

COMPARATIVE EVALUATION OF IFN- γ SERUM LEVELS AND mRNA EXPRESSION IN CONNECTION WITH GALLSTONE PATIENTS: A CASE-CONTROLLED STUDY

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Abstract

IFN- γ is a complex cytokine that plays a major role in immune system regulation. In addition to strengthening host defence, IFN- γ also causes autoantibodies, activates autologous cytotoxic T cells, and kills target cells, all of which contribute to a chain of several diseases. The current study enrolled 30 gallstone patients and 30 healthy controls. Blood and serum concentrations were considered for quantifying relative mRNA expression levels, followed by evaluation of serum concentration for the IFN- γ gene by comparing it with healthy controls. The current study has shown significant expression of mRNA levels for the IFN- γ gene with a 3.4008 fold change difference, having a $P < 0.008$. At the same time, serum concentrations of gallstone patients found high levels of IFN- γ (17.06 vs 33.12) when compared to Healthy controls with a ($P < 0.0001$). Along with these safety parameters like ALP ($P < 0.004$), Bilirubin ($P < 0.00001$), Urea ($P < 0.0010$) serum creatinine ($P < 0.00001$), cholesterol, HDL and VLDL ($P < 0.0005$, $P < 0.0002$ and $P < 0.00001$) and Hematological tests like RBC, Lymphocytes and ESR ($P < 0.0035$, $P < 0.0046$, $P < 0.00001$ and $P < 0.00006$) shown significant increase in patients having gallstone disease when compared with healthy controls. This study has shown a significant mRNA expression and serum levels of IFN- γ in patients when compared to healthy controls. This findings may be a risk aspect for the development of gallstone disease, signifying that it might be utilised as a marker to measure disease activity and pave the way for future disease treatment strategies.

Keywords: Diagnostic Marker; Gallstone disease; Inflammatory Cytokine; mRNA expression

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Introduction

Gallstone disease is commonly known as Cholelithiasis, which is an established gastrointestinal disorder. It can form due to cholesterol-rich bile, cholesterol crystal formation, and abnormal gallbladder emptying. Gallstones are common in Western countries, with estimates ranging from 15 to 20%. In the United States, approximately 35% of gallstone cases require cholecystectomy, with more than 750,000 cases performed each year. A 2007 survey found that 6.3% of Iranians had gallstone disease (Ghorbani *et al.*, 2023). During their lives, almost 20% of GSD patients will experience symptoms including infection or colic (Lammert *et al.*, 2016).

Gallstone progression is mostly caused by inflammation (Shabanzadeh *et al.*, 2017). Up to 15% of people in prosperous countries suffer from gallbladder inflammatory and degenerative disorder. One of the first sign of gallstone formation is gallbladder inflammation. Gallstones are the solid deposits of bile fluid found in the gallbladder (Bekov *et al.*, 2023). These gallstones can be separated into two groups according to their composition: cholesterol stones comprises of more than 90% of all gallstones, and other stones that have brown and black colours. Age, ethnicity, genetics, female gender, and lifestyle are risk factors for gallstone formation, which has been demonstrated to be multifactorial (Stinton and Shaffer, 2012; Di Ciaula *et al.*, 2018).

In the recent times everywhere in the world, gallstone disease is a serious public health issue because Insulin resistance, obesity, metabolic syndrome, and diabetes are also associated with gallstone disease, and food is a chief factor in these conditions. High intake of fatty acids, cholesterol, carbs, and legumes has been linked to an increased risk of gallstone disease, according to several research studies. In contrast, the risk of this disease may be lessened by consuming calcium, fibre, coffee, ascorbic acid (vitamin C), and unsaturated fat (Davidović *et al.*, 2011; Stinton *et al.*, 2012). Apart from that it can also cause acute pancreatitis, cholecystitis, and cholangitis, among other clinical conditions with severe symptoms in later stages. Gallstones are a significant contributing factor for the development of gallbladder cancer, as evidenced by the fact that at least 90% of patients with gallbladder cancer have a history of gallstones diseases (Liu *et al.*, 2018). Few reports were identified that gallstones can also be developed due to a combination of environmental and genetic factors, and the risk factors which leads to the development of stones (Lammert *et al.*, 2016).

