Port site infection was reported in 3.49% among patients in infundibular group while 0.58% in CVS group, however, the difference did not reach a significant level. Thirteen patients (7.58%) in infundibular group developed intra-abdominal collection compared to only 2 patients (1.16%) among CVS group, with a highly significant difference. The frequency of bile leak in infundibular and CVS groups was 12 (6.98%), 3 (1.74%) respectively with a significant difference. Although chest infection was more frequent among patients in infundibular group (6.98%) compared to CVS group (1.74%), the difference was not a significant as shown in table 2.

**Table 2. Postoperative complications in infundibular and critical view of safety groups**

| Complications | Infundibular (N=172) N (%) | CVS (N=172) N (%) | P value |
|---|---|---|---|
| Port site infection | 6 (3.49) | 1 (0.58) | 0.056 |
| Intra-abdominal collection | 13 (7.58) | 2 (1.16) | 0.004 |
| Bile leak | 12 (6.98) | 3 (1.74) | 0.017 |
| Chest infection | 11 (6.4) | 5 (2.91) | 0.125 |

The mean hospital stay in infundibular group was (3.14±2.8) days (range 1-14 days), which was significantly higher than that reported for CVS group (1.8±2.7) days, range 1-8 days). In the same context, 16 patients (9.3%) among infundibular group required prolonged hospital stay compared to only 2 patients (1.16%) in CVS group who needed such period, with a highly significant difference (Table 3).

**Table 3. Hospital stay in infundibular and CVS groups**

| Hospital stay (Days) | | Infundibular (n=172) | CVS (n=172) | P vale |
|---|---|---|---|---|
| Mean±SD | | 3.14 ± 2.8 | 1.8 ± 2.7 | 0.011 |
| Range | | 1-14 | 1-8 | |
| | | N (%) | N (%) | |
| Prolonged hospital stay | No | 156 (90.7) | 170 (98.84) | 0.001 |
| | Yes | 16 (9.3) | 2 (1.16) | |

## Discussion

The present study aimed to assess the CVS technique in relation to classical infundibular technique for LC. The criteria used for this assessment were operative time, perioperative and postoperative complications and hospital stay.

Mean operative time for infundibular group was 67.8±20.9 minutes, which is significantly higher than that reported for CVS group (46.71±12.6 minutes). In accordance with this result is a retrospective study conducted by Vettoretto et al., in which the authors reported a mean operation time for CVS as (51.5 versus 69.7 minutes) for infundibular approach [(10)]. However, there were no significant differences between the two approaches regarding complications. Almost similar results were reported by Viswanathan et al. among Indian patients, they found a mean operation time in CVS and infundibular approaches of (55.7 and 74 minute) respectively with a significant difference [(11)]. In another study, Zarin et al. reported that the operative time was significantly reduced in CVS technique compared with infundibular technique (50 versus 73 minutes) [(12)].

The reduced operation time in CVS compared to the traditional method even in less experienced surgeons can be attributed to the principle of CVS, which allows the ability to identify adequate anatomical structure with safe dissection as well as make operative decisions easier without risking patients [(13)].

In the current series, each of bleeding, bile leak and sub hepatic drain insertion were reported more frequently in infundibular approach compared with CVS with highly significant differences, all these add more operative time. Compared with local and international studies, these results seemed very reasonable. In a retrospective local study, Hamad et al. indicated high incidence of BDI among patients undergoing open or LC utilizing classical infudibular technique. The most common presentations of those patients were biliary fistula (36%) and jaundice (28%) [(14)]. Singh and Brunt conducted a prospective study including 1340 patients (CVS - 700, Infundibular technique -640). There was no bile leak or BDI among patients operated with CVS. Whereas in traditional method, 32 operations were converted to open surgery, due to BDIs, out of which, 3 were major BDIs. The authors concluded that no doubt of CVS being safe, feasible and superior to infundibular technique in preventing BDI [(15)]. In Egypt, Safwat et al. conducted a small prospective study on 30 patients with chronic cholecystitis treated surgically with either CVS or infundibular technique. Despite the small number of patients, the study revealed a significant difference in drain insertion between the two techniques in favor of CVS [(16)].

However, some studies did not report such variation between the two approaches. For example, Vettoretto et al. concluded that CVS technique has a similar rate of biliary and hemorrhagic complications [(10)].

Generally, studies which did not found such advantages for CVS may be questioned regarded their attaining of this technique. In this regard, a Dutch study involving video reviewing for 1108 consecutive patients who had claimed to be undergone a LC with CVS technique. The study showed that 8.8% of patients developed complications and 1.7% had bile duct injuries. Reviewers of video found that CVS was really achieved in only 10.8% of the cases, and CVS was not performed in any of the patients who had biliary injuries [(17)]. These findings suggest that although a surgeon may have stated or believed that CVS was reached, as documented in the operative note, this was not the case, and even those who claimed to perform CVS may actually not apply all criteria of technique properly.

The main postoperative complications in the present study were intra-abdominal collection, bile leak and chest infection, which were more frequent among patients in infundibular group compared with CVS group. These results agree with most literatures. In a local prospective study, Al-Saffar and Al-Khaqany found that CBD injury was reported in only one patient (0.2%), while bile leak was reported in only 2.4% [(18)].

In the present study, mean hospital stay in infundibular group was (3.14±2.8) days, which was significantly higher than that reported for CVS group (Table 3). Furthermore, 9.3% and

1.16% of patients in infundibular and CVS group respectively required prolonged hospital with a highly significant difference. In a similar study, Kaya et al. found that all their patients who were operated with CVS were discharged on first, second or third day post-operative day [19]. However, in the Egyptian study, there was no significant difference in the hospital stay between the two techniques, may be because the small number of patients in each group [16].

The remarkable decrease in hospital stay in patients operated with CVS compared with those operated with the traditional method can be explained on the basis of postoperative complications, which have been more frequently among patients in infundibular group.

