STUDIES ON GENETIC AND IMMUNOLOGICAL FACTORS IN CHRONIC SUPPURATIVE OTITIS MEDIA WITH SPECIAL EMPHASIS ON TOLL-LIKE RECEPTOR 4 PATHWAY

Thesis submitted for the award of

DOCTOR OF PHILOSOPHY

degree based on the research carried out in the department of

CELL BIOLOGY AND MOLECULAR GENETICS

Under the Faculty of Allied Health and Basic Sciences

by

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April 2023

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LIST OF ABBREVIATIONS

µg	Micrograms
µl	Microliters
µM	Micro molar
°C	Celsius
cDNA	Complementary-Deoxyribonucleic acid
CLR	C-Lectin-like Receptors
CSOM	Chronic Suppurative Otitis Media
Ct	Cycle threshold
DAMP	Damage Associated Molecular Patterns
DNA	Deoxyribonucleic Acid
dNTPs	Deoxyribonucleotide Triphosphate
dsDNA	Double Stranded DNA
ELB	Erythrocyte Lysis Buffer
ELISA	Enzyme Linked Immunosorbent Assay
FBS	Fetal Bovine Serum
g	Grams
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
h	Hours
H_3PO_4	Phosphoric acid
HCl	Hydrochloric acid
IL	Interleukin

IL-10	Interleukin 10
IκB	Inhibitor kappa B
LAF	Laminar Air flow
mg	Milligrams
MgCl2	Magnesium chloride
ml	Milliliter
mRNA	Messenger RNA
MyD88	Myeloid Differentiation Primary response 88
Na_2HPO_4	Di-sodium hydrogen phosphate
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NCBI	National Center for Biotechnology Information
NFkB	Nuclear factor kappa light chain enhancer of activated B
NIMHANS	National Institute of Mental Health and Neurosciences
NLR	Nucleotide binding and oligomerization domain like Receptor
NOD	Nucleotide binding and oligomerization domain
PAMP	Pathogen Associated Molecular Patterns
PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PRR	Pathogen Recognition Receptor
qPCR	Quantitative Polymerase Chain Reaction

RIG	Retinoic acid- inducible gene
RLR	RIG-like Receptors
RNA	Ribonucleic acid
RPMI	Roswell-Prank Memorial Institute
RT	Room temperature
SDUAHER	Sri Devaraj Urs Academy of Higher Education and Research
SDUMC	Sri Devaraj Urs Medical College
Sec	Seconds
SPSS	Statistical package for social sciences
Taq	*Thermus aquaticus*
TIRAP	Toll/Inteleukin-1 Receptor Domain- Containing Adaptor Protein
TLR4	Toll-Like Receptor 4
TNFα	Tumour Necrosis Factor

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Abstract

ABSTRACT

Bacterial infection of the middle ear is a common problem, which is cleared by an innate immune response referred to as Acute Otitis Media (AOM). The acute response resolves its own, except in a subset of patients who experience progression into chronic suppurative otitis media (CSOM). The chronic response results in inflammatory damage to the middle ear. Uncontrolled inflammation in CSOM has been linked to the overactivation of the Toll-like receptor 4 (TLR4) pathway. This pathway is activated by lipopolysaccharide (LPS) released from the gram-negative bacterial cell wall. Currently, there is limited information about the factors responsible for overactivation of TLR4 pathway in CSOM. One possible factor could be the hypersensitivity of the TLR4 pathway in CSOM patients. Therefore, the present study aimed to evaluate the role of genetic and immunological factors on the TLR4 pathway in CSOM patients.

This is a case-control study, it comprises of CSOM patients (n=63) and healthy controls (n=63). After obtaining the written informed consent, the peripheral blood samples were collected, isolated the peripheral blood mononuclear cells (PBMCs), and cultured for 4 h with and without LPS treatment. The expression levels of *TLR4* and *NFkB* genes were measured in cultured cells by quantitative polymerase chain reaction (qPCR). The levels of TNFα and IL-10 cytokines were measured in the conditioned media using an enzyme-linked immunosorbent assay (ELISA). The genetic variation in the *TLR4* gene promoter region was analysed by using Sangers sequencing method.

LPS-induced fold change in the expression of the *TLR4* gene (2.8 vs. 1.6; $p<0.001$; Student's t-test) and the *NFkB* gene (3.8 vs. 1.4; $p<0.001$; Student's t-test) were higher in the CSOM group than in control group. Furthermore, LPS-induced fold change in TNFα protein production were higher in the CSOM group compared to control group (3.2 vs. 1.1; $p<0.001$; Mann Whitney U test). LPS-induced fold change in IL-10 protein production was lower in the CSOM group compared to control group (0.58 vs. 0.78; $p<0.001$: Mann Whitney U test). The relationship between components of the TLR4 pathway of the three markers in the CSOM group was evaluated by using correlation analysis. There was a positive correlation between *TLR4* and *NFkB* gene expression levels ($r=0.77$, $p=0.01$, Pearson's correlation), and a positive correlation between *NFkB* gene expression and TNFα protein levels were observed ($r=0.37$, $p=0.01$, Spearman's rho correlation). Furthermore, the *TLR4* gene expression was also analysed after subgrouping of CSOM patients based on clinical profile. However, the gender, laterality, and disease duration did not affect the LPS-induced fold change in *TLR4* and *NFkB* gene expression, TNFα and IL-10 protein expression levels. Further, to find weather the *TLR4* gene genetic variation is associated with CSOM, four single nucleotide polymorphisms (SNPs) in the *TLR4* gene promoter region were selected since they are predicted to be transcription factor binding motifs. SNPs in the *TLR4* gene promoter regions such as -2604 G>A, -2570 A>G, -2604 G>A, and -2026 A>G were identified. The genotype and allele frequency distribution showed no statistically significant difference between the two groups ($p>0.05$; Fisher's exact test). This indicates that the *TLR4* gene

promoter region SNPs (-2604 G>A, -2570 A>G, -2604 G>A, and -2026 A>G) may not be associated with CSOM.

The results of the present study support the conclusion that the stimulation of PBMCs with LPS leads to relatively higher levels of *TLR4* and *NFkB* gene expression, and elevated levels of TNFα pro-inflammatory cytokines in CSOM. LPS-induced fold change in *TLR4* gene expression is correlated with *NFkB* gene expression and the *NFkB* gene expression is correlated with TNFα production. These results indicate that the TLR4 pathway is intrinsically hypersensitive to LPS in CSOM. This suggests that the CSOM may arises when a person with intrinsic hypersensitivity to the TLR4 pathway infected with bacteria in the middle ear. Therefore, future studies need to explore the TLR4 pathway for developing drugs that can mitigate the inflammatory damage in CSOM.

Introduction

1. Introduction

Otitis Media (OM) is an infection of the middle ear that causes inflammation and accumulation of fluid behind the eardrum. Based on the severity of the infection, OM is classified as Acute Otitis Media (AOM) and Chronic Supportive Otitis Media (CSOM). AOM is an infection of rapid onset that usually presents ear pain and no tympanic membrane perforation. But, CSOM presents persistent infection and more than three episodes in a month with tympanic membrane perforation [Bhutta et al., 2017; Acuin et al., 2007].

The global estimate of the prevalence rate of CSOM cases with discharging ears ranges between 1-46%. According to World Health Organization (WHO), the highest prevalence of CSOM was observed in Western Pacific countries, 2.5-43%, followed by 0.9-7.8% in South East Asia, 0.4-4.2% in South Africa, 3% in Central America, 1.4% in Eastern Mediterranean, and 0.4% in Europe [Muftah et al., 2015]. A recent study from India also showed that the prevalence of CSOM is 5.2% [Bellad et al., 2019]. CSOM leads to serious health complications such as brain abscess, hearing loss, meningitis, facial nerve paralysis, etc. [Hutz et al., 2018]. Understanding the pathophysiology is required for the better management of patients with CSOM.

CSOM involves inflammatory damage to the middle ear mucosa [Schilder et al., 2016]. Inflammation of the middle ear (swelling, fluid collection, and blockage of the Eustachian tube in the middle ear) is caused by bacteria and viruses [Bakaletz et al., 2010]. Certain immunological factors include innate

defense molecules, such as complement factors, cytokines, lysozyme, defensins, and chemokines are responsible for initiating a response to pathogens [Massa et al., 2003, Bluestone et al., 2003]. These immunological factors potentially have a pathogenic role in causing the disease [Mittal et al., 2014]. Persistent inflammations were observed in CSOM patients [Morris et al., 2012; Mittal et al., 2015]. Current evidence indicates that the inflammatory cytokine (IL-8, TNF, IL-1, and IL-6) were increased in the middle ear mucosa of CSOM patients [Elmorsy et al., 2010; Si et al., 2014]. These cytokines are produced by the activation of Pattern Recognition Receptors (PRRs); one of the significant PRRs is Toll-like receptors (TLRs). Activation of TLRs triggers the release of several pro-inflammatory cytokines [Trune et al., 2015; Leichtle et al., 2011].

Exaggerated inflammation, mainly *via* the Toll-like receptor 4 (TLR4) pathways, was shown to be one of the major influencing factors involved in the development of OM [Jung et al., 2021]. Middle ear infection results in the activation of inflammation to clear the infectious agents. These results in a condition called AOM. However, in some patients, the inflammation does not resolve after the clearance of infectious agents and becomes chronic. Chronic inflammation then results in damage to the middle ear. The factors responsible for the progression of Acute OM to CSOM are currently unclear. Therefore, this study aimed to evaluate genetic and immunological factors role in the TLR4 pathway in CSOM patients.

Aim, Objectives, Rationale, and Significance

2. Aim, Research question, Objectives, Rationale, and Significance

2.1. Aim of the study

To evaluate genetic and immunological factors role in the TLR4 pathway in CSOM patients.

2.2. Research questions

1. Does alterations in the TLR4 pathway lead to CSOM?
2. Does genetic variation in the *TLR4* gene and gene expression increase the TLR4 activity in CSOM?

2.3. Objectives of the study

1. To quantify the effect of LPS on *TLR4* gene expression levels in the PBMCs of CSOM patients and healthy controls.
2. To quantify the effect of LPS on *NFkB* gene expression levels in the PBMCs of CSOM patients and healthy controls.
3. To assess the effect of LPS on TNFα and IL-10 cytokines levels in the PBMCs of CSOM patients and healthy controls.
4. To evaluate the influence of genetic variation in promoter region *TLR4* gene in CSOM patients and healthy controls.

2.4. Rationale

In CSOM patients, the TLR4 pathway may be more sensitive. A potent TLR4 agonist can cause an excessive cellular response. The key receptor and transcription factors of the TLR4 pathway, such as TLR4 (receptor) and NFkB (transcription factor), may be upregulated as a result of this hypersensitivity and secretes of pro-inflammatory cytokines [Guijarro-Muñoz et al., 2014]. This study evaluated this phase by comparing the LPS-treated expression of *TLR4* and *NFkB* genes and the production of pro-inflammatory and anti-inflammatory cytokines. TNFα and IL-10 are the two most important cytokines associated with CSOM [Kuczkowski et al., 2011]. LPS is a representative ligand to activate the TLR4 pathway. TLR4 is known to be expressed on blood cells such as monocytes, macrophages, dendritic cells, and epithelial cells [Zmonarski et al., 2019]. These PBMCs of CSOM patients and healthy controls can be used as a model to study the TLR4 pathway in CSOM. The function of the TLR4 pathway in PBMCs is probably similar because intrinsic factors are genetically determined. Therefore, the aim of this study was to investigate the components linked to the TLR4 pathway in CSOM patients.

This study hypothesized that LPS treatment of PBMC samples would result in the upregulation of *TLR4* and *NFkB* gene expression and elevated TNFα production in CSOM patients compared with healthy controls. In other words, samples from CSOM patients are likely to show hyper-sensitivity to LPS compared to healthy controls.

2.5. Significance of the study

CSOM is a multifactorial disease with a genetic component, and less is understood about the pathogenesis of CSOM. Therefore, understanding the pathophysiological basis of CSOM is necessary to uncover novel therapeutic targets. The results of this study will also contribute to elucidating the molecular mechanism by which the TLR4 pathway plays a role in the pathogenesis of CSOM and alterations in the TLR4 pathway lead to CSOM. Then it can be considered as a drug target. This study provides the mechanistic role of the TLR4 pathway in CSOM patients. The knowledge obtained from this study might be helpful in developing therapeutic strategies to treat/better management of CSOM.

If the TLR4 is hyperresponsive to prevent the development of AOM to CSOM, anti-TLR4 therapies can be designed. This study will also clarify the function of *TLR4* and *NFkB* gene expression and enhanced TNFα cytokine production, which will contribute to understanding the mechanistic basis for the elevated TLR4 levels in CSOM. The information generated from this study might help to develop therapeutic strategies for ameliorating the progression of AOM to CSOM.

Review of Literature

3. Review of Literature

3.1. Clinical features of Otitis Media

3.1.1. Signs and symptoms of OM

The common symptoms of OM include irritability, difficulty sleeping or staying asleep, pulling at one or both ears, fever, fluid draining from the ear, hearing problems, and ear pain [Sharma et al., 2015]. The common signs of OM are the infection in the middle ear, granulation of tissue often seen in the middle ear space, and the external auditory canal may be oedematous.

3.1.2. Classification of Otitis Media

Otitis media is classified based on the severity of the disease into two subtypes.

1. Acute Otitis Media (AOM)
2. Chronic Supportive Otitis Media (CSOM)

AOM starts with a middle ear infection with a fever. CSOM involves persistent infection and continuous ottorrhea for more than three episodes in a month with a tympanic membrane perforation [Mittal et al., 2015; Levi et al., 2022].

3.1.3. Burden of CSOM

CSOM affects 5.2% of the Indian population [Bellad et al., 2019]. The global estimate of the prevalence rate ranges between 1-46% of cases with discharging ears. According to World Health Organization (WHO), the highest

prevalence of CSOM was observed in Western Pacific countries (2.5-43%) [Muftah et al., 2015]. These high prevalence shows that CSOM is a major public health burden in India. An understanding of the anatomical structure of the middle ear is necessary for the better management of CSOM.

3.2. Anatomy of the ear

The external, middle, and inner ears are the three interconnected parts of the ear (Figure 3.1) [Hawkins et al., 2023]. The cochlea, sometimes known as the organ of hearing, is located in the inner ear, which also houses the body's organ of balance. On the other hand, the external and middle ears are primarily concerned with the transmission of sound. The external ear canal, also known as the external auditory canal or external auditory meatus (EAM), is about 2.5 cm long and coated with skin. The outer part of the pinna is made up of cartilage, and the skin lining has hair follicles and wax-producing glands made up of ceruminous, and sebaceous glands. At the end of the external ear canal, the tympanic membrane (eardrum) is present. The middle ear has an irregular shape and is lined with mucosa. Although commonly thought of as an air-filled space, it is filled with nitrogen-rich gas [Hussain et al., 2016]. The external and middle ear are separated by the tympanic membrane, which has a slightly oval shape and a maximum diameter of 9-10 mm. Most of this membrane, located at the bottom portion of the tympanic membrane, is made up of the pars tensa. There are three layers:

1. Epithelium continuous with the external ear canal
2. Fibrous middle layer

3. Mucosal layer lining the whole of the middle ear and upper respiratory tract

Above the pars tensa, in a triangular region without a fibrous layer, is the pars flaccida. This increases its susceptibility to cholesteatoma, an ear condition brought on by a buildup of dead skin cells in the middle ear that results in discharge and hearing loss. The inner ear consists of the cochlea (organ of hearing) and the peripheral vestibular apparatus (organ of body balance). The cochlea is a thick, two and third-turn structure that resembles a snail. It is oriented sideways and contains the organ of the Corti. The inner ears spiral canal ranges from 29-40 mm. The peripheral vestibular system controls balance and coordinates head and eye movement [Harkin et al., 2021].

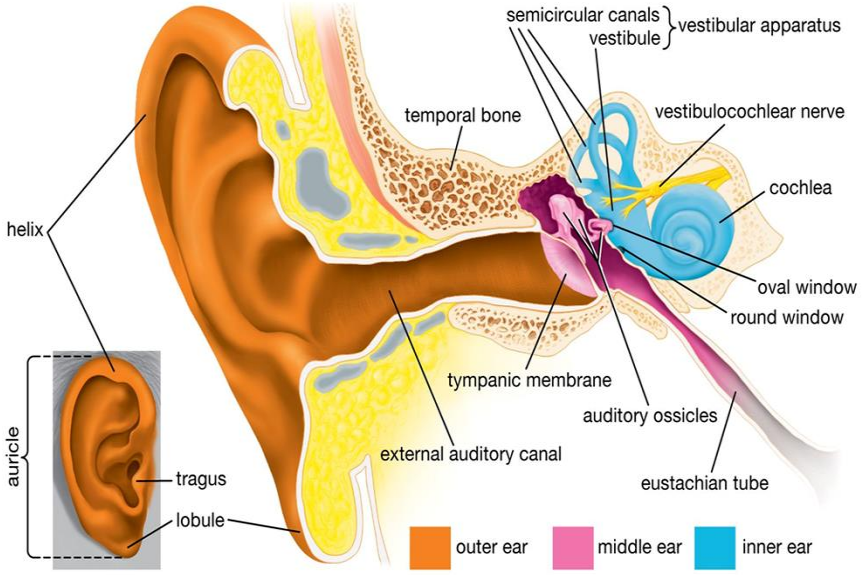


Figure 3.1: Ear anatomy (**Source**: *Hawkins et al., 2023).*

3.3. Pathogenesis of CSOM

CSOM typically develops after an AOM. The infected nasopharyngeal secretions are either aspirated, insufflated, or refluxed via the tube into the middle ear when the tympanic membrane raptures and an upper respiratory tract

infection develops and results in CSOM. Bacteria such as *Pseudomonas aeruginosa, Proteus mirabilis, Escherichia coli*, and *Klebsiella* species are commonly found in the middle ear fluid of CSOM patients [Afolabi et al., 2012]. The pathogenesis of CSOM is also associated with one or more risk factors such as upper respiratory tract infection, host factors, impaired immunologic state, familial predisposition form of feeding, and environmental [Kong et al., 2009].

3.4. Bacteriology of CSOM

Middle ear bacterial infection is the most common cause of OM. Major bacterial species such as the primary causes of AOM include *Streptococcus pneumoniae, Haemophilus influenza,* and *Moraxella catarrhalis*[Sierra et al., 2011; Qureishi et al., 2014]. The most common aerobic microbial isolates in CSOM patients are *Pseudomonas aeruginosa* and *Staphylococcus aureus*, followed by *Proteus Vulgaris* and *Klebsiella pneumoniae* [Khan et al., 2020; Prakash et al., 2013; Sattar et al., 2012; Aduda et al., 2013]. According to numerous studies from different nations, including India, Nepal, Singapore, and Nigeria, *P. aeruginosa*, followed by S. aureus, are the highly prevalent bacterial species in CSOM [Sharma et al., 2010; Dayasena et al., 2011; Madana et al., 2011; Afolabi et al., 2012; Asish et al., 2013]. Studies from Pakistan (Gilgit), Iran, and Saudi Arabia, however, demonstrated that P. aeruginosa and S. aureus were the two most prevalent pathogens associated with CSOM [Ettehad et al., 2006; Mariam et al., 2013]. The differences in the patient populations and geographic variation may be responsible for the differences in the various studies (Table 3.1).

Table 3.1: List of the bacteria observed in CSOM patients.

S. No	Bacteria	Frequency (%)	Reference
1	*Pseudomonas aeruginosa*	33.0	Khan et al., 2020
	Staphylococcus aureus	30.0	
	Pseudomonas spp	17.1	
2	*Staphylococcus aureus*	48.7	Prakash et al., 2013
	Pseudomonas aeruginosa	19.9	
3	*Pseudomonas aeruginosa*	38.0	Satter et al., 2012
	Staphylococcus aureus	22.0	
4	*Proteus spp*	32.7	Aduda et al., 2013
	Enterococcus	28.6	
	Staphylococcus aureus	12.8	
	Pseudomonas spp	11.3	
5	*Pseudomonas aeruginosa*	77.0	Sharma et al., 2010
6	*Pseudomonas species*	29.5	Dayasena et al., 2011
	staphylococcus	20.5	
7	*Pseudomonas aeruginosa*	32.0	Madana et al., 2011
	Proteus mirabilis	20.0	
	Staphylococcus aureus	19.0	
8	*Pseudomonas aeruginosa*	31.3	Afolabi et al., 2012
	Klebsiellaspp	23.9	
	Staphylococcus spp	16.4	
	Proteus mirabilis	14.9	
	Streptococcus spp	11.2	
9	*Pseudomonas aeruginosa*	33.0	Asish et al., 2013
	Staphylococcus aureus	25.8	
	Proteus mirabilis	20.6	
	Enterobacteraerogenes and Streptococcus spp	4.1	

10	*Staphylococcus aureus*	38.4	Ettehad et al., 2006
	pseudomonas aeruginosa	29.6	
	Proteus	10.5	
11	*Staphylococcus aureus*	65.2	Mariam et al., 2013
	Pseudomonas aeruginosa	15.2	
	Proteus mirabilis	13.0	
	Escherichia coli	6.2	

3.5. Cellular and molecular basis of inflammation

Pathogens and poisonous substances are two examples of the many things that can cause tissue damage, and inflammation is an innate immune response that repairs it [Chen et al., 2017]. The inflammatory response is the coordinated activation of signaling pathways that control the amount of inflammatory mediators in both native tissue and inflammatory cells [Sugimoto et al., 2016]. The following summarizes the common mechanism responsible for the inflammatory response.

1. Recognition of stimuli by cell surface pattern receptors
2. Activation of inflammatory pathways
3. Release of inflammatory markers and chemotaxis
4. Resolution of inflammatory damage

3.5.1. Recognition of stimuli by cell surface pattern receptors

The "Recognition of infection or injury" sets off the inflammatory cascade. This is typically accomplished by the Pattern Recognition Receptors (PRRs) detecting Pathogen Associated Molecular Patterns (PAMPs) and Damage Associated Molecular Patterns (DAMPs) [Zindel et al., 2020]. The PRR

signaling pathway activation is followed by transcription factors and the production of pro-inflammatory cytokines [Liu et al., 2017].

3.5.2. Activation of inflammatory pathways

The ligand binds to its receptor and activates the downstream signaling molecules in various pathways such as the TLR4 pathway, TLR2 pathway, and RAGE pathway. The activation of these pathways transduces the signal that activates the transcription factor NFkB, which is present in all cell types and remains dormant when coupled to its inhibitor protein, Ik-B. When a signal is received, NFkB is released from Ik-B and moves to the nucleus, which binds to certain promoter areas to start transcription of pro or anti-inflammatory cytokines such as IL-1-, IL-6, and TNFα [Yu et al., 2020].

3.5.3. Release of inflammatory cytokines and chemotaxis

The release of pro-inflammatory cytokines and chemokines impacts the cellular activities of other cells. The cytokines and chemokines activate the immune cells, such as neutrophils and monocytes, to reach the site of an injury or infection. These cells further release harmful substances from their cytoplasmic granules, resulting in the creation of a cytotoxic environment. The degranulation process and the rapid release of chemicals necessitate the consumption of both glucose and oxygen, known as a "respiratory burst." Both pathogens and host cells are destroyed by these substances [Kany et al., 2019]. These interactions combine to produce the classic symptoms of local

inflammation, including heat (calor), swelling (tumor), redness (rubor), and pain (dolor).

3.5.4. Resolution of inflammatory damage

The last stage of inflammation is the resolution of infectious agents, essential for preventing host collateral damage. After the initial hours of inflammation, tissue-resident and recruited macrophages initiate a coordinated program of resolution [Watanabe et al., 2019]. These cells produce pro-inflammatory prostaglandins and leukotrienes during acute inflammation but quickly switch to lipoxins, which prevent the recruitment of more neutrophils and encourage the infiltration of primed macrophages crucial for clearance of the bacteria [Sugimoto et al., 2016].