According to the previous studies role of inflammatory biomarkers and the risk of gallstone disease are significantly correlated (Shabanzadeh *et al.*, 2017; Liu *et al.*, 2018). Certain foods and dietary habits may be linked to gallstone disease due to their possible anti- or pro-inflammatory properties (Sadri *et al.*, 2022). Although inflammation is known to play a key role in gallstone formation, the precise processes are yet unknown. Augmented bile salt levels are one of the principal factors for gallstone development because inflammation can disrupt the metabolism of several proteins and lipids, which can then affect the metabolism of cholesterol and bile acids (Liu *et al.*, 2018).

Past studies described that TNF- α , CRP, IL-1 α , IL-6, IL-8, IL-10, IL-12, IL-13, and other circulating inflammatory proteins and cytokines were all significantly connected to an advanced risk of gallstone disease (Liu *et al.*, 2018). Patients with gallstone disease have higher serum levels of proinflammatory cytokines like IL-1 α , IL-6, and TNF- α . It is not well recognised how these proteins implicated in the disease's development are expressed locally (Cheng *et al.*, 2024).

Apart from all these above discussed proinflammatory cytokines, IFN- γ is also one among them, which was very little known concerning gallstone disease. The cytokine IFN- γ is vital for both innate and adaptive immunity against intracellular bacterial and viral infections as well as for tumor organization. An increased production of IFN- γ is linked to certain autoimmune and inflammatory disorders. As part of the innate immunity response, natural killer (NK) cells and CD4⁺ effector T cells, specifically Th1 CD4⁺ T cells, are the main producers of IFN- γ . IFN- γ is crucial to the immune system because it may activate a variety of genes, most of which encode molecules involved in immunoregulation (Cavani and Girolomoni, 1998).

Since a number of cytokines have already been demonstrated to have some significance in regard to gallstone disease. We would like to acknowledge that there aren't many specific discoveries about Interferon- γ in this particular condition. The current study, which has a limited sample size (N=30) and few parameters, aims to investigate the role of interferon gamma in gallstone disease. Based on this findings in the future, we would like to expand this research with a larger sample size by identifying the degree of inflammation in the course of the disease by exploring the role of underlying mechanisms of signalling pathways through follow-up studies along with a few cancer biomarkers.

Materials and Methods

Chemicals

Chemicals were obtained from Himedia, Sigma-Aldrich, Integrated DNA Technologies, BioLegend, Kappa, and Qiagen. Running and sample loading dye from Himedia. Blood RNA separation kit, cDNA and reverse transcription kit from Qiagen, and Sybr green from Kappa Bio Solutions. Agarose powder from Sigma Aldrich, India. The Elisa kit, acquired from BioLegend and Integrated DNA Technologies, provided primers.

Participants

Healthy controls and gallstone patients of the present study were registered in the Research OPD at the National Research Institute of Unani Medicine for Skin Disorders (NRIUMSD) Hyderabad, India. Patients with gallstone were enrolled based on ultrasound scanning, bowel issues, and assessment of the history and condition of the patient, and they were excluded if they had any clinically significant abnormality identified, any surgeries in recent times, any systemic disease, other skin disease, and pregnant or lactating women. Healthy controls were considered based on routine blood, kidney, and liver-related tests. Each participant in the current study will be asked to sign an informed consent form, and this was approved by EC Ref.no.38-18/2018-2019/CRIUM/Hyd/Tech/IEC by the Ethics Committee of NRIUMSD.

Biochemical and Hematological Investigations

A over-all of 5 ml of blood was taken from each participant, and out of 5 ml blood, 2.5 ml was kept for Pax gene blood RNA tubes to extract RNA, and the remaining 2.5 ml was distributed in Plain tubes, EDTA tubes, and sodium citrate tubes for assessing biochemical and pathological investigations. An automated Biochemistry Analyser EM200 was used to investigate renal and liver function tests, including urea and creatinine, as well as bilirubin, ALT, AST, and ALP. After allowing the blood to coagulate for half an hour at room temperature, the serum was centrifuged for 15 min at a speed of about 5,000 rpm kept at -80°C for future analysis. The following haematological markers were also examined: haemoglobin (Hb), erythrocyte sedimentation rate (ESR), white blood

cell (WBC), red blood cell (RBC), platelet counts, and differential leukocyte counts (DLC): polymorphs, lymphocyte, and Eosinophil counts.