Collectively, the present data suggest that practicing the CVS method of identification of vital anatomical structure during LC remarkably decrease the incidence of complications, operative time because of the safe accurate dissection at hepaticocystic triangle that allow the surgeon to proceed without fear of misidentification. Furthermore, the CVS technique associated with shorter hospital stay compared with infundibular technique. The CVS builds self-confidence, and is a simple standardized method both for complicated and uncomplicated gallbladder stone. Accordingly, the CVS approach should be made the standard method of identification of anatomical structures at the HC triangle for all LC operations. This is particularly important when considering trainees or young surgeons, who have scarce experience in biliary anatomical variance and are at risk of causing a major injury.

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## Author contribution

Dr. Noman: data collection and writing the manuscript. Dr. Abdulhassan: Patient examination and supervision of data collection. Dr. Hamdawi: Data analysis.

## Conflict of interest

The authors declare that they have no competing interests.

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# The Prevalence of Diabetes Mellitus Type 2 in Severe and Very Severe Chronic Obstructive Pulmonary Disease Patients

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## Abstract

**Background** Chronic obstructive pulmonary disease (COPD) is the leading cause of morbidity and mortality worldwide. There is evidence to support a connection between COPD and Diabetes mellitus type 2 (T2DM). T2DM affects 2-37% of COPD patients, with results being highly variable between studies.

**Objective** To determine the prevalence of T2DM in patients with severe and very severe stages of COPD and to assess the risk factors affecting the prevalence of T2DM among COPD patients.

**Methods** A cross sectional study was conducted on 140 patients with COPD attending Outpatient and Inpatient of Respiratory Unit at Baghdad Teaching Hospital. The data were collected between the 10th of October 2016 to the 10th of August 2017. These data included demographic parameters such as: age, sex, smoking habit, and respiratory parameters from history and clinical examination. Spirometry was used to assess the severity of COPD patients. Random blood sugar testing was used for identification of COPD patients having T2DM when they are not clear or not known as a case of T2DM previously.

**Results** The prevalence of T2DM among COPD patients was 19.38%; the prevalence of T2DM in those with severe stage was 10.9%, while in very severe stage was 35.4%. In comparison to women, men were more likely to have T2DM.The prevalence of T2DM increased in the elderly COPD patients (>61 years), with high body mass index, and also increased more among current smokers followed by former smokers and never smokers. Lung function tests decline more in COPD patients with presence of T2DM.

**Conclusion** T2DM is more common among COPD patients, and its prevalence rose as the severity of the COPD patients' condition worsened. Additionally, T2DM patients are more likely to experience a decline in pulmonary function.

**Keywords** Diabetes mellitus type 2, chronic obstructive pulmonary disease, prevalence, random blood sugar

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**List of abbreviations:** COPD = Chronic obstructive pulmonary disease, FEV1 = Expiratory volume in first second, FVC = Forced vital capacity, T2DM = Type 2 Diabetes mellitus

## Introduction

Chronic obstructive pulmonary disease (COPD) is a preventable and treatable condition, characterized by airflow limitation that is usually progressive and associated with chronic inflammatory response in airways due to harmful, noxious particles or gases, especially in tobacco smoking exposure. Exacerbations and associated comorbidities with a significant extra pulmonary problem which may contribute to the severity in different patients [(1)].

Between 1.6 and 16% of COPD patients have diabetes, according to reports. Smoking has

been identified as a risk factor for diabetes, similar to COPD, and the risk is reduced by quitting for more than 5 to 10 years. Type 2 diabetes (T2DM) is more prevalent in moderate-to-very severe (but not mild) COPD than in the general population [2]. The evidence for an interaction between T2DM and COPD is supported by studies that demonstrate reduced lung function as a risk factor for the development of diabetes. Inflammatory mediators such as Tumor necrosis factor alpha (TNF-α), Interleukin-6 (IL-6), and C-reactive protein (CRP), which are elevated in COPD, are also increased in diabetes. The impact of parental use of corticosteroids on the management of diabetes during COPD exacerbations and the effect of diabetes control on COPD outcomes are of great clinical concern [3]. According to World Health Organization (WHO) report in 2020, 212.3 million prevalent cases of COPD were reported globally, with COPD accounting for 3.3 million deaths and 74.4 million Disability adjusted life per years (DALYs) [4].

The intersection of T2DM and CPOD may be one of the important epidemiological bridges [3]. Numerous researches have shown that diabetics have significantly lower lung function, and more recent studies have revealed that DM is a common concomitant illness in COPD patients. The strong association between T2DM and COPD is due to multiple interrelated factors and through different mechanisms, including shared risk factors, direct causation in addition to treatment effect. Cigarette smoking is known to be the primary cause of COPD. Additionally, smoking increases insulin resistance, and individuals who smoke have a 30–40% higher risk of developing type 2 diabetes than those who don't [5].

T2DM and COPD share a similar insidious onset, which usually leads to late presentation and diagnosis. Recognition of T2DM and/or COPD may be even more difficult in patients who already diagnosed with the other pathologies, particularly where they are consulted an organ-based specialist [6]. Patients with diabetes who experience chronic pulmonary damage (progressive and irreversible damage) due to diminished lung function and volumes, several functional abnormalities in the respiratory tract, pulmonary autonomic neuropathy, and decreased pulmonary diffusing capacity for carbon monoxide would simply exercise less, occasionally complain of dyspnea with exertion, and have annoyance-inducing seasonal upper respiratory symptoms. All of these symptoms might be inferred by specialist or family doctor to start treatment of COPD, diabetes and lifestyle modification accordingly [7,8].

The study objectives were to determine the prevalence of T2DM in severe and very severe stages of COPD patients. Additionally, to assess the risk factors affecting the prevalence of T2DM among COPD patients.