3.6. Mediators of the inflammation

In general, during tissue injury or bacterial infections, the inflammation can be mediated by several factors. The inflammatory process involves several mediators which can act as ligands (PAMPs and DAMPs), receptors (TLRs), signal transducers (NFkB), and cytokines (pro- and anti-inflammatory) [Liu et al., 2017; Amarante-Mendes et al., 2018].

3.6.1. Pathogen-associated molecular patterns

PAMPs are molecules with conserved motifs linked to pathogen infection that act as ligands for host pattern recognition molecules like Toll-like receptors (TLRs) [Carrillo et al., 2017]. PAMPs start an inflammatory response brought upon by a pathogen [Li and Wu, 2021]. PAMPs are formed from microbial

components and are conserved molecular patterns found in a particular class of microbes, such as lipopolysaccharide and peptidoglycan found in cell walls, DNA, RNA, viral proteins from the envelope, capsid, glycolipids, and glycoprotein, etc.

3.6.2. Damage-associated molecular patterns

Danger-associated molecular patterns/alarmins/danger signals are responsible for the clearance of cell or tissue damage [Roh et al., 2018]. DAMPs are biomolecules capable of inducing "sterile inflammation," a non-infectious inflammatory response [Chen et al., 2017]. DAMPs differ significantly based on the kind of cell and the tissue affected [Vénéreau et al., 2015]. DAMPs denaturate as they transition from a reducing to an oxidizing environment, which alerts immune cells to detect their molecular pattern and induce an immune response [Relja et al., 2020]. Cytosolic or nuclear DAMPs are released into the extracellular environment due to tissue damage [Schaefer et al., 2014]. Examples of protein DAMPs are heat shock proteins, fragments of hyaluronan, and HMGB1 (high mobility box-1), and the non-protein DAMPs are uric acid, DNA, and heparin sulfate.

3.6.3. PRR and its action in cells

The primary receptors involved in inflammation are PRRs [Kumar et al., 2019). The body needs to be informed of the presence of potentially dangerous pathogens when a microbial infection occurs. Specifically designed receptors called PRRs are mostly expressed on immune cells such as cytotoxic natural killer cells, dendritic cells, monocytes, neutrophils, macrophages, and epithelial cells [Gasteiger et al., 2017]. Two groups of molecules, i.e., damage-associated molecular patterns, which are linked to cellular damage, and pathogen-associated molecular patterns, which are linked to microbial infections, can bind to PRRs and transduce the signal inside the cell.

3.6.3.1. Classification of PRRs

The PRRs can be classified into two types (membrane-bound and cytoplasmic) based on their location in the cells. PRRs are specific for PAMPs and DAMPs from several sources and can be expressed on cell surfaces [Li et al., 2021]. TLRs and c-lectin-like receptors (CLRs), are examples of membrane-bound PRRs, and the cytoplasmic PRRs are RIG-1-like receptors (RLRs) and nucleotide binding and oligomerization domain like receptors (NLR).

3.6.3.1.1. Membrane-bound receptors

Specialized protein molecules known as membrane receptors are incorporated into or linked to the cell membrane and can detect PAMPs and DAMPs released by the pathogens and are present in the extracellular environment. The following are the main families of PRRs [Boutrot et al., 2017].

3.6.3.1.1.1. Toll-like receptors (TLRs)

TLRs are type I membrane proteins that have a cytoplasmic tail with a conserved area known as the toll/IL-1 receptor domain and an extracellular domain with leucine-rich repeats (TIR) [Ruysschaert et al., 2015]. There are currently 11 known members of the TLR family "(TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TLR10, TLR11, TLR12, and TLR13). They can recognize PAMPs, which can mediate the inflammatory response [Yarovinsky et al., 2005; Brightbill et al., 2000]. A list of the sources of ligands, PAMPs, and the site of TLR expression is shown in Table 3.2 [Takeuchi et al., 2010].

Table 3.2: List the type of TLRs, ligands and its sources, and localization.

Type of TLR	Ligand	Source of ligand	Localization
TLR1	Triacyl lipoprotein	Bacteria and Mycoplasma	Plasma membrane
TLR2	Lipoprotein	Bacteria (Gram+ve), viruses, and parasites	Plasma membrane
TLR3	dsRNA	Viruses	Endolysosome
TLR4	LPS	Bacteria (Gram-ve) and viruses	Plasma membrane
TLR5	Flagellin	Bacteria	Plasma membrane
TLR6	Diacyl lipoprotein	Bacteria and viruses	Plasma membrane
TLR7 & 8	ssRNA	Bacteria, viruses	Endolysosome
TLR9	CpG-DNA	Viruses, bacteria, and protozoa	Endolysosome

TLR10	Unknown	Unknown	Endolysosome
TLR11	Profilin	Protozoa	Plasma membrane

Toll-like receptor 4 (TLR4) and its signaling pathway: TLR4 is a single-pass type I glycoprotein, one of the best-characterized PRRs, because it was the first member of the TLR family to be found. TLRs and IL-1Rs share a conserved region of ~200 amino acids in their cytoplasmic domain, known as the Toll/interleukin-1 (IL-1) receptor (TIR) domain. There are three conserved regions (boxes) that are essential for signal transduction within the TIR domain. Leucine-rich repeats (LRRs) are found in 19-25 tandem copies in the extracellular domain of TLR4 [Yang et al., 1999: Akira et al., 2004; Korneev et al., 2016] (Figure. 3.2). For ligand recognition, extracellular LRR domains are essential. It recognizes lipopolysaccharide (LPS), a part of the cell wall of gram-negative bacteria. In the study, we are particularly interested in TLR4 because the TLR4 can recognize PAMPs (Ex: LPS) released by bacterial infections and plays a major role in the pathogenesis of CSOM [Jung et al., 2021].

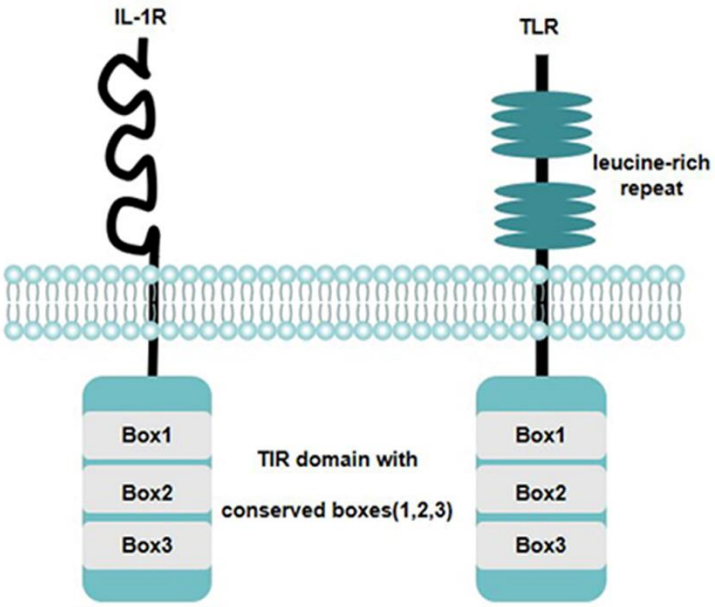


Figure 3.2. The schematic representation of the structure of TLRs (Source: *Yang et al.1999*)**.**

Signaling pathway: The studies using LPS as a ligand provided the most comprehensive understanding of TLR4 activation and the signaling pathway. LPS recognition is initiated by an LPS binding to an LBP protein. Several proteins in the cytoplasm can mediate the activation and transduction of TLR4. This LPS-LBP complex transfers the LPS to CD14. CD14 is a glycosylphosphatidylinositol-anchored membrane protein that binds to the LPS-LBP complex and facilitates the transfer of LPS to MD-2 protein, which is associated with the extracellular domain of TLR4. LPS binding promotes the dimerization of TLR4/MD-2. The conformational changes of the TLR4 induce the recruitment of intracellular adaptor proteins containing the TIR domain, which is necessary to activate the downstream signaling pathway [Lu et al., 2008] (Figure 3.3).

TLR4 signaling is typically split into two major pathways: MyD88-dependent and TRIF-dependent, also referred to as the MyD88-independent pathway. There are four adaptor proteins involved in two major intracellular signaling pathways, including myeloid differentiating primary response gene 88 (MyD88), TIR adaptor protein (TIRAP), TIR-domain-containing adapter-inducing interferon-β (TRIF), and TRIF-related adaptor molecule (TRAM) [O'Neill et al., 2013]. Both of these pathways frequently cause the production of inflammatory cytokines by activating the traditional NFkB pathway. We have concentrated on the MyD88-dependent pathway in this study.

In the MyD88-dependent pathway, TLR4 dimerization causes the association of MyD88 (Figure 3.3). MyD88 then attracts IL-1 receptor-associated kinases (IRAK) 4 by interacting with the death domain (DD) and enabling the attachment of IRAK1. After that, the IRAK4 phosphorylates and activates IRAK1, which prompts TNF receptor-associated factor 6 (TRAF6) to join the receptor complex. The transforming growth factor activated kinase (TAK1), TAK1-binding protein, and phosphorylated IRAK1 and TRAF 6 then separate from the receptor [Merolla et al., 2014]. Ubiquitin-conjugating enzyme 13 (UBC13) and ubiquitin-conjugating enzyme E2(UEV1A) variant 1 are two ubiquitin ligases that the complex containing TRAF6, TAK1, TAB1, and TAB2 binds with after IRAK1 is degraded at the plasma membrane. TRAF6 is then destroyed, and TAK1 is subsequently activated. TAK1, in turn, phosphorylates both the inhibitor of nuclear factor-κB (IκB)-kinase (IKK) complex consisting of IKKα, IKKβ, and IKKγ, then induces the phosphorylation of IκB. The activation leads to its ubiquitination and subsequent degradation, thereby allowing NFkB to translocate to the nucleus and induce the expression of inflammatory cytokines [Akira et al., 2004; Lu et al., 2008; Palsson-McDermott et al., 2004] (Figure 3.3).

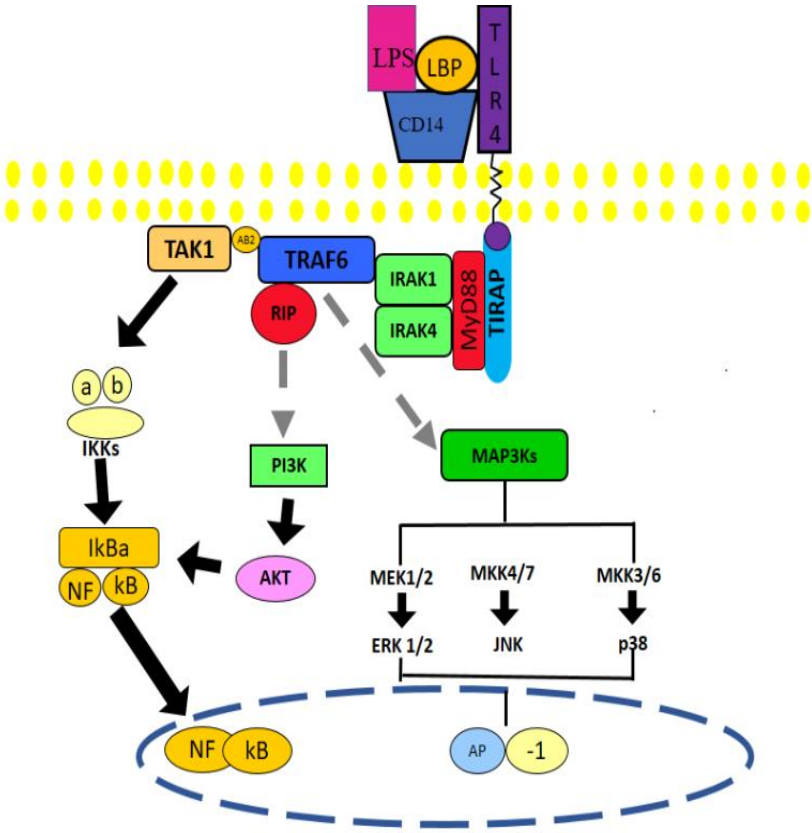


Figure 3.3: TLR4 signaling pathway; Abbreviations: **LBP:** LPS binding protein, **CD14:** a cluster of differentiation 14, **MD-2:** Myeloid differentiation protein 2, **TLR:** toll-like receptor, **MyD88:** myeloid differentiating primary response gene 88, **IRAK:** IL-1 receptor-associated kinase, **TRAF:** Tumor-necrosis-factor receptor-associated factor 6, **TAK:** Transforming growth factor β activated kinase, **TAB:** TAK1-binding protein, **UBC13:** Ubiquitin-conjugating enzyme 13, **UEV1A:** Ubiquitin-conjugating enzyme E2 variant 1, **NFkB:** Nuclear factor κB, **IKK:** Inhibitor of NFkB (IκB)-kinase.

The present study examined the crucial transcription factor NFkB, which binds to DNA and activates post-translational changes in cytokine genes [Smale et al., 2011]. Since NFkB is a transcription factor that pro-inflammatory cytokines and microbial products can activate rather than being cell type-specific, NFkB is regarded as the principal regulator of inflammation [Ben-Neriah and

Karin, 2011]. The functions of NFkB in inflammation are, in fact, exceedingly complex. NFkB may have a direct anti-inflammatory effect by inducing the expression of anti-inflammatory genes and its pro-inflammatory role in the development of short-term inflammation. TNF*α* and IL-10 have been studied in the present study (as representative pro- and anti-inflammatory cytokines).

TNFα: The master of inflammation and a vital component of the cytokine network is TNF*α*, an inflammatory cytokine released by macrophages and monocytes during acute inflammation. It is in charge of a wide spectrum of cell signaling events that result in necrosis or death of cells/pathogens [Idriss et al., 2000]. Two transmembrane receptors (TNFR1 and TNFR2), through which TNF signals, control various vital cell processes, including cell division, proliferation, survival, and death. Activation of TNFR1 seems to be primarily responsible for pro-inflammatory. TNF*α* has the power to stimulate the production of additional pro-inflammatory cytokines, including IL-1 and several chemokines [Parameswaran and Patial., 2010]. Therefore, TNF*α* is a key player in inflammation and is said to be crucial in the emergence of many chronic inflammatory diseases [Fujiwara et al., 2005]. The development of TNF*α* blocking antagonists has completely changed how CSOM and other inflammatory illnesses are treated.

IL-10: A cytokine with strong anti-inflammatory qualities known as IL-10 is essential for controlling the host immune system response to infections, minimizing host injury, and preserving healthy tissue homeostasis [Iyer et al., 2012]. The IL-10 protein is a homodimer; each of its subunits is 178-amino-

acid long [Zdanov et al., 1995]. The IL-10 receptor (IL-10R) is made up of at least two subunits (IL-10R1 and IL-10R2), it will interact with IL-10 to carry out its activity. While IL-10R2 primarily serves as an auxiliary component for signaling, IL-10R1 has a high-affinity ligand-binding role [Slobedman et al., 2009].

3.6.3.1.1.2. C-lectin like receptors

Myeloid cells are the primary source of the expression for the family of transmembrane pattern recognition receptors known as C-type lectin-like receptors (CLRs). Several different types of receptors called CLRs bind to carbohydrates in a calcium-dependent manner [Agier et al., 2018]. Additionally, these receptors function in the identification and removal of fungal infections [Goyal et al., 2018]. Based on their molecular structure, there are three groups of CLRs. Type I transmembrane protein containing several CRDs or CRD-like domains (DEC-205) and macrophage mannose receptor (MMR). Type II transmembrane CLRs typically carry a single CRD domain and include Dectin-1, Dectin-2, macrophage-inducible C-type lectin (Mincle), dendritic cell-specific ICAM3-grabbing non-integrin (DC-SIGN), and DC NK lectin group receptor-1 (DNGR-1). Mannose-binding lectin (MBL), an oligomeric protein that interacts with a variety of carbohydrate patterns on pathogen surfaces, is one type of soluble CLR [Gringhuis et al., 2007].

3.6.3.1.2. Cytoplasmic PRRs

The PRRs localized within the cell in the cytoplasm or enclosed in endosomes are called cytoplasmic PRRs [Hu et al., 2018]. Cytosolic PRRs are crucial in the recognition and clearance of viral nucleic acid. Some major Cytoplasmic PRRs are RIG-like receptors **(**RLRs), new DNA-binding factors, and compounds with nucleotide-binding domains and leucine-rich repeats (NLRs).

3.6.3.1.2.1. Nucleotide-binding and oligomerization domain-like receptors

The primary role of NLRs in inflammasome assembly is the recruitment of inflammatory caspases [Kim et al., 2016]. The innate immune system cytosolic multiprotein oligomers, known as inflammasomes, are in charge of triggering inflammatory reactions. NLRs are divided into five families based on their N-terminal domains: NLRA, NLRB, NLRC, NLRP, and NLRX [Carriere et al., 2021].

3.6.3.1.2.2. RIG-like receptors

A class of cytosolic RNA helicase proteins known as the RIG-I-like receptor (RLR) family of PRRs can recognize viral RNA as nonself by attaching to PAMPs motifs found in RNA ligands that build up as a result of virus infection. Through RLR stimulation of downstream effector molecules, including type I interferon (IFN) and other pro-inflammatory cytokines, which work to induce antiviral and inflammatory gene expression within the local tissue, this connection subsequently triggers an innate antiviral response inside

the infected cells [Ramos et al., 2011]. The three major types of RIGs are cytoplasmic RNA helicases, Melanoma Differentiation Associated Gene 5 (MDA5), Retinoic Acid Inducible Gene I (RIG-I), and Laboratory of Genetics and Physiology 2 (LGP2) [Chen et al., 2017].

3.7. Role of TLR4 pathway in mediating inflammation in OM/CSOM

Recent studies highlight that TLR4 has an important role in the pathogenesis of OM and CSOM. The expression of the *TLR4* gene, downstream signaling pathway genes, and inflammatory cytokines have been reported to be altered in CSOM. In addition to expression, The SNPs in the TLR4 pathway genes were also reported to be associated with CSOM. Hence, we have reviewed and summarized the recent literature on the TLR4 pathway components below.

3.7.1. Expression levels of *TLR4* gene in OM/CSOM

TLR4 expression is associated with the etiology of OM and is expressed in AOM, CSOM with cholesteatoma, and CSOM without cholesteatoma [Trzpis et al., 2014]. Studies have shown that up-regulated *TLR4* gene and *NFkB* gene expression are associated with OM. Hirai et al. (2013) evaluated the histological expression of toll-like receptor 2, 4 in tissue samples from patients with CSOM with cholesteatoma. They observed that the human middle ear tissue samples had higher levels of TLR4 expression [Hirai H et al., 2013]. Si Y et al. (2012) evaluated the expression of TLR2 and TLR4 in normal canal skin, mucosa, granulation tissue, mucosa, granulation tissue, and cholesteatoma epithelium. They observed that the TLR4 protein and mRNA levels were higher in the

mucosa of CSOM and chole OM than in normal canal skin using qRT-PCR [Si Y et al. 2012]. Tuoheti et al., (2021) also showed increased *TLR4* gene expression in the CSOM group, compared to healthy individuals, shows that the TLR4 pathway may play a role in controlling chronic inflammation in controlling chronic inflammation in CSOM and the change from AOM to CSOM. This work established LPS-induced CSOM mouse models and gathered clinical CSOM samples to confirm this. In addition, knock-down of the Nrf2 gene reversed the chronic inflammation to attenuate CSOM by up-regulating TLR4. Therefore, the evidence shows that *TLR4* gene expression contributes to the development of CSOM [Tuoheti et al., 2021]. Aksel et al., (2021) studied the effect of Lipopolysaccharide (LPS) and Lipoteichoic acid (LTA) stimulation on the expression of TLR pathway genes in PBMCs of Akkaraman lambs *in vivo* using qRT-PCR, the results showed that the *TLR4* mRNA expression levels in 24 h LPS with the LTA group increased compared to the control. Therefore, the LPS with LTA stimulated lamb PBMCs more effectively than the separate administration of LPS and LTA at 24 h. These findings suggest that toll-like receptors may play a principal role in human CSOM [Aksel et al., 2021]. The list of the studies and their main findings on the TLR4 pathway in OM/CSOM has been shown in Table 3.3.

Table 3.3: List of studies on the expression of the *TLR4* gene in OM/CSOM.

Objective	Major findings	Reference
Human studies		
To evaluate the histological expression of TLR2 and 4 in tissue samples from patients with CSOM with cholesteatoma	Elevated TLR4 and TLR2 expression in middle ear tissue samples with CSOM with cholesteatoma compared to normal	Hirai H et al., 2013
To investigate the differential expression of *TLR2* and *TLR4* in the CSOM and cholesteatoma	*TLR2/TLR4* gene and protein expression in the mucosa of CSOM and cholesteatoma were higher than normal canal skin	Si et al., 2014
Animal/Human studies		
To evaluate the knock-down of Nrf2 reversed chronic inflammation to attenuate chronic suppurative otitis media by up-regulating TLR4 using LPS-induced AOM, CSOM mouse model, and Middle ear tissue samples	Elevated *TLR4* gene and cytokines in both CSOM and LPS-induced mouse model	Tuoheti et al., 2021
Animal studies		
LPS and LTA treated TLR pathway genes in PBMCs of Akkaraman lambs	*TLR4* mRNA expression levels in 24 h LPS with the LTA group increased compared to the control	Aksel et al., 2021

3.7.2. Expression of inflammatory cytokines in OM/CSOM

The end response of the TLR4 pathway is to produce the cytokines which can clear the infectious agents. The tumor necrosis factor (formerly known as TNFα) and its role in OM have been well documented [Skotnicka et al., 2000]. One of the most potent inducers of inflammation in otitis media is TNFα, which also triggers the release of other inflammatory cytokines. The secretion of inflammatory cytokines frequently occurs in large quantities in middle ear effusions [Catanzaro et al., 1991]. Moreover, OM occurs more frequently and lasts longer when TNFα is expressed in more significant concentrations [wine et al., 2012].

Previous studies were focused on the levels of TNFα in serum and middle ear effusion fluids. Up to a six-fold increase in TNFα and IL-6 has been observed in the serum of CSOM patients compared to the controls [Baike et al., 2017]. Similar differences in the levels of cytokines have been observed in AOM [Chkhaidze et al., 2007]. Furthermore, the levels of TNFα cytokine in OM patients with allergy were higher than in OM patients having no allergy [Jang et al., 2002]. Also, the levels of TNFα in middle ear effusion fluid are higher in culture-positive than negative [Kaur et al., 2015]. Together, these reports indicate that TNFα levels are higher in CSOM. However, the basis for its increase was not fully evaluated in different populations. These results indicate that an increased level may arise due to the intrinsic hyper responsiveness of immune cells in CSOM patients to TNFα activators.

Genetic factors such as up-regulated gene expression appear to underlie the intrinsic hyper-responsiveness of TNFα activators in CSOM patients. Expression of the *TNFA* that codes for the TNFα protein is five-fold higher, and IL-1β, IFN-γ, and IL-6 in the middle ear of CSOM than in controls [Si Y et al., 2014]. Similarly, a higher expression of the *TNFA* gene was seen in the middle ear of AOM patients [MacArthur et al., 2011]. Studies on TNFα expression in CSOM patients with cholesteatoma. A cross-sectional comparative of 16 CSOM patients with cholesteatoma and 16 samples of normal ear skin carried out TNFα gene expression by qRT-PCR, and the results showed that the expression of TNFα in CSOM patients with cholesteatoma was higher than in normal ear skin. Therefore, they concluded that the expression of TNFα and IL-6 in CSOM patients with cholesteatoma was significantly different from patients with normal ear skin [Edward et al., 2019]. The expression levels of TNFα, IL-1α, IL-6, and IL-10 were determined by western blot analysis of tissue samples obtained during ear surgery. The expression levels of TNF-α, IL-1α, and IL-6 in cholesteatoma tissues were substantially higher than those determined in the granulation tissue. The study conclusion revealed an increased expression of TNFα, IL-1α, and IL-6 in chronic otitis media [Kuczkowski et al., 2011]. These findings indicate that the levels of TNFα were elevated in CSOM. Table 3.4 shows the expression of various cytokines after activation of the TLR4 pathway in OM/CSOM.