RNA Isolation and Primer Design

Total RNA was extracted using a Qiagen RNA isolation kit by following kit instructions. The extracted RNA was estimated using the A260/280 ratio. Total RNA was kept at -80°C as soon as it was isolated. Primers for the β -actin and IFN- γ genes were designed by using Primer Blast. The length of the amplified fragment for the designed primers were cross-checked using the serial cloner software Table 1.

PCR Amplification and cDNA Conversion

A cDNA experiment was performed by using extracted RNA from patients and healthy controls. A concentration of 500 ng RNA was taken, and 2 μ l of genomic wipe-out buffer and rest RNAase-free water was added to make up a reaction volume of 14 μ l. Later on, it was then incubated for 2 min at 42°C. The final cDNA sample was kept at -20°C for qRT-PCR experiment

Real-time PCR with SYBR Green Method

Gene expression studies were carried out by Bio-Rad's CFX Real-time system. 2 μ l of prepared cDNA, 6 μ l of nuclease free water, of the forward and reverse primers of 1 μ l of each and rest was added with Sybr green dye. The conditions to perform were like 30 sec of denaturation at 95°C, 30 min of annealing at 60°C, 1 min of elongation at 72°C, 5 min of final elongation at 72°C, and 10 min at 4°C for 35 cycles. The RT data was examined at the end of all 35 cycles. The relative mRNA expression of every gene was quantified by the relative expression equation (RE) by considering the β -actin gene as an internal control.

$$RE = 2^{-\Delta\Delta Ct}$$

Measuring of IFN- γ Serum Levels by ELISA

Enzyme-linked immunosorbent assay (ELISA) was used to estimate the serum levels of IFN- γ for all participants by using the BioLegend kit manual. A Tecan multimode reader was used to note the absorbance readings for all samples at 450 nm.

Table 1. Primers used in the current study

Gene name	Sense	Antisense	Amplicon Length
β -actin	5'CCACGAACTACCTTCAACTCC3'	5'GTGATCTCCTTCTGCATCCTGT 3'	136bp
IFN- γ	5'TAGCAGCATGGCTCTCCTCTTTC3'	5'TTAGTTGGTGTAGGCACTGAGG3'	108bp

Table 2. Demographic data of 30 Healthy controls and 30 Gallstone patients

Characteristics	Healthy controls N=30	Gallstone patients N=30	P-value
Gender (n %)			
Male	16 (53.33)	19 (63.33)	
Female	14 (46.66)	11 (36.66)	
Age (Years)			
	20-60	20-60	
Mean$\pm$SD	38.6 $\pm$ 10.3	37.5 $\pm$ 10.89	0.76
BMI (kg/m²)			
Mean$\pm$SD	26.1 $\pm$ 4.2	25.8 $\pm$ 4.7	0.548
Range	28-33	26-35	

* P- Value <0.05 treated as significant

Table 3. Biochemical Investigations for Healthy controls and Gallstone patients

Parameter	Reference	Healthy controls N=30	Gallstone patients N=30	t-test*	P-Value
FBS (mg/dL)	70-110	92.56 $\pm$ 22.4	124.3 $\pm$ 18.59	1.45	0.32
SGOT/AST (Iu/l)	Up to 35	21.4 $\pm$ 7.2	38.6 $\pm$ 9.1	1.59	0.21
SGPT/ALT (Iu/l)	Up to 45	24.8 $\pm$ 14.1	51.3 $\pm$ 12.6	1.25	0.54
Bilirubin (mg/dL)	0.2-1.2	0.72 $\pm$ 0.22	1.46 $\pm$ 0.58	6.95	0.00001
Alkaline Phosphatase (Iu/l)	30-120	68.6 $\pm$ 18.1	115.2 $\pm$ 12.3	2.16	0.04
Blood Urea (mg/dL)	13-45	32.3 $\pm$ 7.9	47.6 $\pm$ 4.2	3.53	0.0010
Serum Creatinine (mg/dL)	Up to 1.3	0.88 $\pm$ 0.52	1.41 $\pm$ 0.2	6.76	0.00001
Cholesterol (mg/dL)	<200	184.0 $\pm$ 21.6	225.6 $\pm$ 42.3	3.83	0.0005
Triglycerides (mg/dL)	<165	136.8 $\pm$ 40.3	191 $\pm$ 38.9	1.07	0.85
HDL (mg/dL)	<40	43.1 $\pm$ 11	37.6 $\pm$ 5.4	4.14	0.0002
LDL (mg/dL)	100-159	107 $\pm$ 35.1	176 $\pm$ 29.2	1.44	0.32
VLDL (mg/dL)	<=30	28.6 $\pm$ 3.98	35.4 $\pm$ 13.7	11.84	0.00001

* P- Value <0.05 treated as significant

Statistical Analysis

For all demographic, biochemical, and hematological investigations, an openepi online tool was used to calculate Student t test and p value. Gene expression and serum levels for all samples were run in duplicates and Mean $\pm$ SD values were measured in Excel 2016. Anova analysis was performed for $\Delta\Delta$ Ct values and fold change by using GraphPad Prism 8.