## Methods

A cross sectional study was conducted on 140 patients with COPD attending Outpatient and Inpatient of Respiratory Unit at Baghdad Teaching Hospital. A random sample were collected between the 10$^{th}$ of October 2016 to the 10$^{th}$ of August 2017. The data included demographic parameters; age, sex, occupation, smoking habit, and respiratory parameters from history and clinical examination, chest radiography and medications used by the patient currently or previously, symptoms related to respiratory system, duration of illness, level of dyspnea {Medical Research Council range (0-4)}, number of exacerbations. Concerning information about T2DM, history of T2DM, duration of T2DM, taking antidiabetic agents, family history of DM was also reported. The inclusion criteria were post bronchodilator spirometry obstruction defined as expiratory volume in first second (FEV1)/ forced vital capacity (FVC) ratio <0.70. Patients aged more than 40 years, and diagnosed with COPD and classified according to criteria of Global Initiative for COPD into severe and very severe stage [9].

The exclusion criteria included patients with any other lung disease other than COPD, patients on systemic steroid therapy, T1DM patients, patients with incomplete questionnaires, no medical and demographic information, no spirometry, no laboratory tests) or FEV1/FVC ratio >0.70 after administration of bronchodilator. Informed consent was obtained from all the participants for the investigations needed and the purpose of the study.

**Diagnosis of COPD**

All the participants enrolled in this study were subjected to spirometry testing. Several measures of lung function were FEV1, FVC, and post bronchodilator FEV1/FVC ratio.

They were measured by office spirometry in pulmonary function test in Outpatient Clinic at Baghdad Teaching Hospital. Using modification of the criteria developed by the Global Initiative for Chronic Obstructive Lung Disease (GOLD) [9], the subjects were classified according to their GOLD COPD stage as follows:

- Stage 1 (mild): FEV1 ≥80% of predicted; FEV1/FVC <0.70.
- Stage 2 (moderate): FEV1 ≥50 to <80% of predicted and FEV1/FVC <0.70.
- Stage 3 (severe): FEV1 ≥30 to <50% of predicted and FEV1/FVC <0.70.
- Stage 4 (very severe): FEV1 <30% of predicted and FEV1/FVC <0.70.

**Diagnosis of T2DM**

The diagnosis of T2DM was according to American Diabetes Association (ADA), subjects already known to have T2DM were directly enrolled in this study as known cases with COPD patients. The subjects whose diabetes status was unclear underwent random blood sugar (RBS) testing, and if more than 200 mg/dl (11.1 mmol/L) with classic symptoms of hyperglycemia. They were classified as new T2DM cases with COPD. Late onset T1DM (LADA) was ruled out in this cohort in newly diagnosed patient by follow those patients specifically for the definite diagnosis [10].

**Smoking status**

The subjects enrolled in this study were classified according to smoking status into:

- Never smoker: They never smoked or who smoked fewer than 100 cigarettes in their entire lifetime.
- Current smoker: They had smoked at least 100 cigarettes in their entire life and still smoking.
- Former smokers: They smoked at least 100 cigarettes in their life but not currently smoking.

The calculated number of pack-years = years of smoking X number of daily smoked cigarettes/ 20 [11].

**Statistical analysis**

Statistical analysis was performed with the Soft Package Scientific Statistics, version 22.0 (SPSS Inc, Chicago, IL, USA). Continuous variables were presented as mean ± standard deviation (SD) and 95% confidence interval (95% CI) and categorical variables were presented as percentages. Chie-square test was used to determine the associations between the categorical variables. P-value is equal or less than 0.05 was considered as the level of significance was).

## Results

**Sample characteristics**

A total of 140 patients diagnosed as COPD were selected in this study. There were 92 (65.7%) COPD patients with severe and 48 (34.3%) with very severe stage. Their age ranged from 42 to 84 years with a mean±SD of 58.6±8.4 years. Females were 43 (30.7%) and 97 (69.3%) were males, giving a male to female ratio of 2.25:1. There were 62 (44.3%) current smokers, 55 (39.3%) former smokers and 23 (16.4%) never smokers. COPD patients with severe and very severe stage were insignificant different age groups (p>0.05), the rates of patients diagnosed COPD with severe and very severe stage consisted from males (70.1% and 29.9%, respectively). The proportion of COPD patients with severe and very severe stage was

found to be higher in current smokers (41.3% and 50%, respectively). A positive correlation between prolong duration of COPD and increasing severity of COPD with-risk of T2DM (Table 1).

**Table 1. Demographic characteristics of the study group**

| Variable | Categories | All COPD N=140 No. (%) | Severe COPD N=92 (65.7%) No. (%) | Very Severe COPD N=48 (34.3%) No. (%) | P value |
|---|---|---|---|---|---|
| Age group (yr) | 41-50 | 31 (22.1) | 21 (22.8) | 10 (20.8) | 0.952 |
| | 51-60 | 29 (20.7) | 20 (21.7) | 9 (18.8) | |
| | 61-70 | 38 (27.2) | 24 (26.1) | 14 (29.2) | |
| | >70 | 42 (30.0) | 27 (29.4) | 15 (31.2) | |
| Sex | Male | 97 (69.3) | 68 (73.9) | 29 (60.4) | 0.021 |
| | Female | 43 (30.7) | 24 (26.1) | 19 (39.6) | |
| Smoking status | Current | 62 (44.3) | 38 (41.1) | 24 (50.0) | 0.018 |
| | Former | 55 (39.3) | 33 (35.7) | 22 (45.8) | |
| | Never | 23 (16.4) | 21 (22.8) | 2 (4.2) | |
| | | | **Mean±SD** | **Mean±SD** | |
| Respiratory function test | FVC L | | 1.63±0.3 | 1.1±0.6 | <0.001 |
| | FVC% Pred. | | 53.4±10.6 | 36.8±11.2 | <0.001 |
| | FEV1 L | | 1.1±0.8 | 0.69±0.3 | <0.001 |
| | FEV1% Pred. | | 41.7±3.1 | 25.7±5.4 | <0.001 |
| | FEV1/FVC% | | 62.1±3.7 | 49.2±7.4 | <0.013 |
| Duration of COPD (yr) | | | 8.4±4.3 | 11.7±8.2 | 0.011 |
| RBS (mmol/l) | | | 9.6±0.5 | 12.3±1.5 | <0.001 |