Table 3.4: List of the studies on the expression of various cytokines in OM/CSOM.

Objectives	Major findings	Reference
To determine TNFα and IL-6, serum cytokine level in the patient CSOM	Up to a six-fold increase in TNFα and IL-6 cytokine has been observed in the serum of CSOM patients compared to the controls	Baike et al., 2017
To determine TNFα and IL-6 the serum cytokine level in the patient AOM	In AOM patients, as compared with healthy individuals, the concentrations of both TNFα and IL-6 increased	Chkhaidze et al., 2007
To investigate TNFα, IL-4, and IL-6 cytokine levels in the MEEs of children with persistent OM	The levels of IL-4, IL-6, and TNFα in MEE were higher in the positive allergy group than in the negative allergy group in OM.	Jang et al., 2002
To determine the levels of TNFα, IL-6, IL-8, IL-10, and IL1-β in middle ear effusion fluid are higher in culture-positive than negative in OM	Higher levels of TNFα, IL-6, IL-8, and IL1-β in culture-positive than negative in OM middle ear effusion fluid	Kaur et al., 2015
To determine the levels of TNFα, IL-1β, IFN-γ, and IL-6 in human middle-ear mucosae sampled non-OM group and CSOM group	TNFα IL-1β, IFN-γ, and IL-6 cytokines are higher in the middle ear of CSOM than in controls	Si Y et al., 2014

To determine the expression of TNFα and IL-6 in CSOM patients with cholesteatoma in tissue samples	The expression of TNFα in CSOM patients with cholesteatoma was higher than in normal ear skin	Edward et al., 2019
To determine expression levels of TNFα, IL-1α and IL-6 cytokines in tissue samples in CSOM patients compared to normal tissue	The expression levels of TNFα, IL-1α, and IL-6 and less IL-10 in cholesteatoma tissues are higher than in the granulation tissue	Kuczkowski et al., 2011

3.7.3. Single nucleotide polymorphisms of *TLR4* gene in OM/CSOM

In addition to the variation in the expression of TLR4 genes, recent studies have also investigated the *TLR4* gene polymorphisms and its association with several human diseases, including CSOM. In a population, the *TLR4* gene polymorphism exists, and frequencies are >5%. That includes two missense mutations in the TLR4 ectodomain, Asp299Gly (rs4986790) and Thr399Ile (rs4986791). Emonts et al., (2007) studies on TLR4 and its polymorphism in AOM TLR4 SNPs 896 A/G Asp299Gly (rs4986790), they found that the SNPs are associated with AOM ($p<0.05$) in the Dutch population [Emonts et al., 2007]. Jotic et al., (2015) found that the polymorphisms in *TLR4* gene SNPs cSNPs 896 A/G, 196 C/T pSNPs Asp299Gly (rs4986790) Thr399IIe (rs4986791) were association with CSOM [Jotic et al., 2015]. Harfens et al., (2015) studied predisposition to childhood OM and genetic polymorphisms in *TLR4* gene SNPs (NG_011475:1.g.12375A>T, rs5030717, rs1329060, rs1329057) and found that the SNPs were positive association with the OM with a significant difference

(p=0.003, p=0.002, and p=0.003) in finish cohort [Harfens et al., 2015]. Alpay et al., (2010) studied the SNPs (896 A/G and Asp299Gly (rs4986790)) in the *TLR4* gene of tympanosclerosis patients and found that the SNPs were positively associated with OM (p<0.05) in turkey [Alpay et al., 2010]. MacArthur et al., (2014) studied genetic susceptibility to CSOM: candidate gene SNPs in *TLR4* gene 5 (SNPs rs11788138,rs4837494, rs10116253, rs1927914, rs1554973) in the Caucasian population. They found that the SNPs were positively associated with CSOM with significant differences (p=0.008, p=0.031, p=0.007, p=0.023, p=0.021) [MacArthur et al., 2014]. Table. 3.5 summarises the list of studies on SNPs in the *TLR4* gene in OM/CSOM.

Table 3.5: List of studies on the SNPs in the *TLR4* gene in OM/CSOM.

Population	cSNPs	pSNPs	rs number	P value	Reference
Dutch	896 A/G	Asp299Gly	rs4986790	0.05	Emounts et al., 2007
Serbian	896 A/G	Asp299Gly	rs4986790	NS	Jotic et al., 2015
	196 C/T	Thr399IIe	rs4986791		
Finnish	NG_0114 75:1.g.12 375A>T	Intergenic	rs5030717	0.003	Harfens et al., 2015
			rs1329060	0.002	
			rs1329057	0.003	
Turkey	896 A/G	Asp299Gly	rs4986790	0.05	Alpay et al., 2010
Caucasian	Tag SNPs	Intergenic	rs1178818	0.008	MacArthy et al., 2014
		Intergenic	rs4837494	0.031	
		Intergenic	rs1011623	0.007	
		Intergenic	rs1927914	0.023	
		Intergenic	rs1554973	0.021	

3.8. Role of *TLR4* gene expression in other chronic inflammatory diseases

The importance of TLR4 in mediating inflammation has been reported in several other diseases. The upregulation of *TLR4* gene expression was observed in several diseases, such as breast cancer cells, coronary arteriosclerosis, and Alzheimer's diseases [Yang et al., 2010; Geng et al., 2006; Miron et al., 2018; Eißler et al., 2011]. Studies have evaluated the expression of the *TLR4* and *TLR2* genes in breast cancer cell lines using qRT-PCR and observed that the *TLR4* gene expression was the highest expressed TLR in the MDA-MB-231 breast cancer cell line compared to other TLRs. Therefore, the knock-down of the *TLR4* gene could actively inhibit the proliferation and survival of breast cancer cells [Yang et al., 2010]. Studies on the expression of TLR4 protein and mRNA levels in PBMCs of patients with coronary arteriosclerosis disease (CAD) were analyzed using flow cytometry (FCM) and qPCR and observed that *TLR4* gene and protein expressions in the CAD group were significantly higher compared to control. Therefore, the expression levels of the *TLR4* gene in PBMCs are increased in atherosclerotic patients [Geng et al., 2006]. *TLR4* gene expression and pro-inflammatory cytokines in rodents with Alzheimer's were analyzed using qRT-PCR. Alzheimer's disease of human postmortem brains also showed increased expression of *TLR4* and *TNFα* genes. Similarly, a mouse model of hippocampal deafferentation without amyloidosis (i.e., the entorhinal cortex lesioned mouse) also showed a significant increase in the *TLR4* gene expression compared to sham-lesioned mice [Miron et al., 2018]. *TLR4* gene expression in mice with cardiovascular diseases showed an increased *TLR4* gene expression [Eißler et al.,

2011]. Therefore, the literature indicates that the expression of the *TLR4* gene plays a major role in other chronic inflammatory diseases. Table 3.6 shows the studies on *TLR4* gene expression in chronic inflammatory diseases condition**.**

Table 3.6: List of studies on *TLR4* gene expression in inflammatory conditions.

Objectives	Major findings	Reference
Cell lines studies		
To evaluate the *TLR4* gene expression and protein in breast cancer cell lines	*TLR4* gene expression is highest in the MDA-MB-231 breast cancer cell line compared to other TLRs	Yang et al., 2010
Human studies		
To evaluate the *TLR4* gene expression on PBMCs in atherosclerotic	*TLR4* gene in the CAD group was significantly higher than that in the controls	Geng et al., 2006
Animal/Human studies		
To evaluate the *TLR4* and *TNFα* gene expression in Alzheimer's Disease of Rodents	Alzheimer's disease of human Postmortem brains has observed increased expression of *TLR4* and *TNFα* genes	Miron et al., 2018
To evaluate the *TLR4* gene expression was determined in untreated SHR rats compared to normotensive Wistar-Kyoto rats	Elevated *TLR4* gene expression in hypertensive rats (SHR)	Eißler et al., 2011

3.8. Lacunae in knowledge

The literature review shows that the TLR4 pathway is involved in several other inflammatory conditions and CSOM. However, the molecular mechanisms leading to aberrations in inflammatory pathways, particularly the TLR4 pathway in CSOM, are unknown. Middle ear infection results in the activation of inflammation to clear the infectious agents. These results in a condition called Acute OM. However, in some patients, the inflammation does not resolve after the clearance of infectious agents and becomes chronic. Chronic inflammation then results in damage to the middle ear tissue. Currently, the factors responsible for the progression of Acute OM in CSOM are unclear. However, the hyper-responsiveness of the TLR4 pathway has not been explored in CSOM, and the paucity of information on the functional status of the TLR4 pathway in CSOM has yet to be evaluated; therefore, the present study aims and objectives were generated.

Materials and Methods

4. Materials and Methods

4.1. Materials

The materials and methods followed in the present study were as follows.

4.1.1. Chemicals and Reagents

Agarose: Sigma (Cat. # A9539)

Antibiotic and antimycotic solution: HiMedia (Cat. # A002)

Big Dye Terminator v3.1 Cycle Sequencing Kit: Thermo Scientific (Cat. # 4337455)

Chloroform: Merck **(**Cat. # 102447)

Diethyl pyro carbonate (DEPC) Treated Water: Thermo Fisher Scientific (Cat. # AM9915G)

Di-sodium hydrogen phosphate (Na_2HPO_4): (Cat. # CAS 7558-79-4)

DNA ladder (100 bp): Genei (Cat. # 6126526710017300

DNA gel loading dye (6X): Thermo Fisher Scientific (Cat. # R0611)

dNTPs: Genei (Cat. # 61060250005130)

Ethanol: Merck (Cat. # 100983)

Ethidium bromide*:* Sigma Aldrich (Cat. # E7637-25G)

Ethylenediamine tetra acetic acid (EDTA): Thermo Fisher Scientific (Cat. # 17892)

Fetal bovine serum albumin (FBS): Gibco (Cat. # 10270-016)

Ficoll histopaque-1077: Sigma Aldrich (Cat. # 10771)

Gene JET PCR Purification kit: Thermo Scientific (Cat. # K0701)

Hydrochloric acid (HCl): SDFCL (Cat. # 20125L25)

IL-10 ELISA Kit: Krishgen Biosystem (Cat. # KB1072)

IScript cDNA kit: Bio-Rad (Cat. # 1708891)

Lipopolysaccharide (LPS): Sigma (Cat. # 2654)

Methanol: Merck (Cat. # 106009)

Potassium hydrogen orthophosphate (K_2HPO_4): Fisher Scientific (Cat. # CAS 7758-11-4)

Primers for PCR: Sigma-Aldrich

Proteinase K: Genei (Cat. # 612150121001710)

RPMI-1640 medium (Powder form): Gibco (Cat. # 3100022)

So advanced universal master mix: Bio-Rad (Cat. # 1725271)

Sodium bicarbonate ($NaHCO_3$): Thermo Fisher Scientific (Cat. # 25080094)

Sodium chloride (NaCl): HiMedia (Cat. # CAS: 7647-14-5)

Sodium dodecyl sulfate (SDS): Sigma-Aldrich (Cat. # V800386)

Sodium hydroxide (NaOH): Fisher Scientific (Cat. # 15895)

Taq DNA polymerase: Genei (Cat. # 610602500051730)

TNFα ELISA Kit: Krishgen Biosystem (Cat. # KB1145)

Tris (hydroxymethyl) aminomethane ($C_4H_{11}NO_3$): Sigma-Aldrich (Cat. # CAS 77-86-1)

Trizol reagent: Invitrogen (Cat. # 15596026)

4.1.2. Instruments

Centrifuge (Refrigerated): REMI (Model-CM-12)

CO_2 incubator: Thermo Fisher Scientific (Model-371)

Cooling centrifuge: Thermo Fisher Scientific (Model-Sarvall legend XTR)

DNA Sequencer: Applied Biosystem (Cat. # ABI 3130 Genetic analyzer)

Electronic balances: Sartorius (Model-GE 612-1)

Elisa reader: Merilyzer (Cat. # EIAQuant)

Freezer (-80 ˚C): Cryo Scientific (Model- URC-V-700-4)

Gel documentation system: Bio-Rad (Model-Gel Doc XR+ 170-8170)

Gel electrophoresis unit: Genei (Model-ETS-06)

Laminar air flow: Esco (Cat. # LAF 35)

Magnetic stirrer: REMI 15 ml (Model-5 ml HDX)

Microcentrifuge: Tarsons (Model-1000)

Microwave oven: LG Solo (Model-MW2049UM)

Millipore system: Millipore Milli-Q Advantage (Model: A10)

pH meter: Sartorius (Model-PB-11-P10)

Plate centrifuge: BR Biochem (Model-BIDH-100)

qPCR: Bio-Rad (Model-781BR16930)

Rotator (Circular cell mixer): Neo Lab (Model-CM-101)

Spectrophotometer: Perkin Elmer (Model-Lambda 35)

Spin win microcentrifuge: Tarsons (Model-10015140)

Thermal cycler (PCR): Bio-Rad (Model-C1000)

Vertical autoclave: Labline (Cat. # AV-104)

Vertical freezer (-20 ˚C): Bio-Equip (Model-BPS-345S)

Vertical laminar air flow chamber (VLAF): ESCO Biotech (ACB-4A1)

Vortex mixer: REMI (CM-101)

Water bath: Julabo (Model-TW-8)

4.1.3. Glassware

Beakers: Borosil (Cat. # 1002)

Duran bottles: Borosil (Cat. # 3321)

Glass cuvettes: Mettler Toledo (Model- 30675053)

Glass funnel: Glasil (30 mm)

Glass rod: MG Overseas (10 mm)

Measuring cylinders: Borosil (Cat. # 579)

Reagent bottles: Borosil (Cat. # 3321)

Standard flask: Borosil (Cat. # 5640029)

4.1.4. Plastic ware

6-well plate: Mat Tek's (Cat. **#** P06G-0-10-F)

Combs (1.0 mm): Tarsons (Cat. # 7087)

Cryo box (1.8 ml): Tarsons (Cat. # 524010)

Falcon tubes (15 ml): Abdos (Cat. # P10402)

Falcon tubes (50 ml): Tarsons (Cat. # 546041)

Heparin vacutainer: BD (Cat. # 367871)

K2EDTA vacutainer: BD (Cat. # 367856)

Micro centrifuge tube (1.5 ml): Tarsons (Cat. # 500010)

Microtips (0.2-10 µl): Abdos (Cat. # P10115)

Microtips (100-1000 µl): Abdos (Cat. # P10106)

Microtips (2-20 µl): Abdos (Cat. # P10130)

Parafilm: Sigma-Aldrich (Cat. # P7543)

Pasteur pipette: Tarsons (Cat. # 940060)

PCR cooler (96 well): Eppendorf (Cat. # Z606634)

PCR plate sealer: Bio-Rad (Cat**. #** MSB1001)

PCR plates (96 well): Bio-Rad (Cat. # HSP960196)

PCR tubes (0.2 ml): Tarsons (Cat. # 510051)

Syringe filters: HiMedia (Cat. # SF14)

Tube rack (2.0 ml, 15 ml, and 50 ml): Tuffblok (Cat. # 1164M14)

4.1.5. Preparation of reagents

20% sodium dodecyl sulfate (SDS): 20 g of SDS was dissolved in 80 ml of distilled water and made up to 100 ml.

5 M sodium chloride (NaCl): 29.2 g of NaCl was dissolved in 80 ml of distilled water and makeup into 100 ml. The solution was autoclaved and stored at room temperature.

70% Ethanol: 70 ml of ethanol and 30 ml of distilled water were added to the duran bottle and makeup to 100 ml.

70% Isopropyl alcohol (IPA): 70 ml of isopropyl alcohol was mixed with 30 ml of double distilled water.

Biotin conjugated detection antibody (5 ml): Added 5 µl of detection antibody to 4995 µl of (1X) assay diluent to make the final volume 5 ml.

Concentrated streptavidin: HRP conjugate (5 ml): Added 66.67 µl of concentrated streptavidin: HRP to 4995 µl of Streptavidin: HRP diluent to make final volume 5 ml.

Dilution 1: Diluted the recombinant protein by adding 5 µl of reconstituted standard solution in 495 µl of (1X) assay diluent to prepare 0.5 ml of 10 ng/ml.

Dilution 2: Added 50 µl (10 ng/ml) of dilution 1 to 950 µl of (1X) assay diluent to prepare a top standard of 500 pg/ml.

Erythrocyte lysis buffer (ELB): 8.29 g of 155 mM ammonium chloride, 1.002 g of 10 mM potassium bicarbonate, and 200 µl of 0.1 mM EDTA was dissolved in distilled water and adjusted pH=7.4.

Human TNFα and IL-10 standards (10 ng/ml): Reconstituted the lyophilized vail in 20 µl of distilled water to get a 1 µg/ml concentration.

Lipopolysaccharide (LPS) (10 ng/ml): 1 mg/ml of lyophilized powder LPS was dissolved in 1 ml of PBS. 5 µl of 1 mg /ml of LPS were dissolved in 45 µl of PBS.

NaOH (1 N): 4 g of NaOH was dissolved in 100 ml distilled water.

Phosphate buffer saline (pH=7.4): weighed 20.214 g of $Na_2HPO_4 \cdot 7H_2O$ and 3.394 g of $NaH_2PO_4 \cdot H_2O$ added to 800 ml of distilled water. Adjusted the pH=7.4 with 1 N HCl and 1 N NaOH and makeup to final volume 1 L.

Preparation RPMI working media: 80 ml of RPMI media stock + 20 ml of fetal bovine serum (FBS) (20%).

Primers working solution (10 µM): 10 µl of primer stock solution (100 µM) was added to an RNase- and DNase-free tube, and 90 µl of PCR-grade water mix by vertexing and at -20 °C, aliquoted and stored working primer solutions.

Proteinase K (10 mg/ml): 10 mg of proteinase K was dissolved in 1 ml of distilled water.

RPMI-1640 stock: Weighed 2 g of sodium bicarbonate, and 10.4 g of RPMI powder was dissolved in 500 ml of autoclaved distilled water. The pH was adjusted to 7.2 with 1 N HCl and 1 N NaOH and makeup to final volume 1 L.

Tris-acetate EDTA (TAE) buffer (1X) [Stock]: 242 g of Tris base, 100 ml of 0.5 M EDTA, and 57.1 ml glacial acetic acid were dissolved in distilled water and makeup to a final volume of 1000 ml.

Tris-acetate EDTA (TAE) buffer (1X) [Working]: 20 ml of TAE (50X) was dissolved in 990 ml of distilled water and make up to a final volume of 1000 ml.

Tris-EDTA buffer (1X) (pH=8.0): 1 ml of 1 M Tris-HCl (pH=8.0) and 0.2 ml 0.5 M EDTA (pH=8.0) and add to the duran bottle and makeup to 100 ml by adding 98.8 ml of distilled water.

Wash buffer (1X): 5 ml of wash buffer (20X) was reconstituted by adding 95 ml of distilled water.

4.2. Methods

4.2.1. Study design

The present study was carried out by following a case-control study design. A total of 126 subjects, including 63 CSOM patients, were recruited in the present study based on diagnostic criteria. The age of the study participants ranges between 18-60 years. The remaining 63 were age and gender-matched healthy controls without any history of chronic infections in the age group of 18-60 years. All the study participants were examined by otomicroscopic examination by a senior otorhinolaryngologist. Clinical details such as patient history, family history, and demographic parameters (age, gender, disease duration, and unilateral/bilateral) were collected using a structured proforma. The confirmed patients were given standard care of treatment irrespective of their participation in the study. The schematic representation of the overall study design followed in the present study was shown in Figure 4.1.

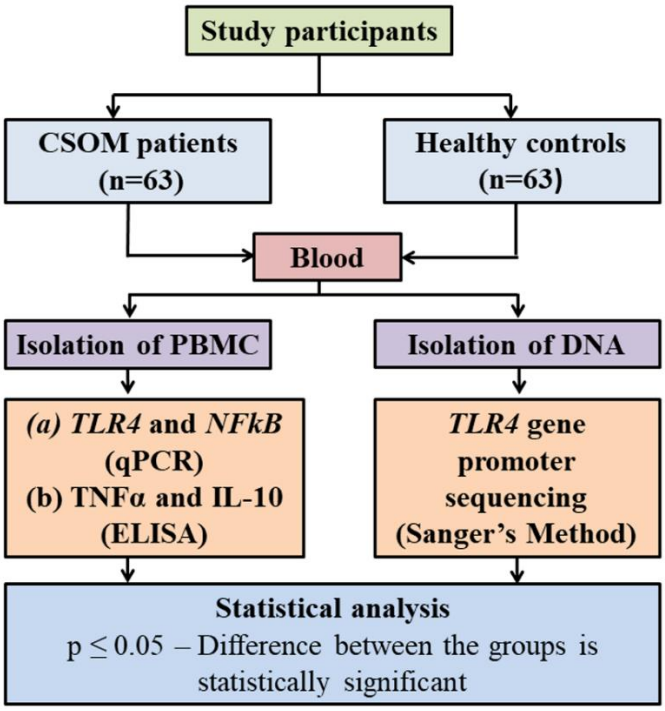


Figure 4.1: Schematic representation of the overall study design followed in the present study.

4.2.2. Ethical clearance

The study was conducted after obtaining approval from the Central Ethical Committee ***(Ref. No SDUAHER/KLR/CEC/01/2017-18)***, Sri Devaraj Urs Medical College, Sri Devaraj Urs Academy of Higher Education and Research, Kolar. CSOM patients and healthy controls were recruited between 2018-2020. Patient and healthy control informed consent was obtained in written form before enrolling into the study.

4.2.3. Selection of the study population

The study population is CSOM patients who are attending the Department of Otorhinolaryngology, which is affiliated with R. L. Jalappa Hospital and Research Centre, Kolar, Karnataka, India. CSOM patients and healthy controls

were enrolled upon satisfaction with the following criteria (Table.4.1-4.2) [Harmes et al., 2013].

Table 4.1: Inclusion and exclusion criteria for selection of CSOM patients

Inclusion	Exclusion
(i) Patients diagnosed with CSOM	(i) Post-traumatic CSOM
(ii) Unilateral and bilateral CSOM	(ii) Otomycosis
(iii) Both genders are in the age group of 18-60 years	(iii) Down's syndrome
	(iv) Cleft lip/palate
	(v) Acute otitis media

Table 4.2: Inclusion and exclusion criteria for selection of healthy control

Inclusion	Exclusion
(i) Willing to participate study	(i) Individual with a known family of genetic disease
(ii) Both genders are in the age group of 18-60 years.	
(iii) Without a history of any chronic infections	

4.2.4. Diagnosis of CSOM

i. Hard of hearing
ii. Chronic ear infection
iii. Tympanic membrane perforation
iv. Pure tone audiometry (PTA)

4.2.5. Sample size calculation

The sample size was calculated based on the difference in TNFα levels between the case and control groups. Previously reported average plasma TNFα levels in OM (11.9 pg/ml) and healthy controls (5.6 pg/ml) were used for the

calculation of the required sample size [Cetinkaya et al., 2019]. The minimum samples required to observe a difference was 6.3 pg/ml with a power of 80%, and 1% α error was determined to be 63/group. The sample size was calculated using the web-based tool OpenEpi version 3.01.