Results

Demographic Data

All participants who were enumerated in the ongoing study were separated by gender and BMI followed by mentioning in the form of Mean $\pm$ SD, which was represented in Table 2.

Biochemical and Hematological Investigations

Laboratory investigations were conducted for all sixty participants. The results indicate that biochemical and hematological investigations of gallstone patients have higher values compared to healthy controls. Alkaline phosphatase (ALP) and bilirubin levels in blood tests were shown higher in patients with gallstones (P<0.004 and 0.00001),

suggesting a possible blockage in the bile duct which indicates that a typical complication of gallstones and shows difficulty with bile flow because of the obstruction induced by the gallstones (Costa *et al.*, 2023). Even though KFT tests like Blood urea and Creatinine have shown a significant increase in gallstone patients (P<0.0010 and P<0.00001) for which former has no role in gallstone and the later has proven to be diagnostic indicator (Rajab *et al.*, 2020). Sugar tests like cholesterol, HDL, and VLDL shows significant in gallstone patients (P<0.0005, P<0.0002 and P<0.00001) which indicates that gallstone patients might be having diabetic related symptoms with a possibility of cholesterol formations stones (Ghorbani *et al.*, 2023). Significant increase in RBC, Lymphocytes and ESR in gallstone patients (P<0.0035, P<0.0046, P<0.00001 and P<0.00006) might indicates that there may be a possibility of having inflammatory cytokine in their blood. (Tables 3 and 4).

RNA Isolation

RNA was extracted from both patient and healthy group. The purity of each sample was measured by using a Nanodrop device, followed by loading the extracted samples on a 1.5 % agarose gel

Table 4. Hematological Investigations for Healthy controls vs Gallstone patients

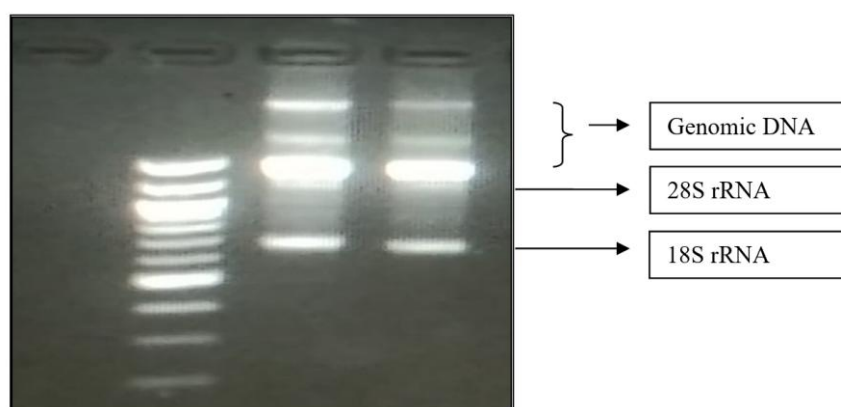
Parameter	Reference	Healthy controls N=30	Gallstone patients N=30	t-test	p-Value
Haemoglobin (gm/dL)	12-16	14.1±1.82	11.3±1.7	1.14	0.71
RBC (m/cu.mm)	4.5-5.5	4.9±0.7	5.1±0.4	3.06	0.0035
WBC (cells/cu.mm)	4,000-11,000	7148±1985	7613±2457	1.53	0.25
Platelets (lakhs/cu.mm)	1.5-4.5	2.7±0.92	4.1±0.9	1.04	0.90
Neutrophils (%)	40-80%	55.8±7.79	70.6±5.5	2.06	0.06
Lymphocytes (%)	20-40%	38.04±4.32	40.5±6.3	2.12	0.046
Eosinophils (%)	01-06%	3.01±1.35	3.87±1.24	1.18	0.65
Monocytes (%)	02-10%	2.5±1.68	5.67±1.4	1.44	0.33
Basophils (%)	00-02%	0±0.0	0±0.0	0	0
ESR (I hour) mm		15.6±2.8	24.5±9.7	12.01	0.00001
ESR (II hour) mm	0- 20 mm/h	22.3±5.6	46.5±12.3	4.82	0.00006