COPD: Chronic obstructive pulmonary disease, FEV1: Forced expiratory volume at first second, FVC: Forced vital capacity, RBS: Random blood sugar

**Prevalence**

The overall prevalence of T2DM detected among COPD patients with severe and very severe stage was found to be 19.28% (27/140). A higher prevalence of T2DM was detected among COPD patients with very severe stage (35.4%) (17/48) when compared to that in COPD patients with severe stage (10.9%) (10/92). A statistically significant difference (P<0.05) was found to exist in this case. This table also showed insignificantly higher prevalence of T2DM among male COPD patients in both severe (7.6%) and very severe stage (25%) than in females (3.3% and 10.4%, respectively) (p>0.05). The prevalence of DM was significantly higher among COPD patients with very sever stage at age group more than 60 years (>60 years) when compared to those below 60 years old (<60 years) (p<0.05) (Table 2).

Table (3) showed the differences in the mean values of pulmonary function tests between the COPD patients with T2DM and without T2DM. It showed a statistical difference between COPD patient with T2DM and without T2DM regarding the pulmonary function tests including FVC L, FEV1 L, FEV1/FVC%, FEV% Predicted, and FVC% Predicted (P<0.05).

**Table 2. Prevalence of Type 2 diabetes mellitus among the study groups according to gender, age and smoking**

| Variable | | Severe COPD with T2DM N=10 No. (%) | Very Severe COPD with T2DM N=17 No. (%) | P value |
|---|---|---|---|---|
| Gender | Males | 7 (7.6) | 12 (25.0) | 0.974 |
| | Females | 3 (3.3) | 5 (10.4) | |
| Age group (yr) | 40-60 | 1 (1.1) | 8 (16.65) | 0.049 |
| | 61-70 | 4 (4.3) | 4 (8.3) | |
| | >70 | 5 (5.4) | 5 (14.4) | |
| Smoking | Never | 1 (1.1) | 3 (6.3) | 0.862 |
| | Former smokers | 4 (4.3) | 6 (12.5) | |
| | Current smoker | 5 (5.4) | 8 (16.7) | |

COPD: Chronic obstructive pulmonary disease, T2DM: Type 2 diabetes mellitus

**Table 3. Mean pulmonary function test values among chronic obstructive pulmonary disease patients with or without presence of type 2 diabetes mellitus**

| Pulmonary function test | COPD with T2DM N=27 Mean±SD | COPD without T2DM N=113 Mean±SD | P-value |
|---|---|---|---|
| FVC L | 0.9±0.2 | 1.4±0.4 | <0.0001 |
| FEV1 L | 0.7±0.3 | 1.2±0.6 | 0.001 |
| FEV1/FVC% | 48.3±6.2 | 63.2± 2.9 | <0.0001 |
| FEV1% pred. | 23.8±5.3 | 40.9±2.8 | <0.001 |
| FVC% Pred. | 35.2±7.3 | 54.1±9.4 | <0.0001 |

COPD: Chronic obstructive pulmonary disease, T2DM: Type 2 diabetes mellitus, FEV1: Forced expiratory volume at first second, FVC: Forced vital capacity

## Discussion

Many previous studies had agreed in predicting an increase in COPD morbidity and mortality. At 2020, COPD is expected to cause over 6 million deaths annually around the world, as a result, it rose to the third-ranking cause of death worldwide [12]. Through understanding the pathophysiology of COPD, and the concept of systemic inflammation, they had been helped to explain the high frequency of major and important co morbidities such as T2DM. In addition to coexisting conditions that one would naturally expect due to the patients' advanced age and due to other associated risk factors, such as smoking, stress, sedentary life style, physical activity, unhealthy diet habit, etc. [13,14].

The present study revealed that the overall prevalence of T2DM among COPD patients with severe and very severe stage was 19.3% (10.9 % in severe stage and 35.4% in very severe stage). It is significantly higher if compared to the overall prevalence of diabetes in the general Iraqi population for adults aged 20-79 years of 9.4% [15].

The prevalence of T2DM in current study is in line with various studies reported from different countries. In a cohort study conducted in Taiwan by Ho et al., 2017, whom found that during a period of 10 years follow

up, 304 (19%) of 1568 COPD patients developed incident DM [16]. This result was also similar to a survey conducted by Stojkovikj et al. 2016, who found that 21% of Macedonian COPD patients with severe and very severe stage reported T2DM [17]. However, current results were lower than that reported by Mahishale et al. study 2015, who found 25.63% of Indian patients with COPD having T2DM and concluded that very severe COPD was associated with a higher risk of T2DM [18].

The possible development of COPD and T2DM, could have evidence in the context of a chronic systemic inflammation with the presence of cardiovascular or other metabolic disorders, known to cause systemic inflammation, increasing the association between COPD and T2DM [19]. There are multiple factors such as inflammation or disease-related inflammation, oxidative stress, hypoxia, reduced physical activity, and smoking habit in addition to hyperglycemia, which may contribute to the higher prevalence of diabetes in COPD. It found that the treatment with corticosteroids is considered to be another factor that may increase the risk of the association between these two diseases [20].