4.2.6. Sample collection and processing

Three ml of venous blood samples were collected from the CSOM patients, and the age, gender matched healthy controls after obtaining written informed consent. One ml of blood was transferred to EDTA vacutainer and Two ml of blood was transferred to heparin vacutainers. Blood samples collected in a heparinized vacutainer was used for the isolation of PBMC using Ficoll-histopaque. The isolated PBMCs were used for cell culture experiments and the isolation of RNA. The blood samples collected in EDTA vacutainer was used to isolate genomic DNA. The isolated DNA and RNA samples were stored at -80 °C until further use. A schematic representation of the entire experimental design followed in the present study was shown in Figure 4.2.

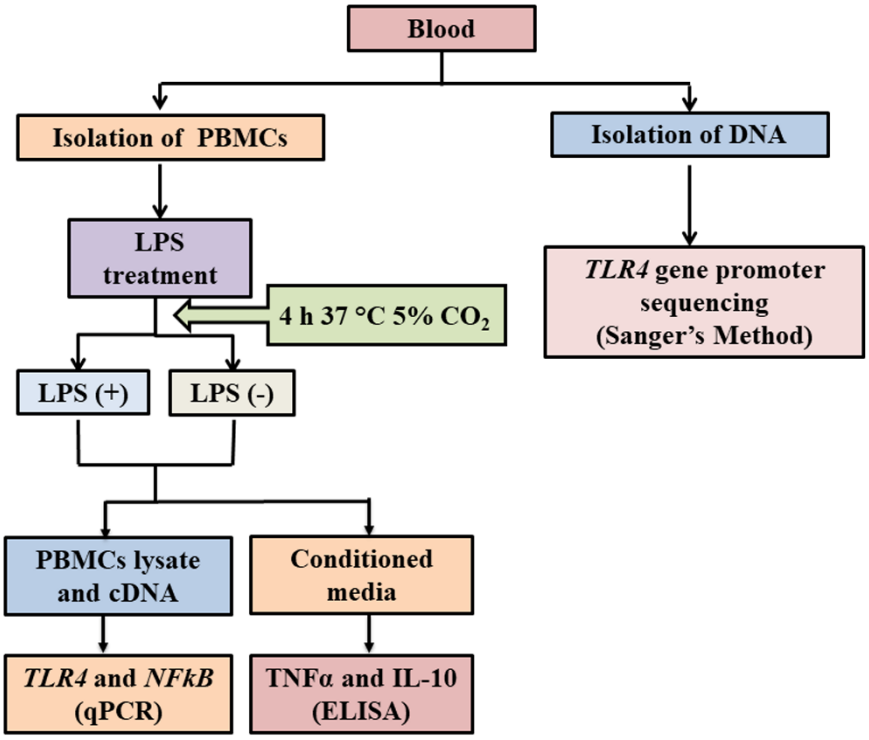


Figure 4.2: Schematic representation of the experimental protocol used in the present study.

4.2.7. Isolation of PBMCs

PBMCs were isolated from whole blood (2 ml) using Ficoll-Histopaque [Jia et al., 2018]. Briefly, 2 ml of heparinized blood sample was layered on an equal volume of Ficoll-histopaque and centrifuged at 2000 rpm for 30 min. After centrifugation, the PBMC layer was collected carefully and transferred to 15 falcon tubes containing sterile PBS. The PBMCs were counted using a Hemocytometer and used for *in vitro* culture and LPS treatment.

4.2.8. PBMC culture with and without LPS

PBMCs were isolated from 2 ml of blood samples from all the study participants. *In vitro* PBMC culture was set up in a 6-well plate by adding the following components: PBMCs ($1x10^5$ cells), 3 ml of working RPMI 1640

medium, fetal bovine serum (20%), antibiotics solution (1%), and lipopolysaccharide (10 ng/ml). The culture plates were incubated at 37 °C and 5% CO_2 for 4 h. After incubation, the cultures were centrifuged, and the supernatant was collected (conditioned medium), the cell pellet was used for RNA isolation and stored at -80 °C until further use [Broekman et al., 2015].

4.2.9. Isolation of total RNA using Trizol method

After 4 h of incubation, PBMC cultures were collected in eppendroff tubes, centrifuged for 10 min at 2000 rpm, and collected the supernatant. The pellet was washed with 1X PBS and used for RNA isolation using Trizol reagent [Chomczynski et al., 1993]. One ml of Trizol reagent was added to the pellet, mixed vigorously, and incubated for 5 min at room temperature. To the tubes, 200 µl of chloroform was added, mixed vigorously, and incubated at room temperature for 3 min. The tubes were centrifuged at 12000 rpm for 15 min at 4 °C. The supernatant was transferred to new eppendroff, added 500 µl of isopropanol and incubated at 4 °C for 10 min. The tubs were centrifuged at 12000 rpm for 10 min at 4 °C, discarded the supernatant, the pellet was washed with 70% ethanol by centrifugation at 12000 rpm at 4 °C for 10 min (repeated the wash step 3 times). The pellet was air dried for 10 min, dissolved in 50 µl RNase-free water, and the concentration and purity of the RNA were measured using a spectrophotometer. All RNA samples were stored at -20 °C until further use.

4.2.10. Conversion of total RNA into cDNA

Total RNA samples were converted to cDNA using a cDNA conversion kit (Bio-Rad) and the manufacturer's instructions. The composition of the reaction mixture for each 20 µl was as follows:

Contents	Volume/reaction (µl)
5X script reaction mix	4
Reverse transcriptase enzyme	1
Nuclease-free water	1

To 6 µl of the reaction mixture, 14 µl of total RNA was added, mixed thoroughly, and performed RT-PCR using the following conditions:

Steps	Temperature (°C)	Time (min)
Priming	25	5
Reverse transcription	46	20
RT inactivation	95	1
	Hold at 4° C	

4.2.11. *TLR4* and *NFkB* gene expression quantification using qPCR

The referential cDNA sequence of the *TLR4* and *NFkB* gene was retrieved from GenBank (Accession NO. NM_138554.5 and NM_001077494.3) Sequence-specific primer pairs were designed to amplify the exon boundary regions of the *TLR4* and *NFkB* genes using Primer Quest tool and IDT DNA software.

The designed primers were used in a real-time polymerase chain reaction (PCR). The composition of the reaction mixture for each 10 µl were as follows:

Contents	Volume/reaction (µl)
SYBR Green Supermix	5.0
Forward primer	0.5
Reverse primer	0.5
RNase-free water	3.0

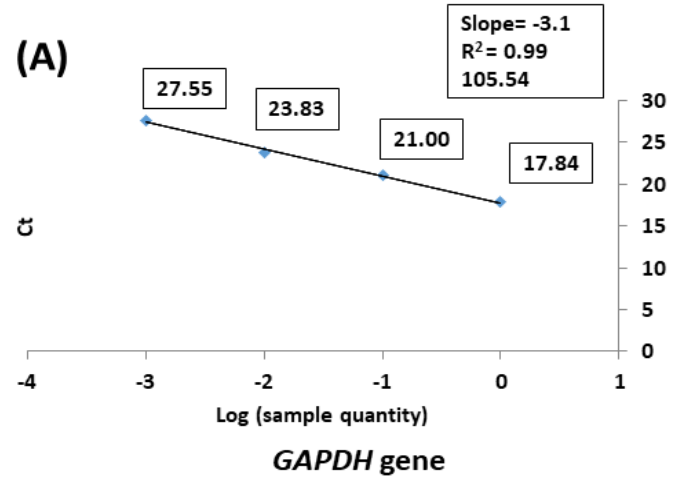
For 9 µl of the reaction mixture, 1 µl of cDNA was added, and performed the real-time quantification using CFX96 touch system. The qPCR reaction conditions were as follows:

Step	No. of cycles	Cycles	Temperatures (°C)	Duration
1	1	Initial denaturation	95	5 min
2	40 cycles	Denaturation	95	30 sec
3*	(steps 2-4)	Annealing	57	30 sec
4*		Extension		

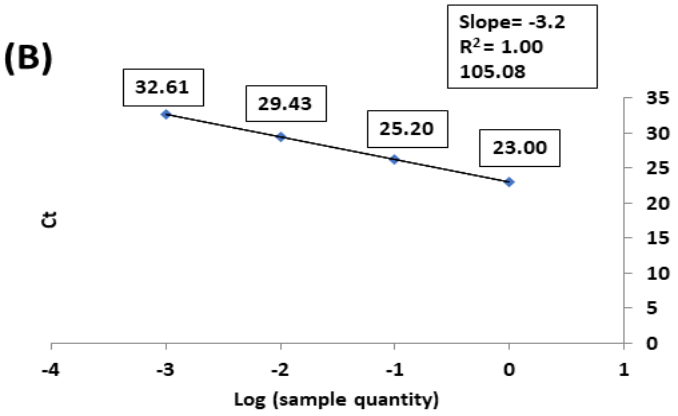
*=Plate read at Steps 3 and 4. Green fluorescence is detected at 57 °C.

Cycle threshold (Ct) values were obtained from the PCR amplification graphs. The gene expression data were normalized using *GAPDH*. The relative expression of the *TLR4* and *NFkB* genes was measured from the normalized Ct values using the ΔΔCt method [Raavi et al., 2019; Livak et al., 2001]. Fold change was calculated from the ΔΔCt values. The last cycle provided the post-PCR run melt curve to assess the specificity of amplification. All the reactions were run as duplicates. All the samples were analysed at a single time to ensure reproducibility and minimize variability. The cycle threshold (Ct) of the *TLR4* gene was 23.00, the *NFkB* gene was 22.00, and *GAPDH* gene was 17.84, and the efficiency of the primers was 105% and 101%, and 105%, respectively (Figure 4.3). The representative images of the amplification graphs, melt peaks, and melt curves obtained from samples were shown in Figures 4.4-4.6, respectively. The schematic

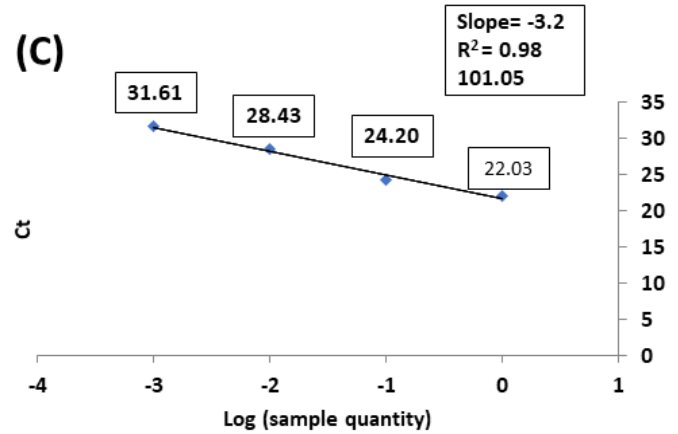
representation of the experimental protocol for quantification of *TLR4* and *NFkB* gene expression using qPCR was shown in Figure 4.7.



***GAPDH* gene**



***TLR4* gene**



***NFkB* gene**

Figure 4.3: Primer efficiency of qPCR condition for quantification: **(A)** *GAPDH* gene transcript; **(B)** *TLR4* gene transcript, and **(C)** *NFkB* gene transcript.

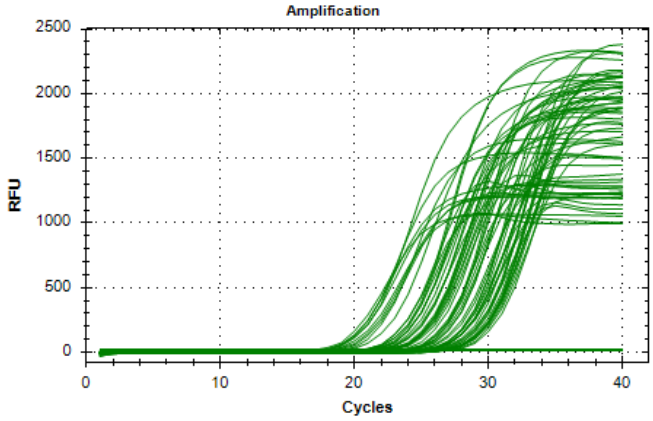


Figure 4.4: Representative graphs of the amplification of the genes in qPCR.

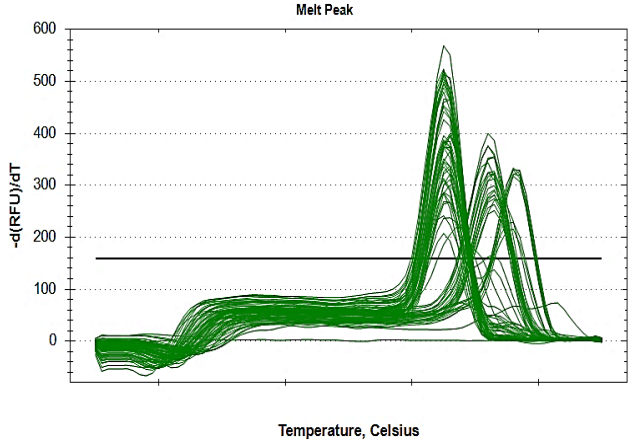


Figure 4.5: Melt peak of the genes in qPCR.

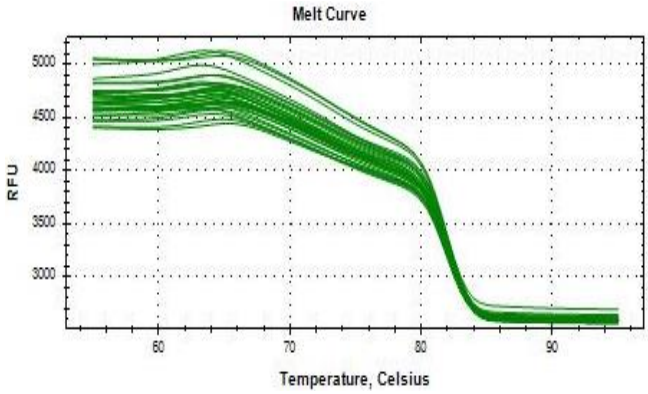


Figure 4.6: Melt curve of the genes in qPCR.

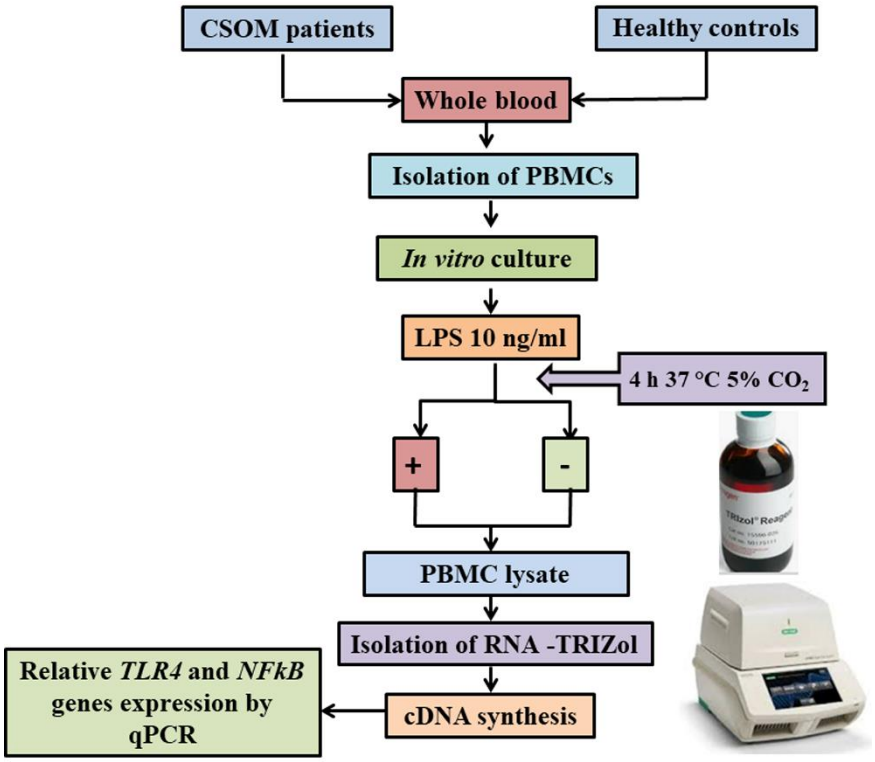


Figure 4.7: Schematic representation of the experimental protocol for quantification of *TLR4* and *NFkB* gene expression by qPCR.

4.2.12. Estimation of cytokines

TNFα and IL-10 cytokines were quantified in the conditioned medium using commercially available ELISA kits (Krishgen Biosystems, India) (Figure 4.8). Briefly, 100 µl of the standard culture supernatants from samples and control were added in different wells of 96 well ELISA plate. To all wells 100 µl of sample diluent (anti-human TNFα and anti-human IL-10, respectively) was added. The plates were incubated for 120 min at room temperature. The liquid portion was removed from all the wells and washed the wells 3 times using 1X wash buffer, added 100 µl of prepared 1X detection antibody to each well. The plates were incubated for 60 min at room temperature, washed 3 times using 1X wash buffer. Added 100 µl of concentrated streptavidin: HRP conjugate into each

well, covered the plate with sealer, and incubated for 60 min at 37 °C. Washed the plate 4 times using 1X wash buffer and added 100 µl of TMB substrate solution to each well. Plates were covered with sealer and incubated in dark for 30 min at 37 °C. After blue shading, 100 µl of stop solution was added to each well and observed the yellow color. Within 30 min after adding stop solution, the absorbance was read using a microplate reader at 450 nm (Figure 4.9).

The levels of TNFα and IL-10 in cell culture supernatants were measured using the standard graphs and expressed as pg/ml as described in the manufacturer instructions. The schematic representation of the experimental design for the estimation of both cytokines was shown in Figure 4.10.

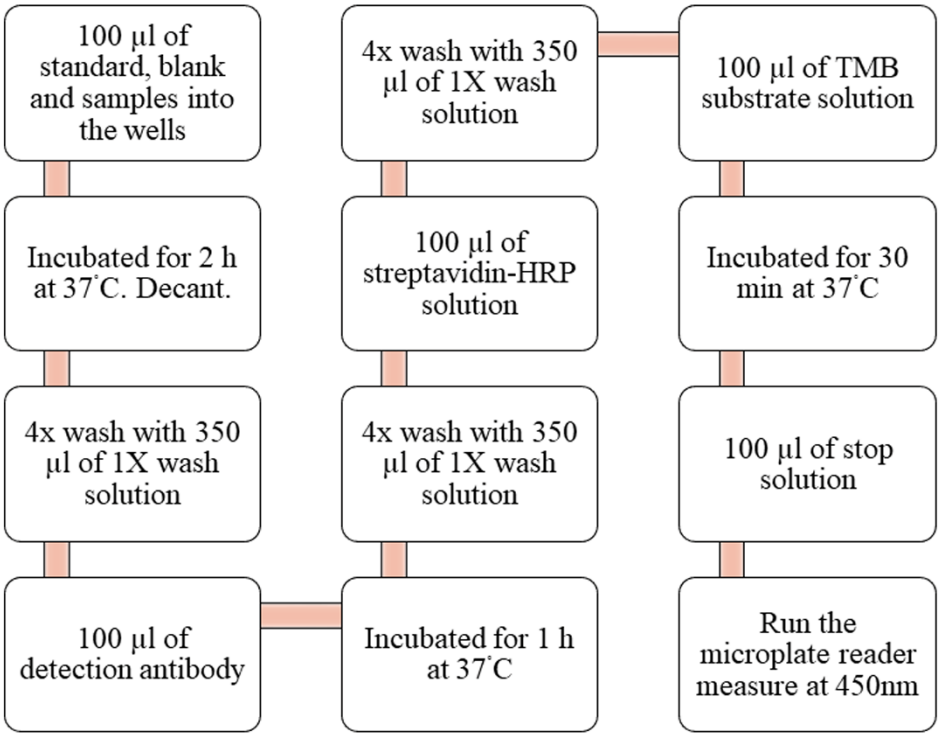


Figure 4.8: Flowchart of ELISA protocol for both TNFα and IL-10 cytokines.

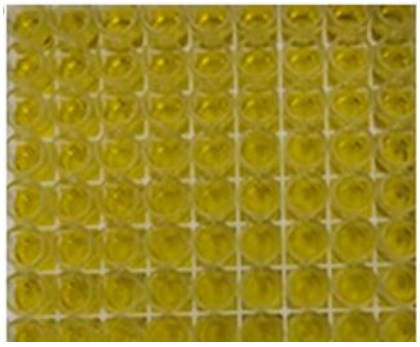


Figure 4.9: Representative image of ELISA plate used to measure the TNFα and IL-10 cytokines in the cell culture supernatant.

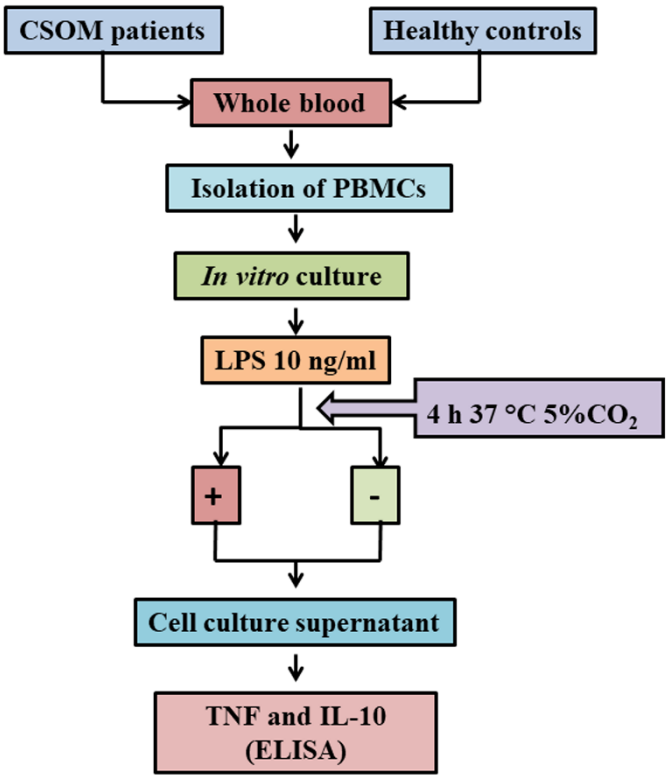


Figure 4.10: Schematic representation of the experimental protocol for estimation of cytokine in the cell culture supernatant.

4.2.13. Isolation of DNA from blood samples

The standard salting-out method for the isolation of DNA from blood samples were followed [Miller et al., 1988]. Blood samples (1 ml) were collected from study participants in a sterile EDTA vacutainer and stored at 4 °C until further processing. To 1 ml of a blood sample, added 4 times of ELB and

vortexed vigorously. The samples were kept on ice for 30 min to facilitate the hemolysis. The samples were centrifuged at 3000 rpm for 10 min, and the supernatant was discarded. The pellet was re-suspended in 2.5 ml of ELB, vortexed, and brought up to 10 ml using ELB. The samples were centrifuged at 3000 rpm for 10 min. The ELB treatment was repeated two more times. The white pellet was suspended in 1.8 ml of ELB and vortexed again. ELB was added to make up the volume of up to 5 ml, 270 µl of 20% SDS and 30 µl of proteinase K (10 mg/ml) were added and mixed. The samples were incubated at 37 °C water bath for overnight.

The next day, 500 µl of 5 M NaCl and an equal volume of isopropyl alcohol were added to the tubes. The tubes were swirled to force the resultant silky and mucoid threads of DNA into a globular mass. The precipitated DNA was transferred to 0.5 ml of freshly prepared 80% ethanol and incubated at room temperature for 15 min. The tubes were centrifuged at 12000 rpm for 5 min, and the supernatant was discarded. This step was repeated for three times to get a purified form of DNA. The DNA was then air-dried, dissolved in 500 µl of Tris-EDTA buffer, incubated at 65 °C for 30 min, and kept on the rotator until dissolved in TE buffer. The dissolved fraction was refrigerated at 4 °C for one day and stored at -20 °C until further use.