* P- Value <0.05 treated as significant

Table 5. Serum Concentrations of IFN- γ (pg/ml) with Gallstone patients vs Healthy controls

Variables		Healthy controls (N=30)	Gallstone Patients (N=30)
IFN- γ (pg/ml)	Mean	17.06	33.12
	SD	4.43	6.925
	p-Value	<0.0001	

* P- Value <0.05 treated as significant

**Figure 1. Patient samples were loaded on 1.5% agarose gel and run for 75 min. Lane 1: No sample; Lane 2:100 bp DNA ladder; Lane 3 and 4: RNA from patients**

at 50 V for 75 min. DNA ladder was added into the agarose wells along with RNA samples and observed RNA bands by using Biochem UVP gel documentation system. Figure 1.

cDNA Conversion and Primer Specificity

RNA sample of 500 ng was used for cDNA conversion which is the prior step before conducting qRT-PCR. Primer specificity was also observed by performing PCR upon adding dNTPs, enzyme buffer, and Taq polymerase enzyme, oligos of forward and reverse. The end products were run at 1.2% for 90 min at 75 volts on the agarose gel. The fragment size of gene was represented in Figure 2.

Relative mRNA Expression for IFN- γ Gene by qRT-PCR

IFN- γ gene expression levels were quantified by CFX96 Bio-Rad qRT-PCR. Average Threshold values (Ct) values were measured for healthy controls. The delta Ct value of each gene was measured by subtracting the internal control gene. At the end these $\Delta\Delta Ct$ values were kept in the relative mRNA equation to get the fold change. According to the results, the IFN- γ gene had a significantly higher level of mRNA expression in gallstone patients when compared with healthy controls, with a 3.4008 fold change difference with a significant P Value <0.008. The fold change result was displayed in Figure 3

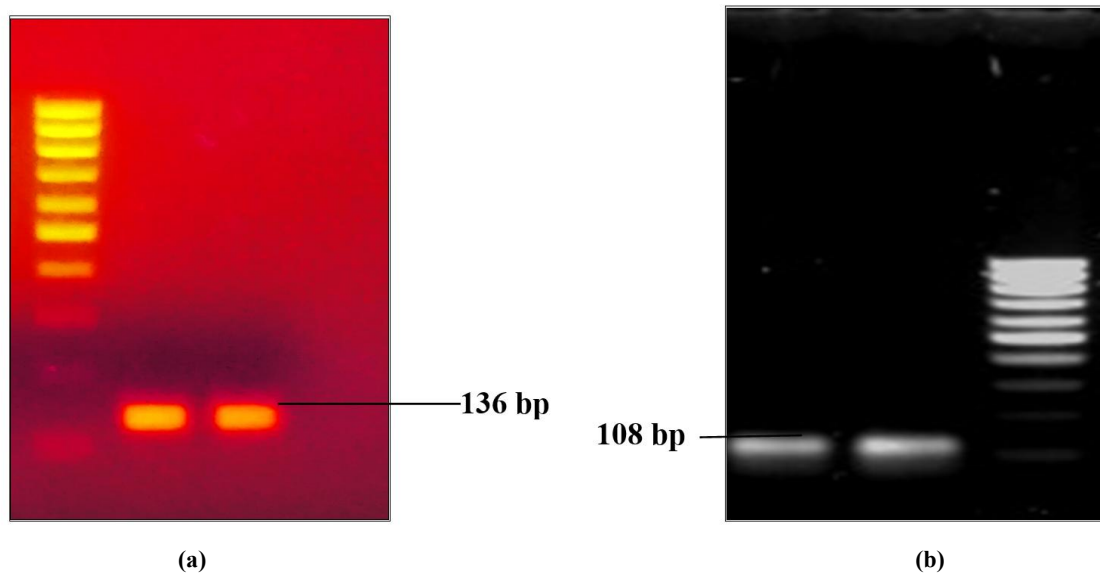


Figure 2. An Agarose gel of 1.2% was used for loading PCR products and ran for 90 min at 75 volts. Specificity of the gene was displayed by single band at the respective fragment length. Figure 2(a) and 2(b) Lane 1&3: DNA ladder of 100 bp; Figure 2(a)-Lane 2&3: β -actin; Figure 2(b)-Lane 1&2: Target gene IFN- γ