In terms of age, this study revealed that T2DM was more common in COPD patients with severe and very severe stages at 60 years. Moreover, it was higher among COPD patients with very severe when compared to that of severe stage. This is in agreement with many studies reported elsewhere by Shen et al. (2014) in Taiwan, who found a higher risk of developing lung cancer and T2DM among COPD patients at age group more than 60 in comparison with COPD patients at age below than 60 years old [21]. In a cross-sectional analytic study, Feary et al. (2010) revealed that there is a decrease in diabetes in older COPD patients; if diabetes is connected with COPD, the biggest effects are seen in the youngest COPD patients who smoke and those between the ages of 45 and 55 who have never smoked. [22]. This could be due to the fact that elderly people are more exposed to risk factors in COPD patients as comorbidities such as hypertension, heart failure, osteoporosis, metabolic disorders, pulmonary hypertension, which may enhance for increasing risk of T2DM in those subjects [23]. Moreover, elderly people are more exposed to frequent infections, systemic inflammations, frequent hospitalizations, high symptoms score, history of frequent and changing in current and previous medications, limited physical activity, poor in their lifestyle, all these factors may play role as the root of many chronic diseases including COPD and T2DM [24].

In a study of 100 people with COPD, Stojkovki et al. (2016) discovered that male patients with severe and very severe COPD had an insignificantly greater prevalence of T2DM than female patients [25]. The differences in the prevalence of T2DM among COPD patients with severe and very severe may be related to the fact that male-female differences are divided into sex-related differences as in (biological variability) and (environmental and socio-cultural factors such as occupational exposure, smoking exposure as active and passive that may have effect on the pulmonary impairment, since the vast majority of our patients were smokers, although some one reported that the development of COPD more prevalent among females because a large percentage of them were smokers and their life style was changed mainly in developed countries [26,27].

Concerning smoking status; this study showed that the prevalence of T2DM in severe and very severe of COPD patients was higher among current smokers (5.4% and 16.7%, respectively) followed in order by former smokers (4.3% and 12.4%), and never smokers (1.1% and 6.3%). This finding suggested that smoking factor may play a role in the increasing the risk for development of T2DM among COPD patients in both the severe and very severe stage. Similarly, many studies have found that T2DM was more prevalent among COPD patients. In subjects who smoke, the adverse effects of T2DM on lung function were even greater. It found that cigarette smokers were 30% to 40% more likely to develop T2DM than non-smokers

and because cigarette smoking increases insulin resistance [14]. Tobacco exposure is common risk factor for both COPD and the co-morbidities including T2DM. The present study as well as many other studies emphasize the urgent need for smoking cessation programs at all levels of healthcare services. It is essential to note that interventions to decrease the prevalence of smoking among patients with COPD (or in the general population) may have an important impact on the prevalence of COPD and comorbidities such as T2DM.

Regarding pulmonary function; the present study revealed that there is a significant declining or impairment in the pulmonary function among COPD patients with T2DM in severe and very stage in comparison with COPD patients without T2DM. This finding was in agreement with many studies carried out in different countries that found a correlation between lung volumes and development of T2DM [12]. It should be noted that some studies found no association between lung function and the presence of T2DM [28-30]. A study conducted by Mekov et al. (2015) found no differences in FVC, FEV1 according to presence of T2DM among COPD patients while there was a significant difference in FEV1/FVC ratio [31]. Subjects with diabetes are at increased risk of several pulmonary conditions including COPD [32]. The direct association between impaired lung function and diabetes is thought to be the result of biochemical changes in the structures of the lung tissue and airways that involve a series of mechanisms likely due to reduced physical activity, smoking habit, systemic inflammation, oxidative stress, and hypoxemia, all may contribute to the higher prevalence of T2DM in COPD patients [33].

The limitations of this study were the small sample size recruited in this study affect the significant differences among COPD specially regarding the association between the prevalence of T2DM and other potential variables. In addition to the diagnosis depended only on RBS of the COPD, which affect the real prevalence of diabetes especially the newly established T2DM among COPD patients.

In conclusion; T2DM was more prevalent in male COPD patients with severe and very severe but there is no significant difference especially current smokers and older age group. In COPD patients with T2DM, declining lung function or reduced lung function was more common.

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## Author contribution

Dr. Al Obaidy: Study conception, critical revision and study design. Dr. Al-Ani: Acquisition of data, analysis, interpretation of data, drafting of manuscript.

## Conflict of interest

None.

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# Maternal Serum Alpha Feto Protein Level May Predict Morbidly Adherent Placenta in Women with Placenta Previa

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## Abstract

**Background** In situations of a morbidly adherent placenta, studies have shown that maternal serum alfa feto protein (AFP) is clinically high.

**Objective** To examine the association of morbidly adherent placenta (MAP) in pregnant women with placenta previa (PP) and their serum AFP biomarker.

**Methods** A prospective observational study that was conducted in the Department of Obstetrics and Gynecology at Azadi Teaching Hospital, Kirkuk, Iraq during the period from Feb. till Nov. 2019. It included 82 pregnant women those between 14-20 weeks of gestation with singleton pregnancy, viable fetus, and diagnosed with low lying placenta, with and without vaginal bleeding. A blood sample from all women was taken to investigate for serum AFP level after confirming the diagnosis of low-lying placenta by early abdominal ultrasound (U/S) scan. All the enrolled pregnant women were followed by Doppler U/S at gestational age 28-32 weeks, underwent targeted screening for MAP and were divided into two groups according to the status of placenta: MAP group included 11 pregnant women and PP group alone included 71 pregnant women.

**Results** In this study, MAP was diagnosed in 13.4% of cases. Mean of serum AFP biomarker in women with MAP was significantly higher than that in women with PP. Serum AFP biomarker in early pregnancy >200 ng/ml is predictive for diagnosis of MAP in late pregnancy.

**Conclusion** Maternal serum AFP biomarker may play an important role at early pregnancy in prediction of placental adherence at late pregnancy in cases of early low-lying placenta.