4.2.14. Quantification of DNA and analysis of purity

The concentration and purity of the isolated DNA was determined by using a spectrophotometer (260 and 280 nm) (Perkin Elmer model Lambda 35,

Waltham, MA, USA). The amount of DNA was estimated using the formula: dsDNA concentration = 50 µg/ml x OD260 x dilution factor. The ratio of absorbance at 260 and 280 nm in the range of 1.7-2.0 was used for the experiments.

4.2.15. Amplification of *TLR4* gene promotor and sequencing

The referential genomic DNA sequence of the promoter region of the *TLR4* gene was retrieved from GenBank (Accession N0. NG_011475.1). Sequence-specific primer pairs were designed to amplify the promoter boundary regions of the *TLR4* gene with the help of the Primer Quest tool and IDT DNA software. PCR was performed using gene-specific primers, and the reactions composition (25 µl) was as follows:

Contents	Volume/reaction (µl)
DNA	4.0
Reverse primer	0.5
Forward primer	0.5
dNTPs	2.5
1.5 mM $MgCl_2$	2.5
10X PCR buffer	1.5
Taq DNA polymerase	0.3
RNase-free water	13.2

The PCR conditions used for the amplification of the *TLR4* gene promotor was as follows:

Step	Temperature (°C)	Time (min)
Initial denaturation	95	5
Cycle denaturation	95	0.5
Annealing	63	0.5
Cycle extension	72	0.5
Go to (steps 2-4)	36 cycles	
Final extension	72	5

The PCR products were purified with a GeneJET PCR Purification kit [Bose et al., 2017]. DNA sequencing was performed for the promoter of the *TLR4* gene with BigDye Terminator v3.1 Cycle Sequencing Kit using ABI-3130 Genetic Analyzer as per the manufacturer's instructions. Briefly, the sequencing reaction mix and the template were subjected to amplification, followed by the cleanup method. This step was followed by adding 125 mM EDTA, 3 M sodium acetate (pH=4.6), and ethanol mix. After incubation at room temperature, spin down for 5 min and added 250 µl of 75% ethanol. Centrifuged for 10 min, decanted the supernatant, and allowed it to dry for 15 min. Hi-dI formamide was added to the tubes, subjecting them to denature and snap chill. Mix the tubes thoroughly and subject them to sequencing. The sequencing mixture compositions (10 µl) and PCR conditions are as follows:

Contents	Volume/reaction (µl)
Big Dye Terminator ready reaction mix	4.0
Template	1.0
Primer	2.0
Milli Q water	3.0

The PCR conditions used for the sequencing of the *TLR4* gene promotor was as follows**:**

Step	Temperature (°C)	Time (min)
Initial denaturation	96	5
Denaturation	96	0.5
Hybridization	50	0.5
Elongation	60	1.5
Go to (steps 2-4)		25 cycles
Final extension	72	5

DNA sequences were analyzed by ABI Variant Reporter software version 1.1 using NG_011475.1 gene sequence as a template for the *TLR4* gene promoter. The schematic representation of the experimental protocol for sequencing of *TLR4* gene promoter region using the Sanger method was shown in Figure 4.11.

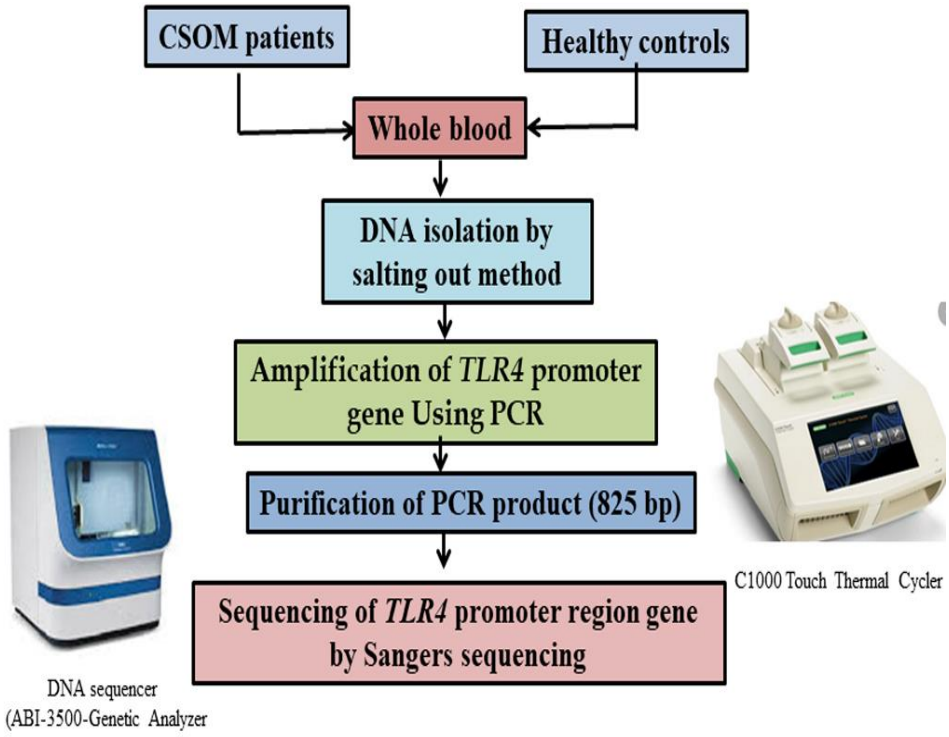


Figure 4.11: Schematic representation of the experimental design for sequencing of *TLR4* gene promoter region by the Sanger method.

4.16. Statistical analysis

The mean and standard deviation is used to represent quantitative variables. Percentages are used to describe qualitative variables. Q-Q plots and normality plots were used to perform the Shapiro-Wilk test. If the data had a normal distribution, the mean was calculated; otherwise, the median was calculated. The means of the two groups were compared using the Student t-test, while the medians were compared using the Mann-Whitney U test. The Pearson correlation and spearman's rho correlation test was used to determine the relationship between the variables. The distribution of both genotype and allele frequency between the two groups were analyzed using fisher's exact test. If $p<0.05$, the difference was considered statistically significant. SPSS statistics V24.0 was used for the statistical analysis of the data. The representative images were prepared using Origin Pro software.

Results

5. Results

5.1. Demographic details

The demographic details of the study participants were represented in table.1. A total of 63 CSOM patients and 63 matched healthy controls were recruited for the study. The age (mean ± SD) of CSOM is 38.0±13.8 and healthy Control is 38.0±13.7. The ratio of male and females are 28 (44.5%) and 35 (55.5 %) between the CSOM patients and healthy controls. Based on the location and tympanic membrane perforation the CSOM patients were divided into three subtypes: (i) Anterior: 07 (11.1%), (ii) Central: 44 (69.9%), and (iii) Posterior:12 (19.0%). The Central perforation (of the tympanic membrane) is more common than anterior and posterior locations. Based on the ear affected, the CSOM patients can be divided into unilateral 26 (41.3%), bilateral 37 (58.70%). The majority of CSOM patients were presented with bilateral involvement of the ear. The duration of the CSOM disease: ≤1 year 10 (15.9%), ≥1 - ≤5 years 37 (58.7%), and ≥ 5 - ≤ 10 years 16 (25.4). CSOM with cholesteatoma was 10 (15.9%) was less commonly seen in the CSOM patients.

Table 5.1: Characteristics of the study participants.

Parameters	CSOM (n=63)	Control (n=63)
Age (Years; mean±SD)	38.0±13.8	38.1±13.7
Gender		
Male	28 (44.5%)	28 (44.5%)
Female	35 (55.5%)	35 (55.5%)
Ear affected		
Unilateral	26 (41.3 %)	NA

Bilateral	37 (58.7 %)	
Duration of disease		
≤ 1 year	10 (15.9%)	NA
>1 to ≤ 5 years	37 (58.7%)	
>5 to ≤ 10 years	16 (25.4%)	
Type of TM perforation		
Central	44 (69.9%)	NA
Anterior	07 (11.1%)	
Posterior	12 (19.0%)	
Presence of cholesteatoma	10 (15.9%)	NA
Presenting symptoms		
Ear discharge	55 (87.3%)	NA
Hearing loss	56 (88.8%)	
Earache	59 (93.6%)	

SD=Standard deviation; **NA**=Not applicable; **TM**=Tympanic membrane; **CSOM**= Chronic Supportive Otitis Media

5.2. Effect of LPS on *TLR4* gene expression

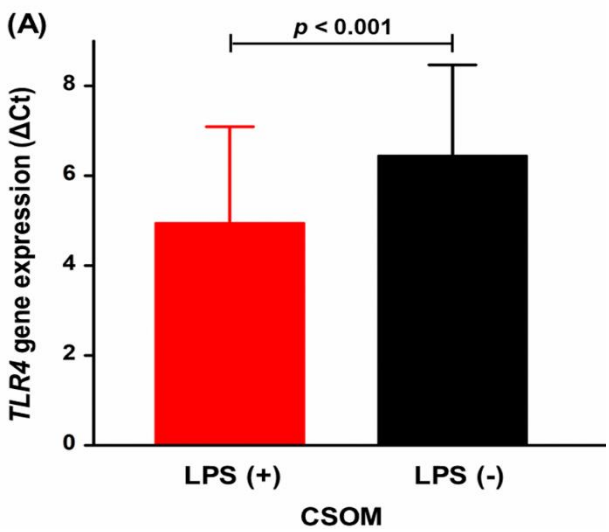
To find whether the *TLR4* gene expression was sensitive to LPS treatment in CSOM patients, the *TLR4* gene expression was quantified using qPCR in the PBMCs after 4 h of treatment with LPS at 37 °C and compared with the expression in healthy controls.

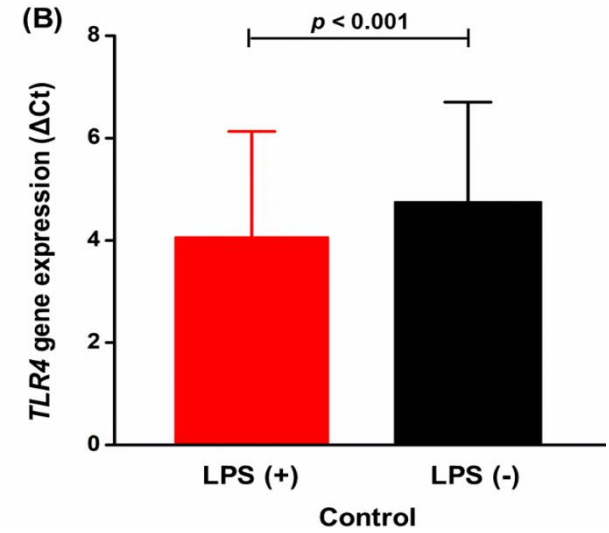
The normalized expression (ΔCt) of the *TLR4* gene showed normal distribution in both CSOM and control groups. The mean±standard deviation was calculated. The results obtained from the experiments were depicted in figure 5.1A-C.

In the CSOM group, the average ΔCt of the *TLR4* gene was 4.95±2.14 in the LPS treated and 6.45±2.02 in the untreated samples. The higher normalized expression of the *TLR4* gene observed in the LPS-treated samples was found to be statistically significant ($p<0.001$; Student's t-test). The average LPS-induced fold change (log $2^{-\Delta\Delta Ct}$) in the *TLR4* gene expression was 2.8 times.

In the control group, the average ΔCt of the *TLR4* gene was 4.06±2.07 in the LPS treated and 4.75±1.95 in the untreated samples. The higher normalized expression of the *TLR4* gene observed in the LPS-treated samples was found to be statistically significant ($p<0.001$; Student's t-test). The average LPS-induced fold change (log $2^{-\Delta\Delta Ct}$) in the *TLR4* gene expression was 1.6 times.

The LPS-induced fold change of the *TLR4* gene in the CSOM and control groups were compared by statistical methods. The difference between the groups was found to be significant ($p<0.001$; Student's t-test). Therefore, these results indicate that *TLR4* gene expression shows higher sensitivity to LPS-induced upregulation in CSOM patients.


(A)
p < 0.001
TLR4 gene expression (ΔCt)
0
2
4
6
8
LPS (+)
LPS (-)
CSOM


(B)
p < 0.001
TLR4 gene expression (ΔCt)
0
2
4
6
8
LPS (+)
LPS (-)
Control

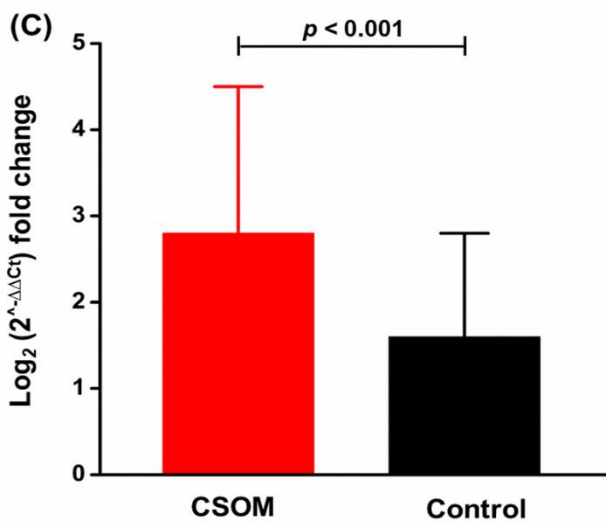
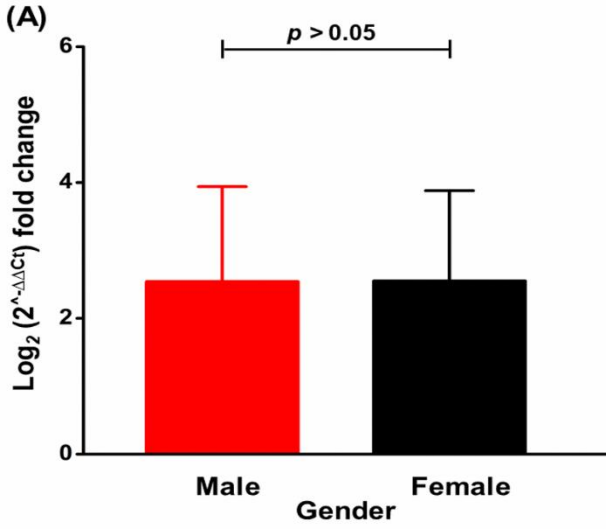
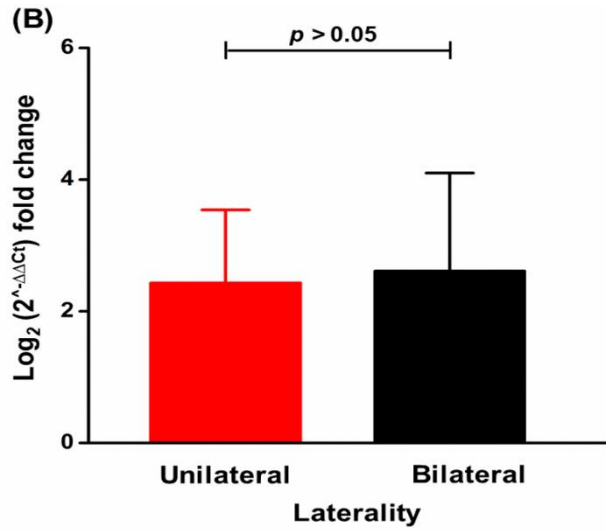


Figure 5.1: Effect of LPS on *TLR4* gene expression in the study groups: (**A)** Normalized gene expression in the CSOM group, (**B**) Normalized gene expression in the control group, and (**C)** Comparison of LPS-treated fold changes in *TLR4* gene expression between the study groups (n=63/group).

Furthermore, to find the effect clinical variables such as gender, laterality and disease duration on LPS induced *TLR4* gene expression levels in CSOM patients, subgroup (Table 5.1) analysis were performed. The gender (Male *vs.* Female), laterality (Unilateral *vs.* Bilateral), and disease duration ((<4 *vs*. >4 years) did not show any impact on LPS-induced fold change *TLR4* gene expression levels. The results obtained from the experiments were depicted in figure 5.2A-C.


(A)
p > 0.05
6
4
2
0
Log2 (2^-ΔΔCt) fold change
Male
Female
Gender


(B)
p > 0.05
6
4
2
0
Log2 (2^-ΔΔCt) fold change
Unilateral
Bilateral
Laterality

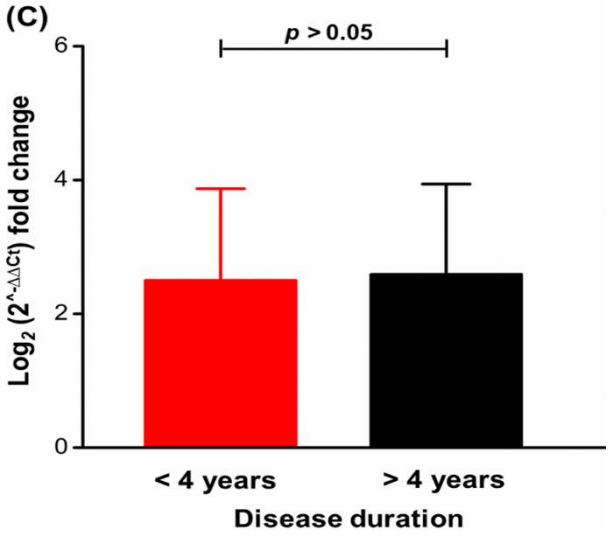


Figure 5.2: Effect of LPS on *TLR4* gene expression in the study subgroups: (**A**) Comparison of LPS-treated fold changes in *TLR4* gene expression between gender (Male *vs.* Female) in the CSOM groups, (**B)** Comparison of LPS-treated fold changes in *TLR4* gene expression between laterality (Unilateral *vs.* Bilateral) in the CSOM groups, and (**C)** Comparison of LPS-treated fold changes in *TLR4* gene expression between disease duration (<4 *vs*. >4 years) in the CSOM groups.

5.3. Effect of LPS on *NFkB* gene expression

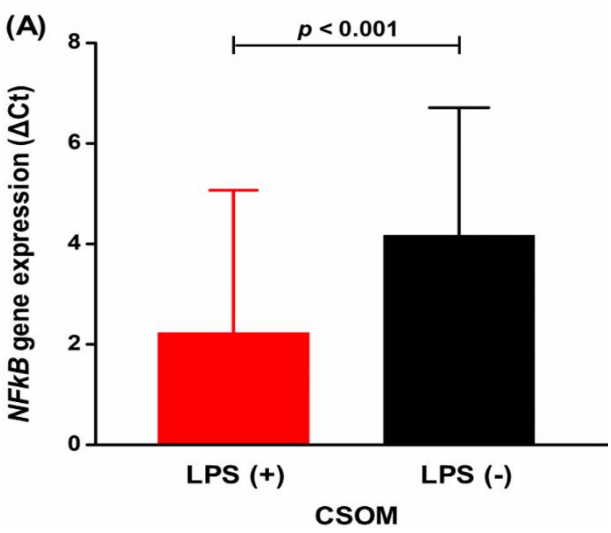
To find whether the *NFkB* gene expression was sensitive to LPS treatment in CSOM patients, the *NFkB* gene expression was quantified using qPCR in the PBMCs lysate after 4 h of treatment with LPS at 37 °C and compared with the expression in healthy controls.

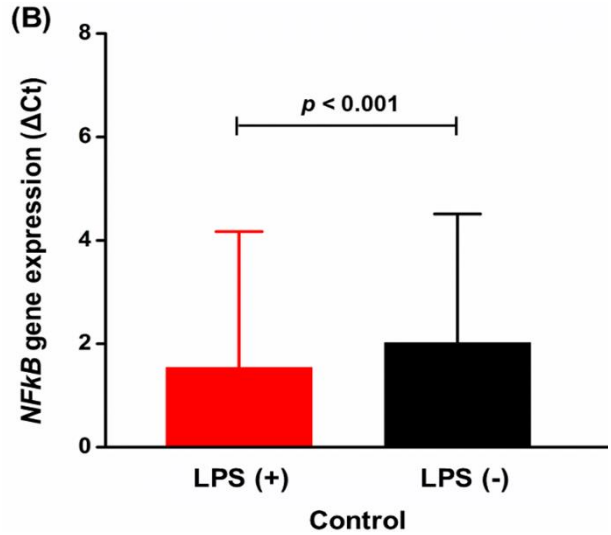
The normalized expression (ΔCt) of *NFkB* gene showed normal distribution in both CSOM and control groups. The mean±standard deviation of the expression was calculated. The results obtained from the experiments were depicted in figure 5.3A-C.

In the CSOM group, the average ΔCt of the *NFkB* gene was 2.23±2.84 in the LPS treated and 4.17±2.54 in the untreated samples. The higher normalized expression of the *NFkB* gene expression observed in the LPS-treated samples was found to be statistically significant ($p<0.001$; Student's t-test). The average LPS-induced fold change (log $2^{-\Delta\Delta Ct}$) in the *NFkB* gene expression was 3.8 times.

In the control group, the average ΔCt of the *NFkB* gene was 1.54±2.63 in the LPS treated and 2.02±2.49 in the untreated samples. The higher normalized expression of the *NFkB* gene observed in the LPS-treated samples was found to be statistically significant ($p<0.001$; Student's t-test). The average LPS-induced fold change (log $2^{-\Delta\Delta Ct}$) in the *NFkB* gene expression was 1.4 times.

The LPS-induced fold change expression of *NFkB* gene in the CSOM and control groups were compared by statistical methods. The difference between the groups was found to be significant ($p<0.001$; Student's t-test). Therefore, these results indicate that *NFkB* gene expression shows higher sensitivity to LPS-induced upregulation in CSOM patients.


(A)
p < 0.001
NFkB gene expression (ΔCt)
8
6
4
2
0
LPS (+)
LPS (-)
CSOM


(B)
p < 0.001
NFkB gene expression (ΔCt)
8
6
4
2
0
LPS (+)
LPS (-)
Control

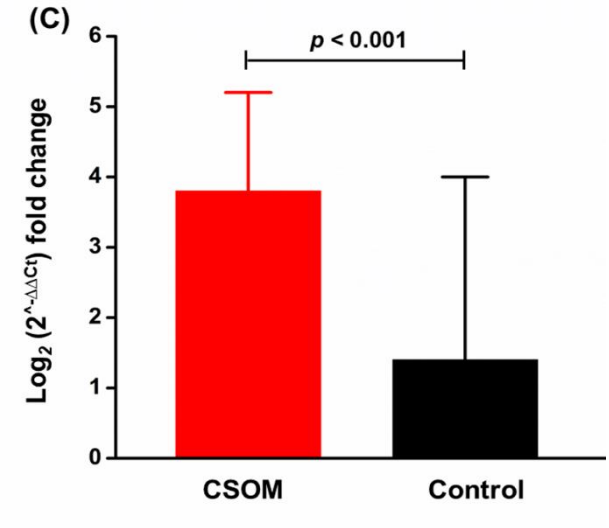
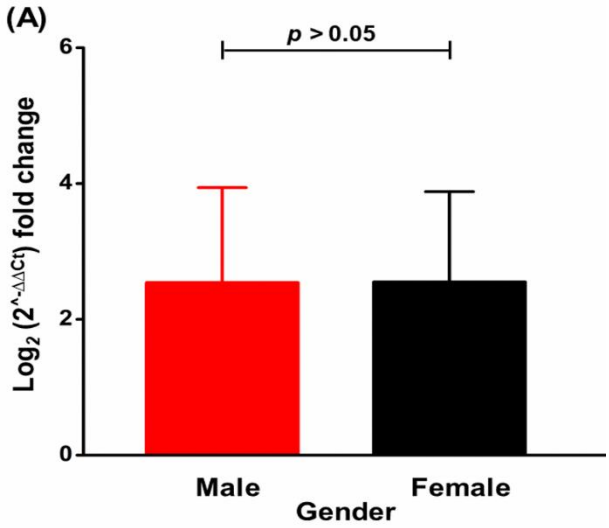
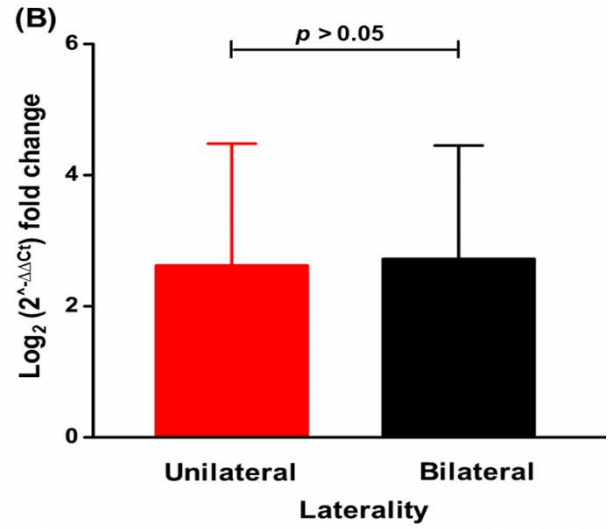


Figure 5.3: Effect of LPS on *NFkB* gene expression in the study groups: (**A**) Normalized gene expression in the CSOM group, **(B)** Normalized gene expression in the control group, and (**C**) Comparison of LPS-treated fold changes in *NFkB* gene expression between the study groups (n=63/group).