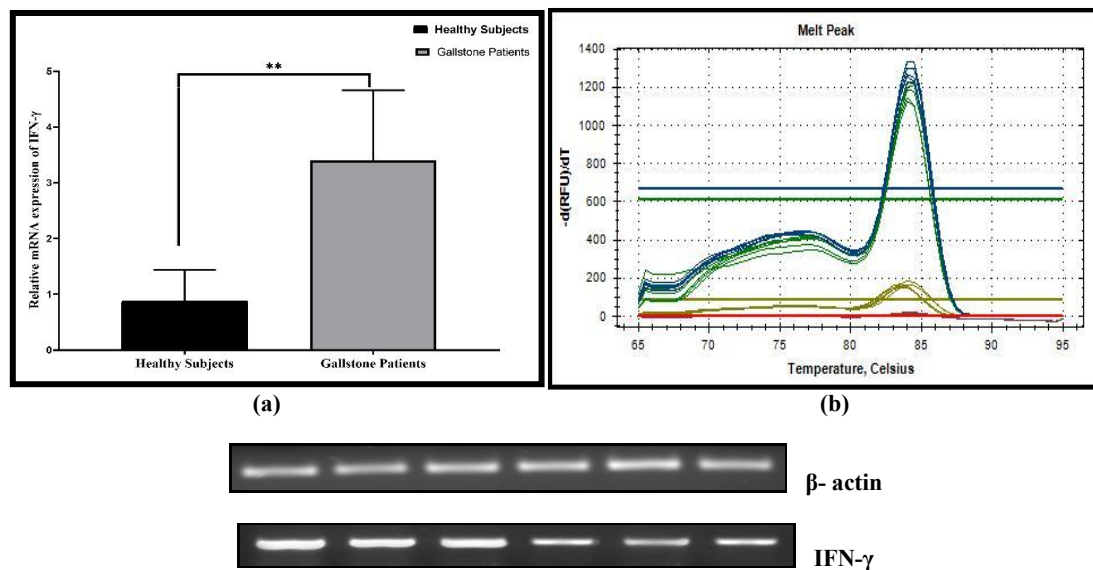


Figure 3. mRNA expression was quantified by comparing healthy and gallstone patients for the IFN- γ gene. When compared to the internal β -actin gene, gallstone patients displayed a significant increase in fold change of 3.4008 with a $P < 0.008$. Figure 3(b): Melt peak defines the specificity of the gene by showing single peak for every amplification cycle

Measuring of IFN- γ serum levels by ELISA

While collecting blood samples from each participant serum samples were preserved at -80°C . ELISA was performed for all 60 participants by following the kit instructions. The results showed a remarkable $P < 0.0001$ value in the mean serum of gallstone patients when compared to the control group (17.06 vs 33.12). It was established that

nearly twice the serum intensity levels of IFN- γ found were in patients than in controls Figure 4 and Table 5. From this findings protein levels were fall under same line as expression studies. From these findings, we can conclude that there must be some connection with IFN- γ protein marker in the progression of gallstone disease.

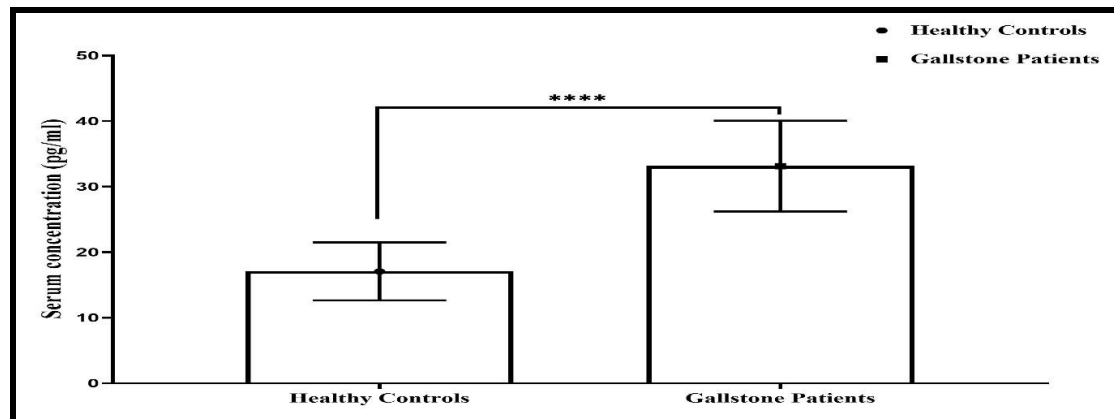


Figure 4. Assessment of serum levels from both healthy and gallstone patients was represented. The serum levels of patients were found to have high IFN- γ levels with respect to the Healthy controls with a significant $P < 0.0001$. Data was expressed in Mean $\pm$ SD and performed in duplicates