**Keywords** Morbidly adherent placenta, alpha feto protein, previa, pregnancy, Iraq

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**List of abbreviations:** AFP = Maternal serum α-fetoprotein, AUC = Area under curve, BMI = Body mass index, C/S = Cesarean section, DCR = Damage control resuscitation, MAP = Morbidly adherent placenta, MRI = Magnetic resonance imaging, PP = Placenta previa, ROC = Receiver operating characteristics, SPSS = Statistical package for social sciences, TAS = Transabdominal sonography, TVS = Transvaginal sonography, U/S = Ultrasound

## Introduction

The morbidly adherent placenta previa (MAP) (placenta accreta, increta, and percreta) is a developing obstetric issue, and a link has been found between this kind of placentation and previous lower segment cesarean section [(1)]. It is one of the most dangerous pregnancy problems. Women with a morbidly adherent placenta have been documented to have maternal morbidity in up to 60% of cases and mortality in up to 7% of cases. Another risk factor is the mother's age. The increased rate of caesarean births around the world has resulted in an increase in the incidence of both placenta previa (PP) and MAP [(2)]. The term "placenta accreta" refers to the

trophoblastic attachment of the placenta to the myometrium without the presence of an intervening decidua. A placenta increta occurs when the trophoblast invades the myometrium, and a percreta occurs when the trophoblast invades the myometrium beyond the serosa and into neighboring structures such as the bladder. The word "placenta accreta" is frequently used to describe a wide range of disorders, including accreta, increta, and percreta, as well as cases of clinically evident MAP [3,4]. The incidence of placenta accreta varies from 1 in 300 to 1 in 2000 pregnancies. This large range of results could be due to diagnostic challenges (histopathology) in distinguishing between difficult manual removal and abnormally attached placentas [5]. Currently, the prenatal course of women with MAP is compared to that of women with PP. The ability to diagnose MAP antenatally using imaging [ultrasound (U/S) color Doppler and magnetic resonance imaging (MRI)] allows for multidisciplinary planning in the hopes of reducing maternal and newborn morbidity and mortality. Interventional radiological techniques aid in the preservation of the uterus and thus the patient's future reproductive prospects [2]. The traditional surgical therapy for MAP is a intrapartum hysterectomy following the delivery of the fetus through an upper segment incision that leaves the placenta in place, however conservative treatment, particularly in women who want to preserve their reproductive potential, is also an option [1]. In addition to the lengthy surgical operations necessary, MAP is linked to significant bleeding during birth, which frequently necessitates substantial blood transfusions, putting the mother at risk for hypothermia, acidosis, and coagulopathy, among other catastrophic problems [6]. Recently, resuscitation in MAP shifted toward Damage Control Resuscitation (DCR) protocols, which is used in massive trauma and consists of three steps: abbreviated surgery to control the hemorrhage and contamination, resuscitation in the intensive care unit, and planned re-operation with definitive surgery. This protocol includes early use of packed red blood cell and fresh frozen plasma in 1/1 ratio to avoid over-infusion with crystalloids [6]. Doppler U/S has been a standard practice for a long time. It is a simple, precise, and safe method to visualize the placenta that can often be used in conjunction with transvaginal sonography (TVS) when available [7]. In cases of suspected PP on transabdominal sonography (TAS), the patient should undergo TVS to more accurately delineate the relationship between the placenta and the endocervical os [3]. Maternal serum alpha fetoprotein (AFP) is being utilized to predict fetal quality. It is among the most widely used diagnostic biomarkers, based on its use for screening for malignancies and prenatal abnormalities. Its presence in the amniotic fluid could indicate anencephalus or neural tube abnormalities. Furthermore, studies have shown that in situations of a MAP, maternal serum AFP is clinically high, and it is a secondary indicator of PP [8].

The aim of this study was to examine the association of MAP in pregnant women with PP and their serum AFP marker.

## Methods

### Study design, setting, and time

This was a prospective observational study that was conducted in the Department of Obstetrics and Gynecology at Azadi Teaching Hospital, Kirkuk, Iraq, during the period of 10 months from February till November 2019.

### Study population and sample size

The study included initially 96 pregnant women between 14-20 weeks gestational age, first attended the outpatient clinic or the emergency department with singleton pregnancy, viable fetus, and diagnosed with low lying placenta, with and without vaginal bleeding. Patients who had fetus with aneuploidies, neural tube defects, abdominal wall defects, patients with multiple pregnancy, maternal hepatic disease, and maternal ovarian cancer were excluded from the study.

Gestational age was calculated at the time of presentation according to first day of last menstrual period and confirmed by early abdominal U/S examination as having low-lying placenta at gestational age 14-20 weeks and blood was taken for AFP examination at first visit then they followed up by abdominal U/S scan at 2nd visit for checking the persistence of PP between 24-27 weeks gestation, then after by Doppler U/S in 3rd visit at 28-32 weeks Gestation. Fourteen participants were lost to follow up, so the total number of participants included in the analysis was 82. All the patients signed an informed consent that allows us to review their medical records for research purposes as long as the patient anonymity and confidentiality of their medical records are maintained. Detailed history by questionnaire, obstetrical history, past medical and surgical histories were taken for all patients. Information about previous pregnancies (number, abortion, outcome, and mode of delivery either vaginal delivery or cesarean section) were taken. Ultrasound findings (2D U/S, Color Doppler U/S) as position of the placenta and grade of PP were recorded. A general (vital signs) and abdominal examinations were done for all study patients.

**Sample collection and serum AFP marker test procedure**

A five ml of blood was drawn from the volar surface of the forearm from all study participants at presentation to test for serum AFP marker. The test used the sandwich immunodetection method; the detector antibody in buffer binds to antigen in sample, forming antigen-antibody complexes. The cut-off (reference range): ≤10.9 ng/ml.