Furthermore, to find the effect clinical variables such as gender, laterality and disease duration on LPS induced *NFkB* gene expression levels in CSOM patients, subgroup (Table 5.1) analysis were performed. The gender (Male *vs.* Female), laterality (Unilateral *vs.* Bilateral), and disease duration ((<4 *vs*. >4 years) did not show any impact on LPS-induced fold change *NFkB* gene expression levels. The results obtained from the experiments were depicted in figure 5.4A-C. List of primers used for quantification of *TLR4*, *NFkB*, and *GAPDH* genes in qPCR were shown in the table.5.2.


(A)
p > 0.05
6
4
2
0
Log2 (2^-ΔΔCt) fold change
Male
Female
Gender


(B)
p > 0.05
6
4
2
0
Log2 (2^-ΔΔCt) fold change
Unilateral
Bilateral
Laterality

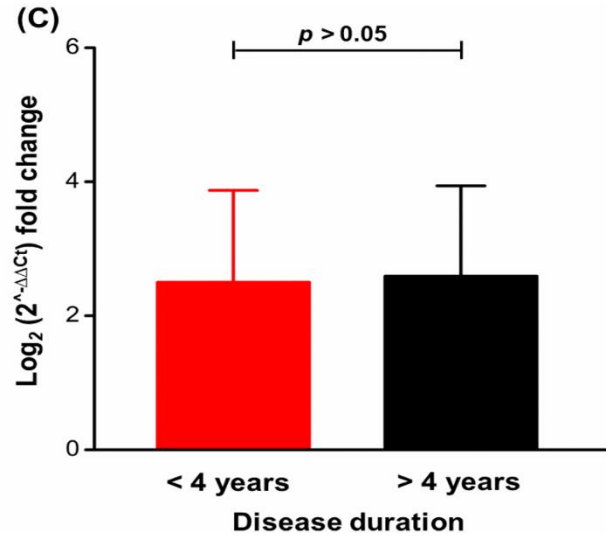


Figure 5.4: Effect of LPS on *NFkB* gene expression in the study subgroups: (**A)** Comparison of LPS-treated fold changes in *NFkB* gene expression between gender (Male vs. Female) in the CSOM groups, (**B)** Comparison of LPS-treated fold changes in *NFkB* gene expression between laterality (Unilateral *vs.* Bilateral) in the CSOM groups, and (**C)** Comparison of LPS-treated fold changes in *NFkB* gene expression between disease duration (<4 *vs*. >4 years) in the CSOM groups.

Table 5.2. List of the forward and reverse primers used to quantify the expression of *TLR4* and *NFkB* genes.

Genes	Primer sequences (5' – 3')	Amplicon size (bp)
GAPDH	FP: GATCATCAGCAATGCCTCCT RP: GACTGTGGTCATGAGTCCTTC	110
NFkB	FP: TAC CGA CAG ACA ACC TCA CC RP: CAG CTT GTC TCG GGT TTC TG	150
TLR4	FP: GAACCTGGACCTGAGCTTTAAT RP: GTC TGG ATT TCA CAC CTG GAT AA	105

5.4. Effect of LPS on TNFα production

The expression of *TLR4* and *NFkB* genes was upregulated in the PBMC of CSOM patients. Further, the study was extended to investigate the levels of effector cytokines such as TNFα and IL-10 in conditioned media obtained from the PBMC cultures treated with and without LPS. To find whether the TNFα production was sensitive to LPS treatment in CSOM patients, the TNFα production was measured using ELISA in the PBMCs conditioned medium after 4 h of treatment with LPS at 37 °C and compared with the expression in healthy controls.

The TNFα protein levels were measured along with standards, calculated the expression in CSOM patients using the formula y=0.0005x+0.1688, expressed as pg/ml (Figure 5.5), and compared with healthy control group.

The expression of TNFα did not show the normal distribution in both CSOM and control groups. The median and Inter quartile range (IQR) was calculated. The results obtained from the experiments were depicted in figure 5.6A-C.

In the CSOM group, the average (range) levels of TNFα were 466.0 (137.2-778.8) pg/ml in the LPS treated and 142.3 (97.1-313.1) pg/ml in the untreated samples. The higher expression of the levels of TNFα observed in the LPS-treated samples was found to be statistically significant ($p<0.001$; Mann-Whitney U test). The average LPS-induced fold change in TNFα expression was 3.2 times.

In the control group, the average TNFα expression levels were 243.9 (149.1-371.9) pg/ml in the LPS treated and 140.3 (82.3-271.1) pg/ml in the untreated samples. The higher expression levels of TNFα observed in the LPS-treated samples were found to be statistically significant (p<0.001; Mann-Whitney U test). The average LPS-induced fold change in the TNFα expression levels was 1.1 times.

The LPS-induced fold change of the TNFα expression levels in the CSOM and control groups was compared by statistical methods. The difference between the groups was found to be significant (p<0.001; Mann-Whitney U test). Therefore, these results indicate that TNFα expression levels show higher sensitivity to LPS-induced upregulation in CSOM patients.

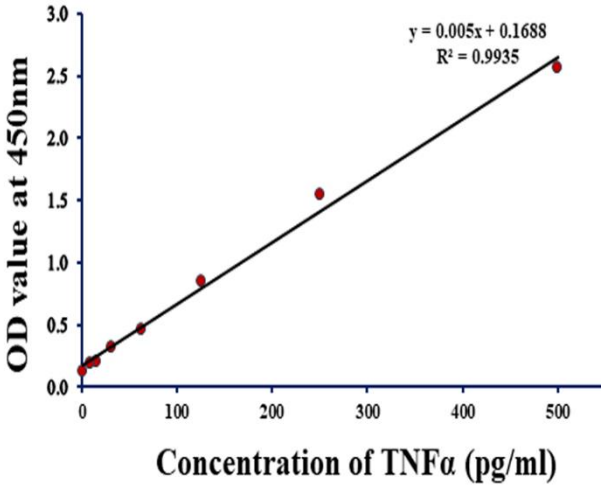
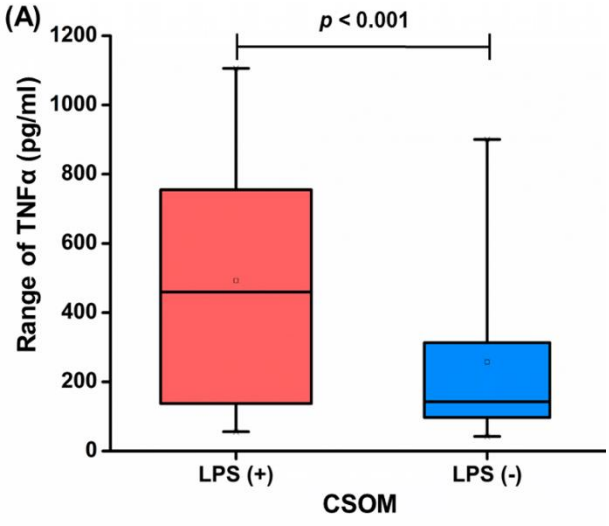
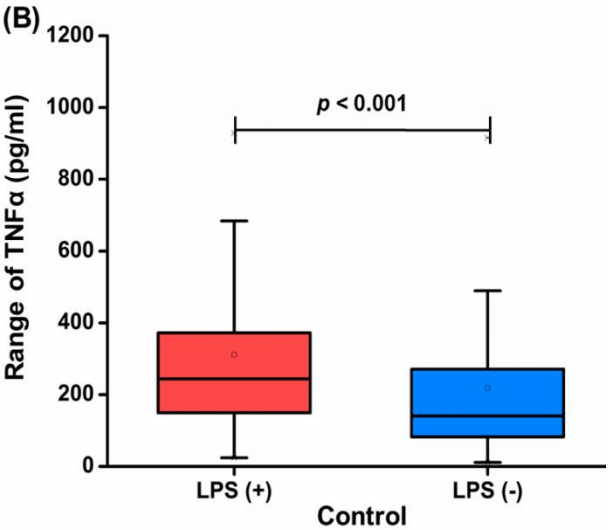


Figure 5.5: Standard graph for the levels of TNFα protein in conditioned medium of CSOM patients and healthy controls.


(A)
p < 0.001
Range of TNFα (pg/ml)
1200
1000
800
600
400
200
0
LPS (+)
LPS (-)
CSOM


(B)
p < 0.001
Range of TNFα (pg/ml)
1200
1000
800
600
400
200
0
LPS (+)
LPS (-)
Control

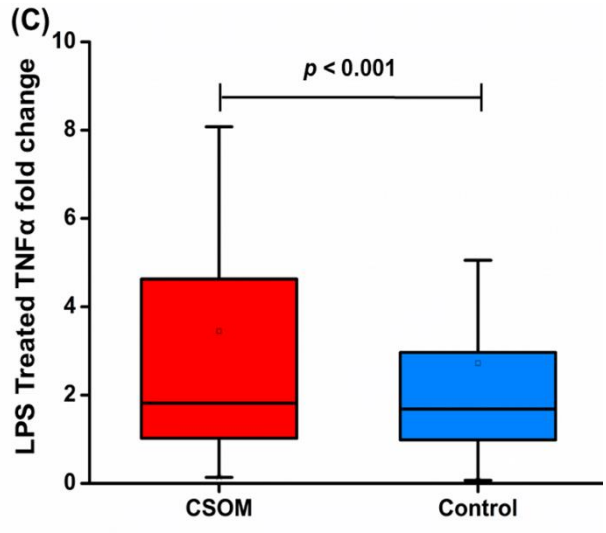
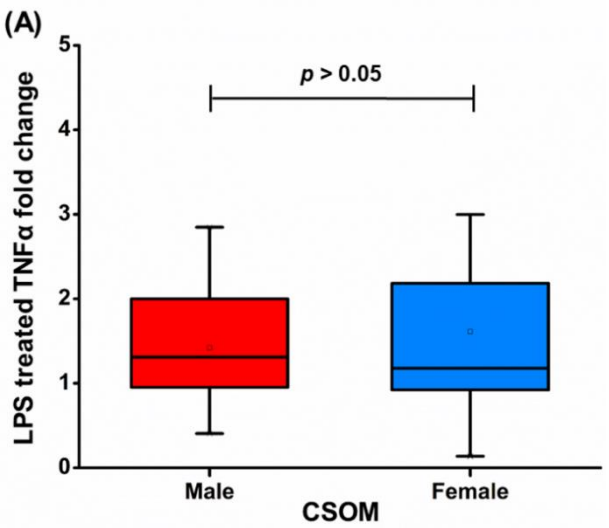
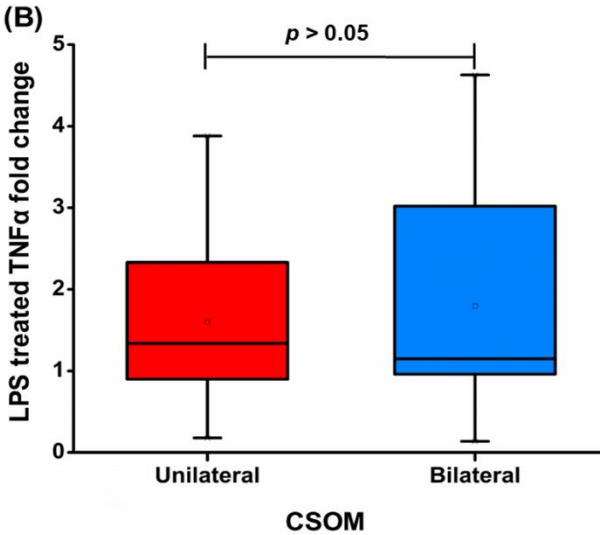


Figure 5.6: Effect of LPS on TNFα production in the study groups: (**A)** TNFα levels in the treated and untreated conditioned media in the CSOM group, **(B)** TNFα levels in the treated and untreated conditioned media in the control group, and **(C)** Comparison of LPS-treated TNFα fold change between CSOM and control groups (n=63/group).

Furthermore, to find the effect of clinical variables such as gender, laterality and disease duration on LPS induced TNFα protein expression levels in CSOM patients, subgroup (Table 5.1) analysis were performed. The gender (Male vs. Female), laterality (Unilateral vs. Bilateral), and disease duration ((<4 vs. >4 years) did not show any impact on LPS-induced TNFα protein fold change expression. The results obtained from the experiments were depicted in figure 5.7A-C.


(A)
LPS treated TNFα fold change
p > 0.05
0
1
2
3
4
5
Male
Female
CSOM


(B)
LPS treated TNFα fold change
p > 0.05
0
1
2
3
4
5
Unilateral
Bilateral
CSOM

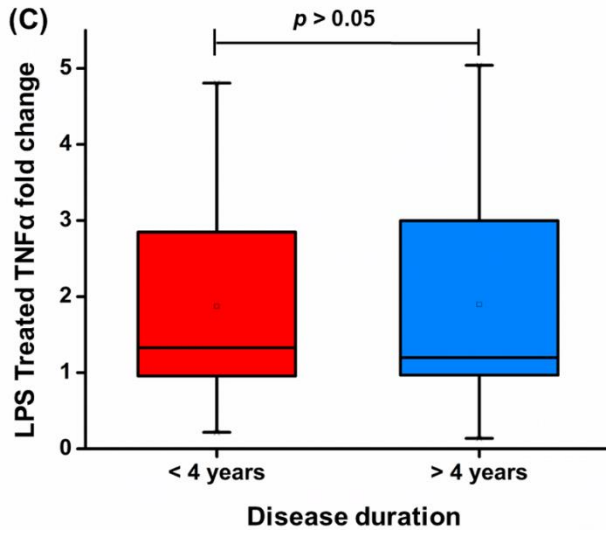


Figure 5.7: Effect of LPS on TNFα production in the study subgroups: (**A**) Comparison of LPS-treated fold changes in TNFα production between gender (Male *vs.* Female) in the CSOM groups, (**B**) Comparison of LPS-treated fold changes in TNFα production between laterality (Unilateral *vs.* Bilateral) in the CSOM groups, and (**C**) Comparison of LPS-treated fold changes in TNFα production between disease duration (<4 *vs*. >4) in the CSOM groups.

5.5. Relationship between components of TLR4 pathway

The LPS-induced fold change in the gene expression of *TLR4* was correlated with the fold change in *NFkB* gene expression in CSOM group. The correlation results are presented in figure 5.8. The correlation was positive with strong strength (r=0.77, *p*= 0.01, Pearson's correlation). This indicates that an LPS-induced increase in *TLR4* gene expression may be responsible for the upregulation of the *NFkB* gene.

Furthermore, the LPS-induced fold change in the gene expression of *NFkB* and TNFα fold change was checked for correlation in the CSOM group. The correlation results are presented in figure 5.9. The correlation was positive with

moderate strength (r=0.37, *p*=0.01, spearman's rho correlation). This indicates LPS-induced increase in *NFkB* gene expression may be responsible for increased TNFα production.

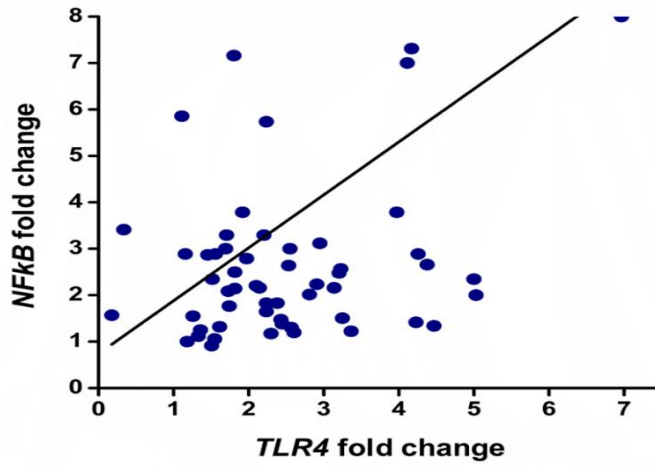


Figure 5.8: Correlation between LPS-induced *TLR4* gene expression and *NFkB* gene expression in CSOM patients.

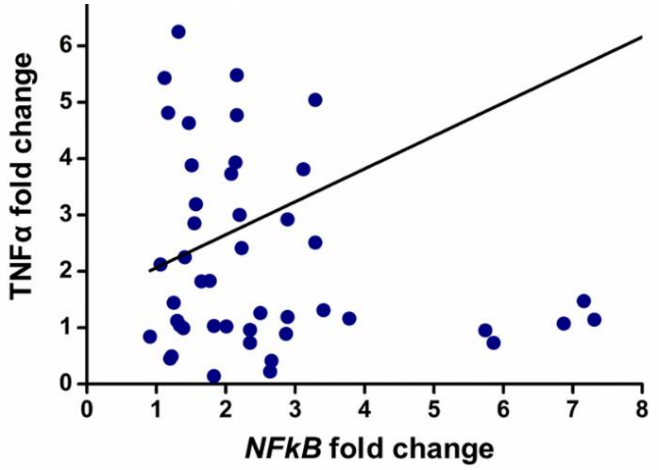


Figure 5.9: Correlation between LPS-induced *NFkB* gene expression and TNFα production in CSOM patients.

5.6. Effect of LPS on IL-10 production

The TNFα production was significantly increased in the condition medium of PBMC cultures from CSOM patients when compared to healthy controls. Further, to find whether the IL-10 production was sensitive to LPS treatment in CSOM patients, the IL-10 production was measured using ELISA in the PBMCs conditioned medium after 4 h of treatment with LPS at 37 °C and compared with the expression in healthy controls.

The IL-10 protein levels were measured along with standards, calculated the expression in CSOM patients using the formula y=0.0045x+0.4035, expressed as pg/ml (Figure 5.10), and compared with healthy control.

Expression levels of IL-10 did not show the normal distribution in both CSOM and control groups. The median (IQR) was calculated. The results obtained from the experiments were depicted in figure 5.11A-C.

In the CSOM group, the average (range) levels of IL-10 were 92.1 (65.4-110.7) pg/ml in the LPS treated and 146.7 (107.6-228.1) pg/ml in the untreated samples. The lower levels of IL-10 expression observed in the LPS-treated samples were found to be statistically significant (p<0.001; Mann-Whitney U test). The average LPS-induced fold change in the IL-10 levels was 0.58 times.

In the control group, the average (range) IL-10 levels were 206.7 (116.5-388.5) pg/ml in the LPS treated and 317.0 (161.8-470.7) pg/ml in the untreated samples. The lower levels of IL-10 observed in the LPS-treated samples were

found to be statistically significant (p<0.001; Mann-Whitney U test). The average LPS-induced fold change in the IL-10 levels was 0.78 times.

The LPS-induced fold change of the IL-10 levels in the CSOM and control groups was compared by statistical methods. The difference between the groups was found to be significantly less in CSOM group (p<0.001; Mann-Whitney U test). Therefore, these results indicate that IL-10 expression levels did not appear to be sensitive to LPS-induced upregulation in CSOM patients.

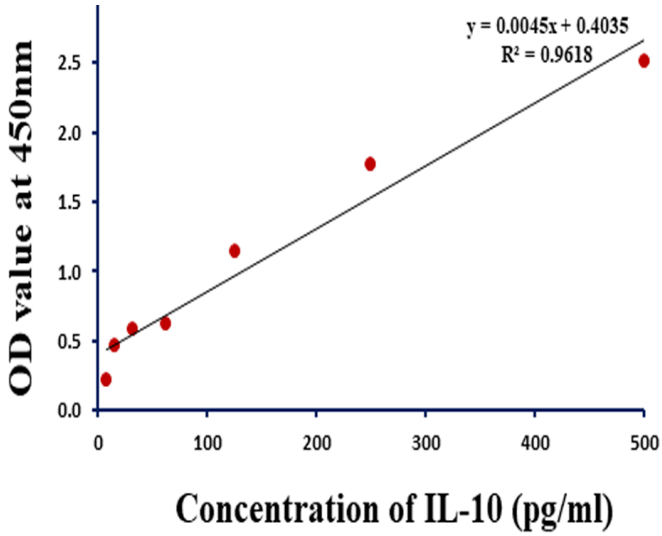
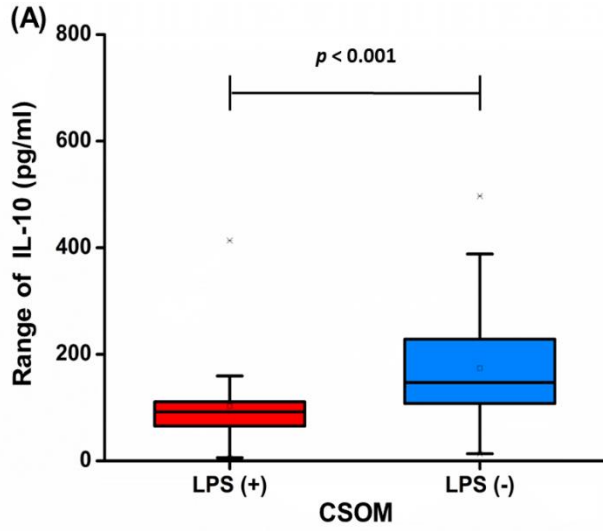
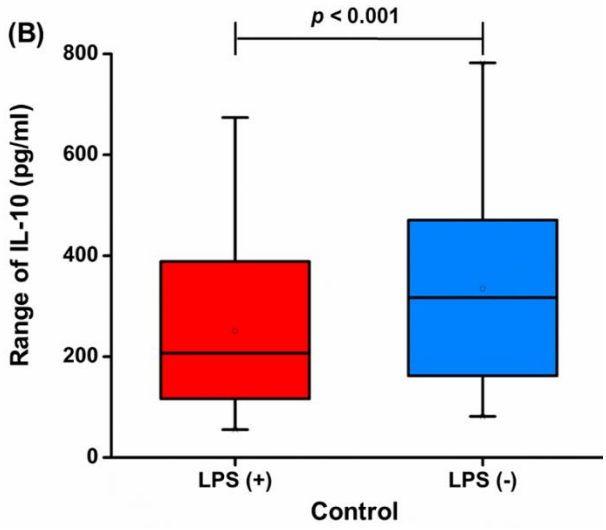


Figure 5.10: Standard graph for the levels of IL-10 protein in conditioned medium of CSOM patients and healthy controls.