Discussions

The prevalence of gallstone disease has climbed recently and is widespread in the general population. It is estimated that between 10 and 15 percent of people in the United States, Europe, and other wealthy nations have gallstone disease (Di Ciaula *et al.*, 2018). Worldwide, 5% to 15% of adults suffer from gallstone disease, making it a common condition in the general population (Everhart *et al.*, 1999; Shaffer, 2005; 2006; Chen *et al.*, 2006). Gallstones can exacerbate cardiovascular disease and are linked to it. According to the one of the clinical studies, it has also been linked to an increased risk of coronary artery disease, cerebrovascular issues, atrial fibrillation, hyperlipidaemia, T2DM, and hypertension (Sun *et al.*, 2022).

Furthermore, it has been suggested that inflammation is a crucial factor in the development of gallstones, as evidenced by the fact that in both human and animal models, histopathology changes in the gallbladder wall that show inflammation occur before the production of cholesterol gallstones (Rege, 2000). The likelihood of cholesterol gallstones is also linked to a number of inflammation-related disorders, including diabetes, obesity, and infections (Fremont-Rahl *et al.*, 2013). The relationship between inflammatory proteins and gallstone risk, however, has not been thoroughly studied (Shengelia *et al.*, 2012).

Gallbladder epithelial cells showed different patterns of mRNA expression for different cytokines and chemokine's. They also changed how they expressed specific cytokine and chemokine RNAs and secreted more TNF- α protein in response to LPS treatment. Cholangitis, cholestasis, and

cholelithiasis are among the biliary inflammation and disorders that are significantly impacted by the biliary epithelial cell's capacity to regulate endogenous cytokine and chemokine production (Savard *et al.*, 2002).

Cytokines may sometimes have a systemic influence when inflammation is taken into account. The development of the inflammatory process is linked to proinflammatory cytokines, which support the recruitment of neutrophils and macrophages at the site of infection, enhance their phagocytic and bactericidal capabilities, and initiate the antigen-specific immune response process (Wertz *et al.*, 2018). TNF- α is recognized to be important in the initiation of inflammation by stimulating the production of other inflammatory mediators, including IL-6, which influences the gallbladder's absorption, secretion, and smooth muscle contractility (Gilloteaux *et al.*, 2003). Ebadi *et al.* claim reported that TNF- α causes gallbladder inflammation and accelerates the formation of gallstones, IL-4 prevents gallstones from forming (Ebadi *et al.*, 2013). Compared to IL-1, TNF- α , and IL-6, patients with gallstones showed substantially reduced levels of IL-4 (Issa and Muthena, 2014).

A research study from Shanghai, China, demonstrated that there is a substantial correlation between the inflammation proteins measured in bile and those in circulation, indicating that blood samples may be used to better understand the relationship between inflammation and gallstone risk (Koshiol *et al.*, 2016). A few past studies reported that Gallstone development is linked to several human gene polymorphisms, including those in the apo B, apo A1, LDL receptor, and apo E genes (Mittal and Mittal, 2002; Jaime *et al.*, 2010). However, these genetic variables are not exclusive

to gallstone formation. Cytokine is another element that plays a part in the production of gallstones. Hepatocytes, gallbladder epithelium, and different immune cells have all been proven to produce cytokines, which are small molecular weight protein mediators (Rosen *et al.*, 1997). Biliary tract and gallbladder epithelial cells (GBEC) have been demonstrated to produce cytokines such IL-6, IL-8, TNF- α , and MCP-1 and to exhibit IL-6 and TNF- α receptors (Reynoso-Paz *et al.*, 1999; Savard *et al.*, 2002).