**Follow up**

All the enrolled pregnant women were followed up by U/S and Doppler U/S in 3rd visit at gestational age between 28-32 weeks, underwent targeted screening for morbidly adherent placenta and were divided into two groups according to the status of placenta:

1. MAP group: Included 11 pregnant women diagnosed with morbidly adherent placenta during the 3rd trimester of pregnancy or during delivery.
2. PP group: Included 71 pregnant women diagnosed with PP.

**Diagnosis of MAP**

It was done by:

- U/S study which was performed by experienced ultra-sonographer in Azadi Teaching Hospital and reported findings associated with placental invasion in the 2nd and 3rd trimesters (gray-scale abdominal U/S) [9] include:
  1. The presence of PP.
  2. Echolucent space between the placenta and uterus with myometrial thinning.
  3. Multiple placental lacunae.
  4. Extension of the villi into the myometrium or beyond.
  5. Interruption of the posterior bladder wall-uterine interface.
  6. Hypervascularity of the adjacent bladder wall.
  7. Decreased retroplacental space (less than 1 mm).
- Color Doppler findings associated with MAP include [9,10]:
  A. Turbulent lacunar blood flow.
  B. Increased subplacental vascularity.
  C. Vessels bridging the placenta to the uterine margin, and gaps in myometrial blood flow.

The sensitivity and specificity of both 2nd- and 3rd-trimester U/S for the identification of MAP has been reported to be as high as approximately 80-90% [9,10].

**Statistical analysis**

The data analyzed using statistical package for social sciences (SPSS) version 26. The data presented as mean, standard deviation and ranges. Categorical data presented by frequencies and percentages. Independent t-test (two tailed) was used to compare the continuous variables accordingly. Receiver operating characteristic (ROC) curve analysis was constructed for serum AFP marker level as a predictor of MAP. Pearson's correlation test

(r) was used to assess correlation between continuous variables accordingly. A level of P – value less than 0.05 was considered significant.

## Results

Study participants' age was ranging from 19-42 years with a mean of 31.8±6.3 years. The highest proportion of study participants was aged ≥35 years (40.2%), 41.5% were presented <18 weeks of gestation; 47.6% of them had more than three children; 47.6% were obese; 11% were current smokers, and 8.5% were hypertensive (Table 1).

The rate of previous C/S in MAP was higher than PP alone but this difference was not significant (P= 0.201) as shown in table (2).

**Table 1. Distribution of study participants by general characteristics**

| Variable | | Number | Percentage |
|---|---|---|---|
| Age (Year) | <25 | 19 | 23.2 |
| | 25-34 | 30 | 36.6 |
| | ≥35 | 33 | 40.2 |
| Parity | Nulliparous | 12 | 14.6 |
| | 1-3 | 31 | 37.8 |
| | >3 | 39 | 47.6 |
| Gestational age (weeks) | > 18 | 34 | 41.5 |
| | $18\text{-}27^{+6}$ | 32 | 39.0 |
| | 28-32 | 16 | 19.5 |
| Smoking | Yes | 9 | 11.0 |
| | No | 73 | 89.0 |
| BMI Level | Normal | 16 | 19.5 |
| | Overweight | 27 | 32.9 |
| | Obese | 39 | 47.6 |
| Medical history | No | 71 | 86.6 |
| | Hypertension | 7 | 8.5 |
| | Diabetes Mellitus | 4 | 4.9 |
| Type of placenta | Morbidly adherent placenta | 11 | 13.4 |
| | Placenta Previa | 71 | 86.6 |

N=82, BMI=Body mass index

**Table 2. Association between status of placenta and previous cesarian section**

| Previous cesarian section | Morbidly adherent placenta N=11 N (%) | Placenta Previa N=71 N (%) | Total N=82 N (%) | P Value |
|---|---|---|---|---|
| Yes | 8 (72.7) | 37 (52.1) | 37 (45.1) | 0.201 |
| No | 3 (27.3) | 34 (47.9) | | |

Means of parity and serum AFP marker in women with MAP were significantly higher than that in women with PP (5.92 versus 2.24 weeks, P= 0.001; and 286.34 versus 134.1 ng/ml, P= 0.001 respectively). No significant differences (P ≥ 0.05) in means of age and BMI between study groups as shown in table (3).

**Table 3. Comparison in certain parameters according status of placenta**

| Variable | Morbidly adherent placenta N=11 Mean±SD | Placenta Previa N=71 Mean±SD | P value |
|---|---|---|---|
| Maternal age (years) | 33.5±5.2 | 30.9±6.7 | 0.084 |
| BMI (kg/m$^2$) | 31.22±4.8 | 29.6±5.2 | 0.176 |
| Parity | 5.92±1.27 | 2.24±1.7 | 0.001 |
| Serum AFP marker (ng/ml) | 286.34±52.3 | 134.1±81.0 | 0.001 |

BMI=Body mass index, AFP=Alpha feto protein

ROC curve analysis was constructed for serum AFP marker level as a predictor of MAP. As shown in figure (1), the cut point of serum AFP marker was 200 ng/ml, so serum AFP marker in early pregnancy >200 ng/ml is predictive for diagnosis of MAP in late pregnancy as a large significant area under the curve (AUC = 91.8%) indicating significant association between higher level of serum AFP marker and diagnosis of MAP. The sensitivity was 100% and the specificity was 85.2%.

No significant correlations (P ≥ 0.05) between AFP marker and both of age and BMI as shown in table (4).