(A)
800
600
400
200
0
Range of IL-10 (pg/ml)
p < 0.001
LPS (+)
LPS (-)
CSOM


(B)
800
600
400
200
0
Range of IL-10 (pg/ml)
p < 0.001
LPS (+)
LPS (-)
Control

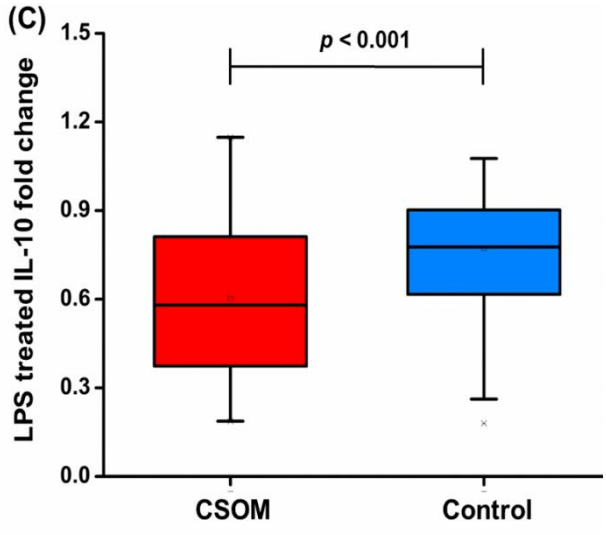
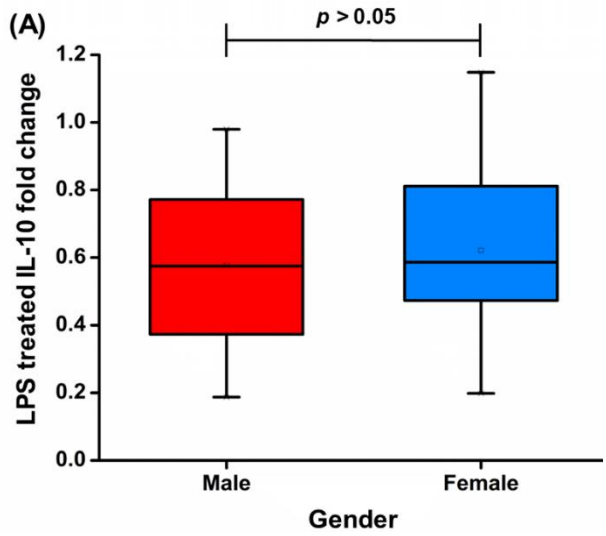
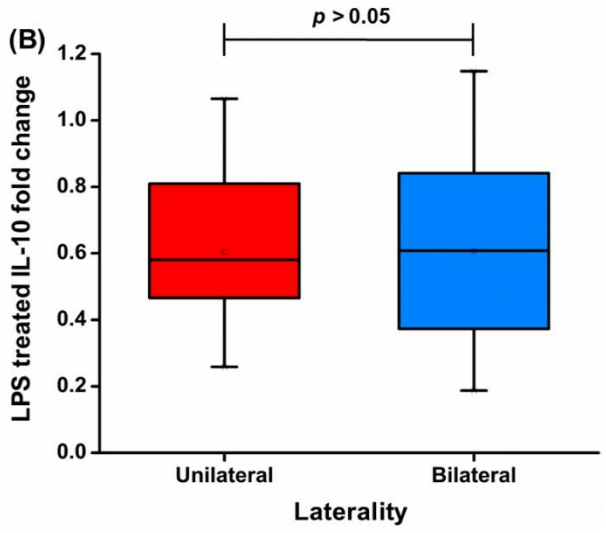


Figure 5.11: Effect of LPS on IL-10 production in the study groups: **(A)** IL-10 levels in the treated and untreated conditioned media in the CSOM group, **(B)** IL-10 levels in the treated and untreated conditioned media in the control group, and **(C)** Comparison of LPS-treated IL-10-fold change between CSOM and control groups (n=63/group).

Furthermore, to find the effect of clinical variables such as gender, laterality and disease duration on LPS induced IL-10 protein expression levels in CSOM patients, subgroup (Table 5.1) analysis were performed. The gender (Male vs. Female), laterality (Unilateral vs. Bilateral), and disease duration (<4 vs. >4 years) did not show any impact on LPS-induced IL-10 expression levels. The results obtained from the experiments were depicted in figure 5.12A-C.


(A)
p > 0.05
LPS treated IL-10 fold change
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Male
Female
Gender


(B)
p > 0.05
LPS treated IL-10 fold change
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Unilateral
Bilateral
Laterality

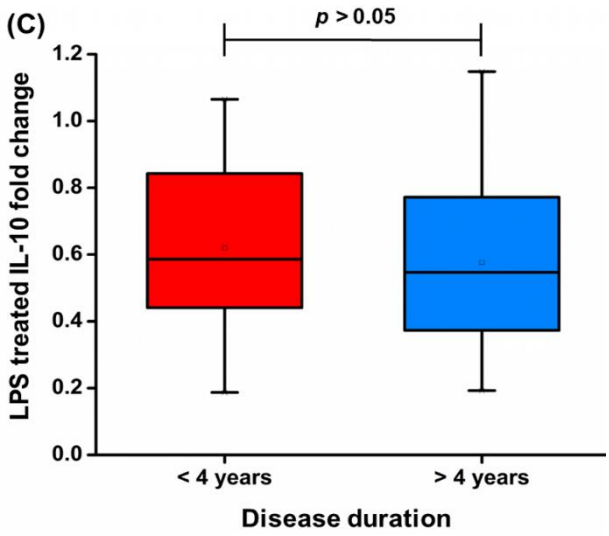


Figure 5.12: Effect of LPS on IL-10 production in the study subgroups: (**A)** Comparison of LPS-treated fold changes in TNFα production between gender (Male *vs.* Female) in the CSOM groups, (**B)** Comparison of LPS-treated fold changes in IL-10 production between laterality (Unilateral *vs*. Bilateral) in the CSOM groups, (**C**) Comparison of LPS-treated fold changes in IL-10 production between disease duration (<4 *vs*. >4years) in the CSOM groups.

5.7. Influence of genetic variation in promoter region *TLR4* gene

To find whether *TLR4* gene promoter SNPs were associated with CSOM patients, Four SNPs in the *TLR4* gene promoter region were selected (since they are predicted to be transcription factor binding motifs): -2604 (GATA2, CdxA), -2570 (tst-2, v-myb), -2604 (N-Myc, Lost), and -2026 (Oct-1, Lost). The selected SNPs were shown in the figure 5.13 [Ragnarsdóttir et al., 2020]. SNPs in *TLR4* gene promoter regions -2604 G>A, -2570 A>G, -2604 G>A, and -2026 A>G were identified. The *TLR4* gene promoter region primers sequences were shown in table 5.3 and the representative band pattern of PCR amplification (825 bp) were shown in the figure 5.14. The distribution of both genotype and allele

frequency of all 4 SNPs in the *TLR4* gene promoter region showed no statistically significant difference between the CSOM and control groups (p>0.05; Fisher's exact test). This indicates that the *TLR4* gene promoter region SNPs (-2604 G>A, -2570 A>G, -2604 G>A, and -2026 A>G) maybe not associated with CSOM. The sequencing results obtained from the both CSOM and control genotype and allele frequencies of 4 SNPs were shown in the table 5.4-5.7.

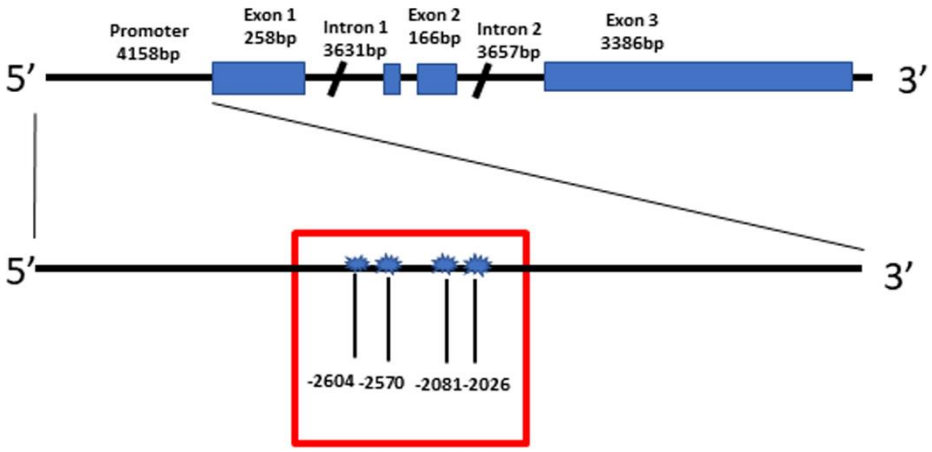


Figure 5.13: The *TLR4* gene promoter region SNPs -2604, -2570, -2604, and -2026 analysed in the present study.

Table 5.3. The sequence of primers used to study the SNPs in promoter region of *TLR4* gene in blood samples of CSOM patients and healthy controls.

Gene	Primer sequence (5' – 3')	Amplicon size (bp)
TLR4	FP: CATTGGTAGCACCAGAGTCC RP: CTGGAAAGTAGCAAGTGCAATG	825

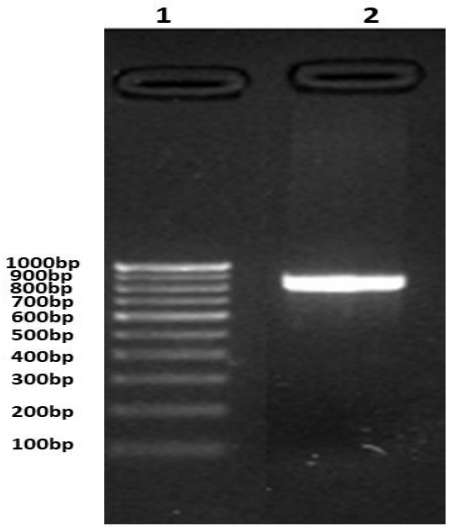


Figure 5.14: Representative PCR band for *TLR4* gene promoter region: **Lane 1**: represents the 100 bp ladder. **Lane 2:** represents PCR amplicon (825 bp).

Table 5.4: Genotype and allele frequencies of *TLR4* gene promoter region SNP-2604 G>A in the CSOM patients and healthy controls.

Genotype/ Allele	Controls (n=22)	CSOM (n=22)	P- value*	OR# (0.95 CI)
GG	10	12	0.27	NA
GA	12	8		
AA	0	2		
G	32	32	0.59	1 (0.39 – 2.55)
A	12	12		

GG: Homozygous (major allele); **AA**: Homozygous (minor allele); **GA**: Heterozygous; * Chi square, one-tailed (Fisher's exact test); # **OR**: Odds ratio; **CI**: Confidence intervals; **NA**: Not Applicable.

Table 5.5: Genotype and allele frequencies of *TLR4* gene promoter region SNP-2570 A>G in the CSOM patients and healthy controls.

Genotype/ Allele	Controls (n=22)	CSOM (n=22)	P- value*	OR# (0.95 CI)
AA	15	11	0.34	NA
AG	07	10		
GG	0	1		
A	37	32	0.000	0.07 (0.02 – 0.20)
G	7	12		

AA: Homozygous (major allele); **GG**: Homozygous (minor allele); **AG**: Heterozygous; ***** Chi square, one-tailed (Fisher's exact test); # **OR**: Odds ratio; **CI**: Confidence intervals; **NA**: Not Applicable.

Table 5.6: Genotype and allele frequencies of *TLR4* gene promoter region SNP--2081 G>A in the CSOM patients and healthy controls.

Genotype/ Allele	Controls (n=22)	CSOM (n=22)	P- value*	OR# (0.95 CI)
GG	19	15	0.29	NA
GA	03	06		
AA	0	1		
G	41	36	0.06	3.03 (0.74 – 12.32)
A	3	8		

GG: Homozygous (major allele); **AA**: Homozygous (minor allele); **GA**: Heterozygous; * Chi square, one-tailed (Fisher's exact test); # **OR**: Odds ratio; **CI**: Confidence intervals; **NA**: Not Applicable.

Table 5.7. Genotype and allele frequencies of *TLR4* gene promoter region SNP-2026 A>G in the CSOM patients and healthy controls.

Genotype/ Allele	Controls (n=22)	CSOM (n=22)	P- value*	OR# (0.95 CI)
AA	17	12	-	NA
AG	7	10		
GG	0	0		
A	41	34	0.23	5.6 (0.59 – 5.01)
G	07	10		

AA: Homozygous (major allele); **GG**: Homozygous (minor allele); **AG**: Heterozygous; * Chi square, one-tailed (Fisher's exact test); # **OR**: Odds ratio; **CI**: Confidence intervals; **NA**: Not Applicable.

Discussion

6. Discussion

The present study aimed to evaluate the role of genetic and immunological factors on the TLR4 pathway in CSOM patients.

The main findings of this study are as follows:

a) LPS-treated fold change of *TLR4* and *NFkB* gene expression were higher in CSOM patients than healthy controls.

b) LPS-treated fold change of TNFα production was higher and the IL-10 production was lower in CSOM patients than healthy controls.

c) SNPs in the promoter region of *TLR4* gene may not be associated with CSOM.

The inferences and the significance of each findings were discussed individually below.

Inference 1: Upregulated *TLR4* and *NFkB* gene expression in the LPS treated PBMCs of CSOM patients

LPS treatment of PBMCs revealed an increased *TLR4* gene expression in CSOM patients compared to healthy controls. LPS is a TLR4 agonist [Hernandez et al., 2019]. The activation of the downstream signaling cascade leads to the upregulation of the *TLR4* gene. As a result, an increase in the synthesis of NFkB protein is brought about by the binding of LPS to the TLR4 receptor. This protein is a transcription factor that controls the expression of the inflammatory gene by moving from the cytoplasm to the nucleus [Kawai et al., 2007]. This study

showed an increased expression of *TLR4* and *NFkB* genes, suggesting that CSOM patients are more hypersensitive to LPS.

LPS-treated *TLR4* and *NFkB* gene expression levels were compared between clinical subgroups such as gender, laterality, and disease duration. The gender, laterality, and disease duration did not affect the LPS-treated *TLR4* and *NFkB* gene expression levels in CSOM patients. Also, in CSOM patients, LPS-treated *TLR4* gene expression exhibited a strong positive connection (strong magnitude) with the *NFkB* gene expression levels; as a result, *TLR4* gene expression increased, likewise, *NFkB* gene expression.

To the best of our knowledge, this is the first study to compare the effect of LPS on the TLR4 pathway markers (*TLR4* and *NFkB*) genes in CSOM patients. Previous studies involved measuring the *TLR4* and *NFkB* gene expression directly from the patient samples. Upregulation of the *TLR4* gene in CSOM has been reported in various samples, such as serum, middle ear mucosa, and middle ear tissue [Si et al., 2012; Hirai et al., 2013; Jotic et al., 2015]. Si et al., (2012) compared the *TLR4* gene and protein expression in CSOM patients and healthy controls using qPCR and immunohistochemistry, respectively. The *TLR4* gene and protein expressions were higher in the middle ear mucosa of CSOM when compared to normal canal skin [Si et al., 2012]. A similar *TLR4* gene upregulation was also observed by Hirai et al., (2013) and Jotic et al., (2015) in middle ear tissue samples [Hirai et al., 2013; Jotic et al., 2015]. Jesic et al., (2014) compared the *TLR4* and NFkB protein expression in CSOM middle ear tissues samples and normal canal skin using immunohistochemistry. The TLR4 protein

expressions were higher and NFkB protein expression were lower in the middle ear mucosa of CSOM when compared to normal canal skin. However, the upregulation of NFkB protein expression observed herein contradicts the previous report [Jesic et al., 2014]. This present study shows that LPS may be responsible for elevated *TLR4* and *NFkB* gene expression. The results of this present study prove that the TLR4 pathway is hypersensitive to LPS because of the upregulation of *TLR4* and *NFkB* gene expression upon LPS stimulation.

Inference 2: LPS induced TNFα production was higher in LPS treated PBMCs (conditioned medium) of CSOM patients

In this study, LPS treatment of PBMCs (in conditioned medium samples) resulted in increased TNFα production and decreased IL-10 levels in CSOM patients compared to healthy controls. TNFα production was the highest, and IL-10 production was the lowest in the CSOM group. This finding indicates that TNFα production is hypersensitive and IL-10 is less sensitive to LPS in CSOM patients. The hypersensitive TNFα production to LPS treatment may be due to increased *NFkB* transcription factor gene expression in the study. As a result, patients show elevated TNFα production levels in CSOM patients compared to healthy controls.

Furthermore, LPS-induced *NFkB* gene expression was compared to TNFα production in the CSOM group. A moderate correlation was observed between the *NFkB* gene and TNFα production. The effect of LPS on TNFα and IL-10 production in clinical variables subgroup analyses were carried out. Results

showed that the gender, laterality, and disease duration did not alter the levels of TNFα and IL-10 production in CSOM patients.

Previous studies have mainly focused on measuring the TNFα levels in serum and middle ear mucosa in CSOM patients [Kuczkowski et al., 2011; Baike et al., 2017; Edwards et al., 2019]. To the best of our knowledge, this is the first study to examine the effect of LPS on TNFα and IL-10 production in CSOM patients. Kuczkowski et al., (2011) reported a higher TNFα protein expression in the middle ear mucosa tissue samples of the CSOM patients than in healthy controls [Kuczkowski et al., 2011]. In addition, Baike et al., (2017) reported six-fold higher serum levels of TNFα in CSOM patients than healthy controls [Baike et al., 2017]. Also, a recent study by Edward et al., (2019) showed a higher TNFα gene expression levels in the middle ear tissue samples of CSOM patients than normal canal skin [Edwards et al., 2019].

The present results proves that the TLR4 pathway is hypersensitive to LPS because of the upregulation of *TLR4* and *NFkB* gene expression, and TNFα production upon LPS stimulation of PBMCs. Therefore, these results indicated that the levels of TNFα expression was higher sensitive to LPS-induced upregulation in CSOM patients.

Inference 3: Association of *TLR4* gene promoter genetic variation (SNPs) with CSOM patients

SNPs in *TLR4* gene promoter regions -2604 G>A, -2570 A>G, -2604 G>A, and -2026 A>G were identified in the both CSOM patients and healthy controls. The distribution of both genotype and allele frequency showed no statistically

significant difference between the two groups (p>0.05; Fisher's exact test). This indicates that the *TLR4* gene promoter region -2604 G>A, -2570 A>G, -2604 G>A, and -2026 A>G SNPs may not be associated with CSOM. Four SNPs in the *TLR4* gene promoter region were selected since they are predicted to be transcription factor binding motifs [Ragnarsdóttir et al., 2015]. Previous studies have shown that the SNPs in *TLR4* gene exons and introns region were associated with CSOM [MacArthur et al., 2013]. The present study evaluated the SNPs in *TLR4* gene promoter regions in CSOM patients. Unlike the SNP in the exons and introns, the SNPs in promoter region were not associated with CSOM condition. This may be due to the small sample size used for the genetic study or the genetic makeup of the study participants.

Final conclusion: The TLR4 pathway is hypersensitive to LPS in CSOM patients, according to the present study findings. The *TLR4* and *NFkB* gene expression, and TNFα production was elevated in the LPS treated PBMCs. Together, these findings lend credence to the hypothesis that the TLR4 pathway may function improperly in CSOM patients, most likely as a result of inadequate regulation. This is the first attempt to look at how CSOM patients naturally respond to LPS. These findings demonstrate the inherent propensity of CSOM patients to overproduce inflammatory cytokines in response to infection.

The results of this study hold translational promise for therapeutic development. Antagonists of the TLR4 pathway may be explored for the amelioration of CSOM. Several anti-TLR4 molecules are currently under clinical

trial for various clinical conditions [Ain et al., 2020]. The successful molecules may be used for the management of CSOM.

Summary and Conclusion

7. Summary and Conclusion

- The aim of this present study was to evaluate the role of the TLR4 pathway in the development of CSOM.
- The study was carried out by adopting a case-control study design. The case group comprised CSOM patients, whereas the control group comprised healthy volunteers. There were 63 participants in each group.
- The first objective of the study was to compare the levels of *TLR4* gene expression in response to LPS treatment in CSOM patients and controls. PBMCs obtained from the study participants were treated with LPS for 4 h along with vehicle control (PBS). *TLR4* gene expression was quantified in the treated cells and untreated cells by qPCR. LPS treatment resulted in the upregulation of expression of the *TLR4* gene in CSOM compared to control (1.75 folds) (p<0.001). These observations indicate that the *TLR4* gene expression shows higher sensitivity to LPS-induced upregulation in CSOM patients.
- The second objective of the study was to compare the levels of *NFkB* gene expression in response to LPS treatment in both CSOM patients and controls. PBMCs obtained from the study participants were treated with LPS for 4 h along with vehicle control (PBS). *NFkB* gene expression was quantified in the treated and untreated cells by qPCR. LPS treatment resulted in the upregulation of expression of the *NFkB* gene in CSOM compared to controls (2.71 fold) (p<0.001). These observations indicate that the *NFkB* gene

expression shows higher sensitivity to LPS-induced upregulation in CSOM patients.

- The third objective of the study was to compare the levels of LPS-treated TNF and IL-10 production in CSOM and controls. PBMCs obtained from the study participants were treated with LPS for 4 h along with a control. TNFα and IL-10 production were quantified in the conditioned cell culture medium by using the ELISA technique. LPS treatment resulted in significantly increased TNFα production in CSOM compared to control (2.9 folds) (p<0.001), and LPS treatment resulted in a significantly decreased in IL-10 production levels in CSOM compared to control (0.74 folds) (p<0.001). These observations indicate that the TNFα production shows higher sensitivity, and IL-10 production shows less sensitivity to LPS-induced upregulation in CSOM patients.

- Correlation analysis was performed between LPS-treated fold change *TLR4* gene expressions compared to *NFkB* gene expression in CSOM patients. LPS-treated fold change in *TLR4* gene expression levels compared to *NFkB* gene expression showed a positive (strong) correlation (r=0.77, p<0.01, Pearson's correlation). These results indicate that the expression of the *TLR4* gene increases, and *NFkB* gene expression increases proportionally. Similarly, LPS-treated fold change *NFkB* gene expressions compared to TNFα production in CSOM patients. LPS-treated fold change *NFkB* gene expression compared to TNFα production showed a positive (moderate) correlation (r=0.37, p<0.01, Spearman's rho correlation). These results indicate that the

expression of the *NFkB* gene increases, TNFα production increases proportionally.

- The fourth objective of the study was to compare *TLR4* gene promoter region SNPs in CSOM patients and controls. Four SNPs in the *TLR4* gene promoter region were selected since they are predicted to be transcription factor binding motifs. SNPs in *TLR4* gene promoter regions such as -2604 G>A,-2570 A>G,-2604 G>A, and -2026 A>G were identified. The distribution of both genotype and allele frequency showed no statistically significant difference between the two groups (p>0.05; Fisher's exact test). This indicates that the *TLR4* gene promoter regions -2604 G>A,-2570 A>G,-2604 G>A, and -2026 A>G SNPs may be not associated with CSOM.
- Overall, this study shows that the TLR4 pathway is hypersensitive to LPS in CSOM patients. Future studies need to explore the TLR4 pathway for developing drugs that can mitigate the inflammatory damage in CSOM.

New Knowledge Generated

8. New Knowledge Generated

- Overactivation of the TLR4 pathway plays an important role in CSOM pathogenesis, the overactivation was linked to bacterial infection of the middle ear.
- The present study shows that the TLR4 pathway is intrinsically hypersensitive to bacterial ligands in CSOM.
- The present study results suggests that CSOM arises may be when a person with intrinsic hypersensitivity of the TLR4 pathway experiences bacterial infection of the middle ear.

Strengths and Weakness

9. Strength and Weakness of the study

Strength

In this study, the responsiveness of the TLR4 pathway was measured using key markers. Whereas previous studies, the key markers were measured in the uninduced state. Because of this approach, host factors responsible for intrinsic predisposition to CSOM are indicated.

Weakness

The selected *TLR4* gene promoter SNPs were not associated with CSOM. The lack of association might be due to small sample size used for the genetic study or the involvement of other SNPs in TLR4 pathway.

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Appendix I

(1) **List of Publications and Presentations**

(2) **Conference certificates**

(3) **Workshop/training certificates**

List of Publications and Presentations

List of Publications

1. J. T. Venkataravanappa, K. C. Prasad, S. Balakrishna. *TLR4* gene expression in patients with chronic suppurative otitis media. ***Ukrainian Biochemical Journal.*** 2021;93-6.