The findings of another investigation mentions that a correlation between gallstone disease in connection with inflammatory cytokines, like IL-1 α , IL-6, IL-8, and tumor necrosis factor TNF- α was far or less strongly associated with gallstone disease compared with gallbladder cancer risk (Shengelia *et al.*, 2012; Koshiol *et al.*, 2016). Although they established a correlation between inflammatory cytokines and gall stone disease but unable to explain how cytokines function in the gallbladder and how they affect the development of gallstones (Reynoso-Paz *et al.*, 1999; Maurer *et al.*, 2009). A few studies has established that IL-10 primarily prevents the generation of pro-inflammatory cytokines, such as IL-12, by lipopolysaccharide (LPS) and bacterial matter (Aste-Amezaga *et al.*, 1998). IL-12 can cause T cells and natural killer cells to produce more TNF- α , which has an immediate impact on gallbladder epithelial cells' activities, secretion, and absorption (Rege, 2000). Since IgE production, which causes allergic inflammation and several disorders, including asthma, can be controlled by interleukin-13, the link between gallstones and asthma warrants more research (Rael and Lockey, 2011). One of the research study reported that Gallstone disease depends on the level of cytokine and IL-4 deficiency might accelerate gallstone formation in mice. The same study also shown that IFN- γ increased in patients with gallstones, contrary to IL-4, which did not affect cholesterol metabolism (King *et al.*, 2002; Kitasato *et al.*, 2007). Furthermore, they made clear that IL-4 has some therapeutic effects on gallstones and biliary diseases, while IFN- γ has harmful effects (Ishige *et al.*, 2008).

A diagnostic study reported that blood levels of CRP, also plays an important role as a blood diagnostic marker in inflammatory process of gallstone disease. The current study also performed safety test for all 60 participants. The results of safety tests were promising with other studies in which LFT parameters like ALP and Bilirubin, KFT tests like Urea and serum creatinine, sugar tests like cholesterol, HDL, and VLDL and Haematology tests RBC, Lymphocytes, and ESR shown significant increase in patients having gallstone

disease when compared with healthy controls. This shows that there may be blockage of the bile duct, due to which there is a chance of an increase in bilirubin, possibility of formation of cholesterol crystals and inflammation of blood cells may be because of inflammatory cytokines. Apart from above tests other investigations were also performed like ALT, AST, Triglycerides, and LDL shows increase levels in gallstone patients but statically not statistically significant with controls.

As of now, atleast to our knowledge, there is no direct evidence of IFN- γ role in gallstone disease. Hence this is the first attempt with control based study trying to establish the role of IFN- γ role in relation of gallstone disease. In order to establish the importance of IFN- γ like previous other cytokines, we performed mRNA expression studies with gallstone patients by comparing them with Healthy controls. The current study found a significant increase mRNA expression levels with a 3.41-fold change for IFN- γ gene in gallstone patients compared with healthy controls with a ($P < 0.001$). This implies that IFN- γ gene might be triggering or over-accelerating through several other inflammatory cytokines like TNF- α , IL-6, IL-10 and IL-12 genes which in turns elevates mRNA levels in gallstone patients. To validate further, the current study also measured the serum concentrations of the IFN- γ gene in both healthy controls and people with gallbladder disease. The results also fall under the same line as earlier studies, that the serum concentrations for the IFN- γ gene showed significant increase in gallstone patient (17.06 ± 4.436 vs 33.12 ± 6.92) when compared with healthy controls. Hence this establishes that there may be some connection between inflammatory cytokine IFN- γ with gallstone disease.

Conclusions

The goal of the current study is to investigate the role of the IFN- γ gene, an inflammatory biomarker that affects many cell types and has wide-ranging effects on the immune system's maturity and functionality. Although IFN- γ can affect several disease processes in addition to gallstone inflammation, its clinical uses are presently limited. IFN- γ plays a part in the development of gallstone disease, much like other inflammatory cytokines do. Nevertheless, this study had certain limitations. Firstly, the study population's sample size was small. Secondly, our study only contained gene expression and serum estimations, thus our findings cannot be extrapolated to identify accurate diagnostic measures. As a result, we take into account all recommendations and remarks, carry out

this investigation, and incorporate some of the aforementioned concerns into further studies. Additionally, we can better understand the course of gallstone disease by doing so with a larger sample size, immunohistochemistry investigations and *in vitro* studies on hepatocellular carcinoma and biliary tract cancer cell lines, and associated with its signaling pathway studies

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Declaration of AI Tools

Quillbot was used to rephrase the sentence in the manuscript and Napkin AI tool was used for making Graphical abstract

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