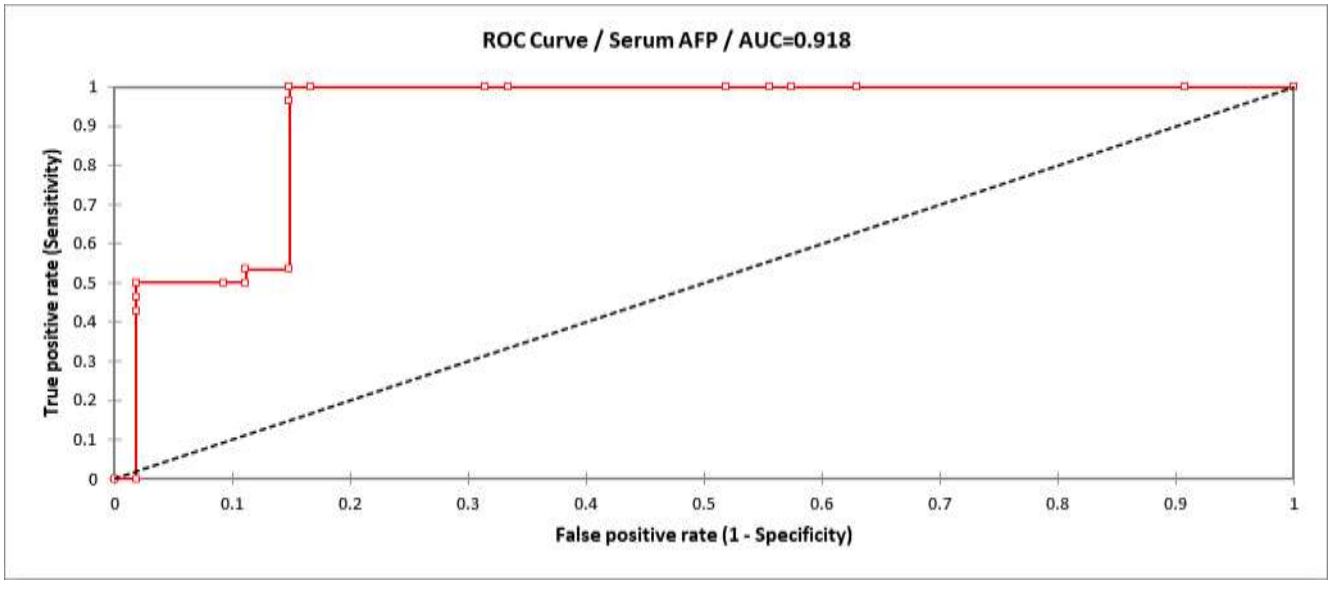


**Figure 1. ROC curve for AFP as a marker of MAP**

**Table 4. Correlation between AFP marker and both of age and body mass index**

| Variable | AFP (ng/ml) | |
|---|---|---|
| | r | P value |
| Age (Year) | - 0.06 | 0.595 |
| BMI (kg/m$^2$) | 0.064 | 0.566 |

BMI=Body mass index

## Discussion

MAP is becoming a more common pregnancy complication, owing to an increase in the rate of C/S during the last 50 years [11]. It is the most frequent indication for peripartum hysterectomy [12]. Furthermore, preterm birth and fetuses that are small for gestational age increase the risk of perinatal problems [13]. Antenatal diagnosis of accreta is critical, as it can reduce maternal morbidity by enabling for a scheduled delivery [14]. Elevated maternal-serum AFP and beta-human chorionic gonadotropin (β-HCG) levels in the second trimester have been linked to MAP [15]. In the current study, MAP was diagnosed in 13.4% of cases, and 86.6% were diagnosed as PP alone, mean of parity was significantly higher in MAP group compared to PP group, and no significant differences in means of age and BMI between study groups. Differently, lower results observed in Lyell et al. study in 2015, in which patients with MAP represented only 5% of the study patients [8]. Results of studies conducted by Berezowsky et al. in 2019 [16] and by Farquhar et al. in 2017 [17] were disagreed to this study results when observed that women with pathological placentation in the form of placenta accreta were more likely to be older. Sample size enrolled in each study, gestational age at presentation, parity, previous cesarean or pelvic surgery and obstetrical morbid conditions can have explained the differences observed among above studies.

In the present study, no significant associations (P = 0.201) between status of placenta and previous C/S, which disagreed by and by Chattopadhyay et al. study in 1993 [18] and Shi et al. study in 2018 [19] when demonstrated that C/S was associated with significantly increased risk of placenta accreta in a subsequent pregnancy complicated with PP. The association between C/S and placental pathology can explained by fact that a uterus during labor has been subjected to contractions, which might shorten the wound, diminish damage to the endometrium, and render the tissue with more potential for healing [20].

In the current study, mean of serum AFP marker in women with MAP was significantly higher than that in women with PP and serum AFP marker in early pregnancy >200 ng/ml is predictive for diagnosis of MAP. Furthermore, no significant correlation between AFP marker and both of age and BMI. These results agreed with results found by Lyell et al. study in 2015 (8), Dreux et al. study in 2012 [15] and Berezowsky A et al study in 2019 [16] when they found that risk for MAP was associated with higher level of maternal-serum AFP. In a small subset of patients at high risk, serum indicators may provide further information (In the second trimester, an unexplained elevation of maternal serum AFP, β-HCG, and/or inhibin-A or a decreased level of maternal serum AFP and/or unconjugated estriol are associated with an increased frequency of adverse obstetrical outcomes). Elevated maternal serum AFP levels in the second trimester have been linked to MAP. Its overexpression could be linked to high levels of placental invasion and MAP. While it isn't known to be linked to poor obstetrical outcomes [21].

In conclusion, maternal serum AFP biomarker may play an important role at early pregnancy in prediction of placental adherence at late pregnancy in cases of early low-lying placenta, ± vaginal bleeding. In women with a low-lying placenta and vaginal bleeding, the maternal serum AFP biomarker represents an early, non-

invasive, and good predictor of MAP that can be used if further investigations confirm it.

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## Author contribution

Dr. Ghalib: Put the research plan. Dr. Hassan: Did the sampling, wrote the manuscript and did the statistical work.

## Conflict of interest

The authors declare there is no conflict of interest.

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