2. J. T. Venkataravanappa, K. C. Prasad, S. Balakrishna. TLR4 pathway is hypersensitive to lipopolysaccharide in patients with chronic suppurative otitis media. 2023: ***Toxicon*** **(Under Review).**

List of Presentations

1. Poster: Jagadish T.V, Prasad K.C, Sharath B. "LPS-induced TNF-alpha secretion is elevated in chronic suppurative otitis media". Poster session presented at: 88th Annual meeting of the Society of Biological Chemists, India (SBCI-2019) and **Conference on Advances at the Interface of Biology and Chemistry**; 31st Oct to 3rd Nov 2019; DAE Convention Centre, Anushaktinagar, Mumbai, India.

2. Poster: Jagadish T.V., Prasad K.C, Sharath B. "TLR4 pathway is dysregulated in Chronic Suppurative Otitis Media". Poster session presented at: 45th meeting of the Indian Society of Human Genetics, Chennai (2020) and **Conference on Advancing Heath care through Genomics**; 13-15 Feb 2020; Sri Ramachandra Institute of Higher Education and Research, Sri Ramachandra Medical College, Chennai, India.

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TLR4 GENE EXPRESSION IN PATIENTS WITH CHRONIC SUPPURATIVE OTITIS MEDIA

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Chronic suppurative otitis media (CSOM) is an infectious disease of the middle ear that involves inflammation and accumulation of fluid behind the eardrum. The pathogenesis of CSOM involves reduced bacterial clearance due to impairment of Toll-Like Receptor (TLR) 4 pathway. TLR4 receptor serves as the molecular sensor for bacterial endotoxin (lipopolysaccharide) and activates inflammatory cell signaling for clearing the bacteria. Previous studies have shown that the expression of TLR4 gene is reduced in middle ear epithelia of CSOM patients. Whether the expression of TLR4 gene is reduced in leukocytes is not known. In our present study we aim to compare the expression of the TLR4 gene in the blood samples of CSOM patients and healthy controls. A case-control study was carried out by involving 16 participants in each group. The levels of the TLR4 gene expression were measured by using the qRT-PCR method. The median (interquartile range) ΔCt values of the TLR4 gene expression was 4.85 (2.61- 8.55) in the patient group and 2.29 (-1.63-4.85) in healthy controls. Expression of the TLR4 gene in the leukocytes of CSOM patient group was reduced by ~5.9 fold compared to the control group and the difference was found to be significant (P = 0.01).

K e y w o r d s: chronic suppurative otitis media, gene expression, toll-like receptor 4 (TLR4).

Otitis media (OM) is a group of complex infectious and inflammatory disease that affects the middle ear cavity. OM can be classified into two subtypes as acute and chronic.acute form of OM (AOM) can lead to chronic suppurative OM (CSOM). Chronic suppurative OM (CSOM) involves persistent infection and continuous ottorrhea for more than 3 episodes in a month with a tympanic membrane perforation [1,2]. CSOM results in serious complications such as brain abscess, hearing loss, meningitis, and facial nerve paralysis [3]. The global estimate of prevalence rate ranges between 1-46% of CSOM [4]. *Pseudomonas aeruginosa* (37.21%) and *Staphylococcus aureus* (27.91%) are the most common bacteria observed in the middle ear fluids of CSOM patients [5].

CSOM is considered to be a multifactorial disease with a genetic component. Animal studies have shown that the *TLR4* gene is involved in the development of CSOM. The *TLR4* gene codes for the type 1 transmembrane protein called Toll-Like Receptor 4 (TLR4) which functions as a pattern recognition receptor specifically for the lipopolysaccharide. The latter molecule is derived from the cell wall of gram-negative bacteria [6,7]. TLR4 is expressed in macrophages, monocytes, dendritic cells, and epithelial cells [8]. The binding of lipopolysaccharide to TLR4 receptor activates the intracellular cell signalling pathway which then results in the secretion of inflammatory cytokines. These cytokines then elicit a protective immune response against bacterial infection. Inactivation of the *TLR4* gene is assumed to compromise the development of a protective immune response and lead to persistent infection [9].

Consistent with the above view, down-regulation of the *TLR4* gene has been reported in the

middle ear epithelia of CSOM patients [10]. It is not known if this pattern is unique to middle ear epithelia or shared by other cell types that express the *TLR4* gene. This study was undertaken to answer this question. The aim of this study was to compare the expression of the *TLR4* gene in the blood samples of CSOM patients and healthy controls.

Materials and Methods

Study design. The case-control design was used to conduct the study. The case group comprised of CSOM patients (n = 16) and the control group comprised of age and gender-matched healthy controls (n = 16). The study was approved (SDUAHER/KLR/CEC/01/2017-18) by the Central Ethics Committee. Patients attending the Department of Otorhinolaryngology, R. L. Jalappa Hospital and Research Centre, Tamaka, Kolar, Karnataka, India, and the patients were enrolled between October 2018-August 2019.

Participant selection. CSOM was diagnosed based an otoscopic examination and the following criteria (i) perforation involving tympanic membrane (ii) chronic ear infection and (iii) hard of hearing. Inclusion criteria for the cases were (i) patients diagnosed with CSOM (ii) both genders in the age group of 18-60 years. Exclusion criteria for the CSOM patients were (i) acute otitis media without effusion, (ii) post-traumatic CSOM, (iii) cleft lip/palate, (iv) down's syndrome, and (v) otomycosis [11]. The patient information sheet and written informed consent form were obtained prior to the recruitment of study subjects. One ml of whole blood samples of both CSOM patients and healthy controls were collected in an EDTA vacutainer and used for quantification of *TLR4* gene expression.

Quantification of TLR4 gene expression by qRT-PCR. The T*LR4* gene expression was performed by using quantitative reverse transcription polymerase chain reaction (qRT-PCR) CFX96 touch system (Bio-Rad, USA). Total RNA was isolated from 1 ml blood samples of both cases (n = 16) and controls (n = 16) using Trizol reagent (GeNei, Bangalore) based on protocol provided by the manufacturer's instruction [12]. The concentration and purity of the total RNA were measured using a spectrophotometer (PerkinElmer UV-VIS (Lambda 35), USA). RNA samples were stored at -20°C until further use. The RNA samples of good quality (260/280 ratio of >2) were used for conversion of cDNA. Total RNA samples were converted to cDNA using iScript cDNA conversion kit (Bio-Rad, USA). Real-time quantification of *TLR4* and *GAPDH* genes were performed using SYBR green method (Bio-Rad, USA).

Following primers were used to quantify the expression of *TLR4* and *GAPDH* (Sigma, USA):

TLR4 gene:

Forward primer: 5′ GAACCTGGACCTGAGCTTTAAT 3′

Reverse primer: 5′ GTCTGGATTTCACACCTGGATAA 3′

GAPDH gene:

Forward primer: 5′ GATCATCAGCAATGCCTCCT-3′,

Reverse primer: 5′ GACTGTGGTCATGAGTCCTTC 3′

The composition of qRT-PCR reaction mixture is as follows: 2 µl of cDNA (1:10 dilution), 0.5 µl of forward and reverse primers, 5 µl of SYBR green, and 2 µl of nuclease-free water. The reaction conditions used for qRT-PCR were as follows: hold at 50°C for 2 min, 40 cycles of hold at 95°C for 10 min, denature at 95°C for 15 sec and anneal/extension at 54.3°C for 60 sec. At the end of the reaction, the Cycle Threshold (Ct) values were obtained from the analysis software. The expression of the *TLR4* gene was normalized using *GAPDH* as endogenous control and the fold change in *TLR4* gene expression was calculated using the $2^{-(\Delta\Delta Ct)}$ method [13].

Statistical analysis. All the statistical analysis was carried out using SPSS Statistics V22.0 (IBM, USA); qualitative variables were represented by percentages. Differences between the two groups were compared by using non parametric test Mann –Whitney U test. P-value less than 0.05 were considered to be statistically significant.

Results

The demographic and clinicalcharacteristics of the study participants were given in Table. The mean age of both CSOM patient and control groups was 31.75 ± 9.89 years. In both groups, 31.25% of the participants were male and 68.75% were female. Majority of the patients showed bilateral involvement of CSOM (68.75%). The common site of tympanic perforation was central (68.75%) followed by posterior (18.75%), and anterior locations (12.05%). Cholesteatoma formation was observed only in few patients (12.50%).

The *TLR4* gene expression was quantified by using qRT-PCR. The resulting ΔCt values did not show normal distribution. Therefore, median and interquartile range (IQR) was calculated for both

the groups. The median ΔCt of *TLR4* gene was 4.85 (2.61 to 8.55) in CSOM group and 2.29 (-1.63 to -4.85) in control group. The difference between the median ΔCt values of the CSOM and control groups was statistically significant ($P = 0.01$; Mann–Whitney U test). The results were represented graphically in Fig. 1. Comparative Ct method used to calculate the fold change in *TLR4* gene expression. The average *TLR4* gene expression in the CSOM group was found to be 0.17 compared to the control group. Thus, the gene expression in the CSOM group was about 5.9 times lower than in the control group.

Further, the *TLR4* gene expression data was analysed after subgrouping the CSOM patients based on the clinical profile. Significant difference was observed between unilateral and bilateral subgroups ($P = 0.03$; Mann–Whitney U test). The results are shown in Fig. 2. Downregulation of the *TLR4* gene expression was severe in the bilateral subgroup than in the unilateral subgroup. There was no significant difference between patients with and without cholesteatoma($P = 0.26$; Mann–Whitney U test).

Discussion

The purpose of this study was to compare the *TLR4* gene expression in CSOM patients and healthy controls. The results of this study show that the levels of *TLR4* gene expression are reduced in CSOM patients compared to healthy controls. This implies that the *TLR4* gene expression is down-regulated in CSOM. To the best of our knowledge, this is the first study to quantify the *TLR4* gene expression in the blood samples of CSOM patients.

The results of this study are in agreement with the pattern reported in the middle mucosa of CSOM patients. Si and co-workers reported that expression

Table. Demographic and clinical parameters of study participants

Parameter	CSOM patients ($n = 16$)	Controls ($n = 16$)
Age (Years) (mean ± SD)	31.75 ± 9.89	31.75 ± 9.89
Gender		
Male	05 (31.25%)	05 (31.25%)
Female	11 (68.75%)	11 (68.75%)
Ear affected		
Unilateral	05 (31.25%)	NA
Bilateral	11 (68.75%)	NA
Perforated TM		
Anterior	02 (12.05%)	NA
Central	11 (68.75%)	NA
Posterior	03 (18.75%)	NA
Disease duration		
≤1 year	06 (37.50%)	NA
>1 year to ≤5 years	06 (37.50%)	NA
>5 year to ≤ 10 years	03 (18.75%)	NA
Since childhood	01 (06.25%)	NA
CSOM		
With Cholesteatoma	02 (12.50%)	NA
Without Cholesteatoma	14 (87.50%)	NA
Presenting symptoms		
Ear discharge	13 (81.25%)	NA
Hearing loss	09 (56.25%)	NA
Ear ache	16 (100.00%)	NA

CSOM – Chronic Suppurative Otitis Media; SD – Standard Deviation; NA – Not Applicable; TM – Tympanic membrane

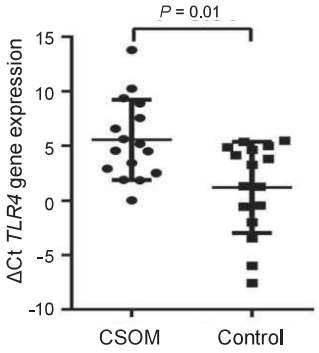

Fig. 1. Differential expression (ΔCt values) of TLR4 gene in CSOM group vs. controls. Median ΔCt_{CSOM} = 4.85; relative gene expression = 0.17; Median $\Delta Ct_{Controls}$ = 2.29; relative gene expression = 1.00

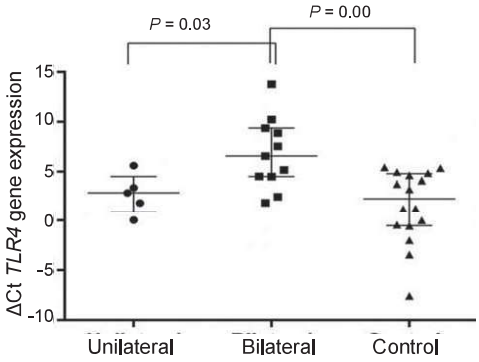

Fig. 2. Differential expression (ΔCt values) of TLR4 gene in bilateral CSOM group, unilateral CSOM group and controls. Median $\Delta Ct_{Bilateral}$ = 6.57; relative gene expression = 0.05, Median $\Delta Ct_{Unilatreal}$ = 2.90; relative gene expression = 0.65); Median $\Delta Ct_{Controls}$ = 2.29; relative gene expression = 1.00

of the *TLR4* gene is lower in middle ear mucosal samples of CSOM patients compared to that of controls [14]. The *TLR4* gene expression levels were not altered in children with a history of OM compared to control in middle ear tissue samples [15]. Also, the *TLR4* gene expression is downregulated in middle ear tissue samples of CSOM patients in comparison to controls [16]. Together these report suggest that *TLR4* gene expression were downregulated in OM.

The role of the *TLR4* gene in the pathogenesis of otitis media has been established with animal studies. Two mice strains (C3H/HeJ and C57BL/6) with knock out of the *TLR4* gene have been developed. Carol and co-workers evaluated the visual changes in the tympanic membrane and auditory brainstem response in 7-8-month-old C3H/HeJ mice. The authors found that the C3H/HeJ mice developed OM spontaneously. Furthermore, increased hearing loss of mixed type and also inflammation in the middle ear were found [17]. Leichtle and coworkers evaluated the C57BL/6 mice and noticed an increase in the middle ear mucosal thickness, cellular infiltration in the middle ear cavity. In addition, they reported that the bacterial clearance in the middle ear cavity was reduced [18]. These reports indicate that genetic variations are the likely cause for the impairment of Toll-Like Receptor 4 in OM. This view is supported by several genetic assocation studies [19-21].

Conclusion. The present study shows that the *TLR4* gene expression is downregulated in the leucocytes of CSOM patients.

Conflict of interest. Authors have completed the Unified Conflicts of Interest form at http://ukr-biochemjournal.org/wp-content/uploads/2018/12/coi_disclosure.pdf and declare no conflict of interest.

Acknowledgments. Thanks to Nagarjuna Reddy MSc student, Sri Devraj Urs Academy of Higher Education and Research for his help in collection of sample.

ЕКСПРЕСІЯ ГЕНА *TLR4* У ПАЦІЄНТІВ ІЗ ХРОНІЧНИМ ГНІЙНИМ СЕРЕДНІМ ОТИТОМ

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Хронічний гнійний середній отит (ХГСО) – інфекційне захворювання середнього вуха, яке супроводжується запаленням та скупченням рідини за барабанною перетинкою. Патогенез ХГСО характеризується зниженням бактеріального кліренсу через порушення сигнального шляху рецептора TLR4, який є молекулярним сенсором бактеріального ендотоксину (ліпополісахариду) і активатором протизапальної сигнальної системи клітин для знищення бактерій. Попередні дослідження показали, що експресія гена *TLR4* знижена в епітелії середнього вуха пацієнтів із ХГСО. Чи знижена експресія гена *TLR4* у лейкоцитах, невідомо. Метою дослідження було порівняти експресію генів *TLR4* у зразках крові пацієнтів із ХГСО та здорових осіб за допомогою кількісної ПЛР. Проведено дослідження "випадок-контроль" за участю 16 осіб у кожній групі. Медіана (міжквартильний розмах) значення ΔCt експресії гена *TLR4* склала 4,85 (2,61-8,55) у групі пацієнтів із ХГСО та 2,29 (-1,63-4,85) у групі здорових осіб. Експресія гена *TLR4* у лейкоцитах пацієнтів із ХГСО була знижена в ~5,9 раза у порівнянні з контрольною групою, і ця різниця виявилася статистично значущою (P = 0,01).

Ключові слова: хронічний гнійний середній отит, експресія генів, рецептор TLR4.

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Toxicon

TLR4 pathway is hypersensitive to lipopolysaccharide in patients with Chronic Suppurative Otitis Media

--Manuscript Draft--

Manuscript Number:	
Article Type:	Research Paper
Section/Category:	Bacterial and fungal toxins
Keywords:	Chronic Suppurative Otitis Media; Lipopolysaccharide; Toll-Like Receptor 4; Tumor Necrosis Factor; Nuclear factor kappa B
Corresponding Author:	Sharath Balakrishna, PhD Sri Devaraj Urs Academy of Higher Education and Research INDIA
First Author:	Jagadish Tavarekere Venkataravanappa
Order of Authors:	Jagadish Tavarekere Venkataravanappa
	Sharath Balakrishna, PhD
	Kothegala Chandrashekaraiah Prasad
Abstract:	Chronic suppurative otitis media (CSOM) is an uncontrolled inflammation in the middle ear due to bacterial infections, it has been linked to the overactivation of the TLR4 pathway. The pathway is activated by lipopolysaccharide (LPS) released from the gram-negative bacterial cell wall. Currently, there is limited information about the factors responsible for overactivation. One possible factor could be the hypersensitivity of the TLR4 pathway in CSOM patients. Therefore, we aimed to evaluate the effect of LPS on the expression of key markers of the TLR4 pathway viz., TLR4, NFkB, and TNFα in CSOM. A case-control study was carried out in the CSOM and healthy participants (n=63). PBMCs from the participants were cultured for 4 h in the presence of LPS. TLR4 and NFkB genes expression was measured in the cell pellet by using qPCR. TNFα cytokine levels were measured in the conditioned media by using ELISA. Fold change expression of genes between LPS-treated and untreated samples were calculated and compared using statistical methods. LPS-induced fold change in the expression of TLR4 (2.8 vs. 1.6; $p<0.001$) and NFkB genes (3.8 vs. 1.4; $p<0.001$) were higher in CSOM group compared to control group. Furthermore, LPS-induced fold change in TNFα production was higher in CSOM group compared to control group (3.2 vs. 1.1; $p<0.001$). Overall results indicate that the LPS treatment resulted in comparatively higher expression of the selected genes, indicates the hypersensitivity of the TLR4 pathway in CSOM patients.
Suggested Reviewers:	Prashanth shetty NITTE KS Hegde Medical Academy drprashanth@nitte.edu.in subject expert in the relevant area
	cyril cyrus India, Imam Abdulrahman Bin Faisal University King Fahd Hospital of the University ccyrus@uod.edu.sa subject expert in relevant area
	Ashis K. Mukherjee institute of advance study in science and technology akm@tezu.ernet.in subject expert
Opposed Reviewers:	

88th Annual Meeting of SBCI and conference on
"Advances at the interface of Biology and Chemistry" (SBCI-2019)
October 31- November 3, 2019

Participation Certificate

This is to certify that Prof. /Dr. /Mr. /Ms. Jagadish T. V. _____ *participated in the 88th Annual Meeting of SBCI and conference on "Advances at the interface of Biology and Chemistry" (October 31 - November 3, 2019), organized by Bio-Science Group, Bhabha Atomic Research Centre, Trombay, Mumbai-400085, held at Anushaktinagar, Mumbai and has delivered an invited lecture/oral presentation/ presented a poster/ been an observer.*

Dr. V. P. Venugopalan	*Dr. H.S. Misra*	*Dr. A. Ballal*
Chairman	*Org. Secretary*	*Convenor*
SBCI-2019	*SBCI-2019*	*SBCI-2019*

45th Annual Meeting of the Indian Society of Human Genetics (ISHG 2020)

Advancing health-Care Through Genomics

13th - 15th February 2020

Certificate

This is to certify that Dr/~~Ms~~/Mr Jagadish T.V.

has participated as ~~Invited Speaker~~ / ~~Chair-Person~~ / Delegate / Presented Poster in the **45th Annual Meeting of ISHG** Organized by the Department of Human Genetics, Sri Ramachandra Institute of Higher Education and Research, Porur, Chennai - 116.

This conference is awarded **12** credit hours by Sri Ramachandra Institute of Higher Education & Research.

Convener / Co-Convener	Dr. Moinak Banerjee	Dr. Subhabrata Chakrabarti	Vice Chancellor
SRIHER	President, ISHG	Secretary, ISHG	SRIHER

Workshops Attended

Certificate of Training

IMMUGENIX BIOSCIENCES
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This is to certify that

Jagadish T V

Has attended and completed virtual hands-on workshop
on

SANGERS DNA SEQUENCE AND MUTATION ANALYSIS

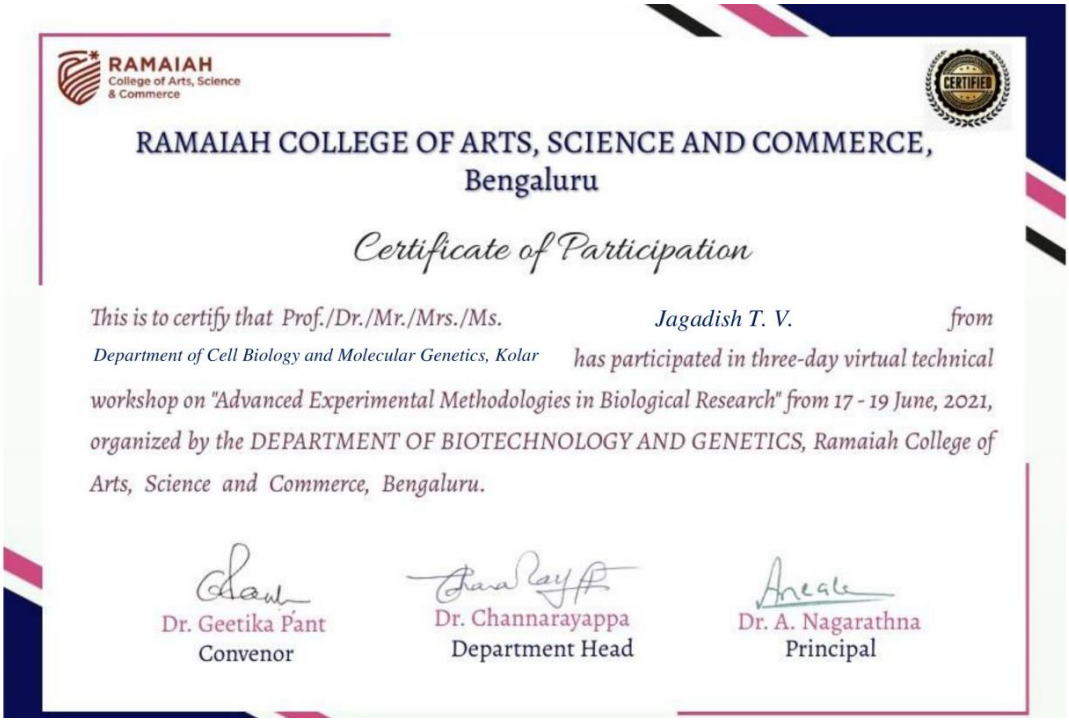
held on

on 20th - 25th September 2021

at

ImmuGenix Biosciences Pvt Ltd, Chennai

Dr. V. Naveen Kumar
Director



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Bengaluru

Certificate of Participation

This is to certify that Prof./Dr./Mr./Mrs./Ms. *Jagadish T. V.* *from Department of Cell Biology and Molecular Genetics, Kolar has participated in three-day virtual technical workshop on "Advanced Experimental Methodologies in Biological Research" from 17 - 19 June, 2021, organized by the DEPARTMENT OF BIOTECHNOLOGY AND GENETICS, Ramaiah College of Arts, Science and Commerce, Bengaluru.*

Dr. Geetika Pant
Convenor

Dr. Channarayappa
Department Head

Dr. A. Nagarathna
Principal

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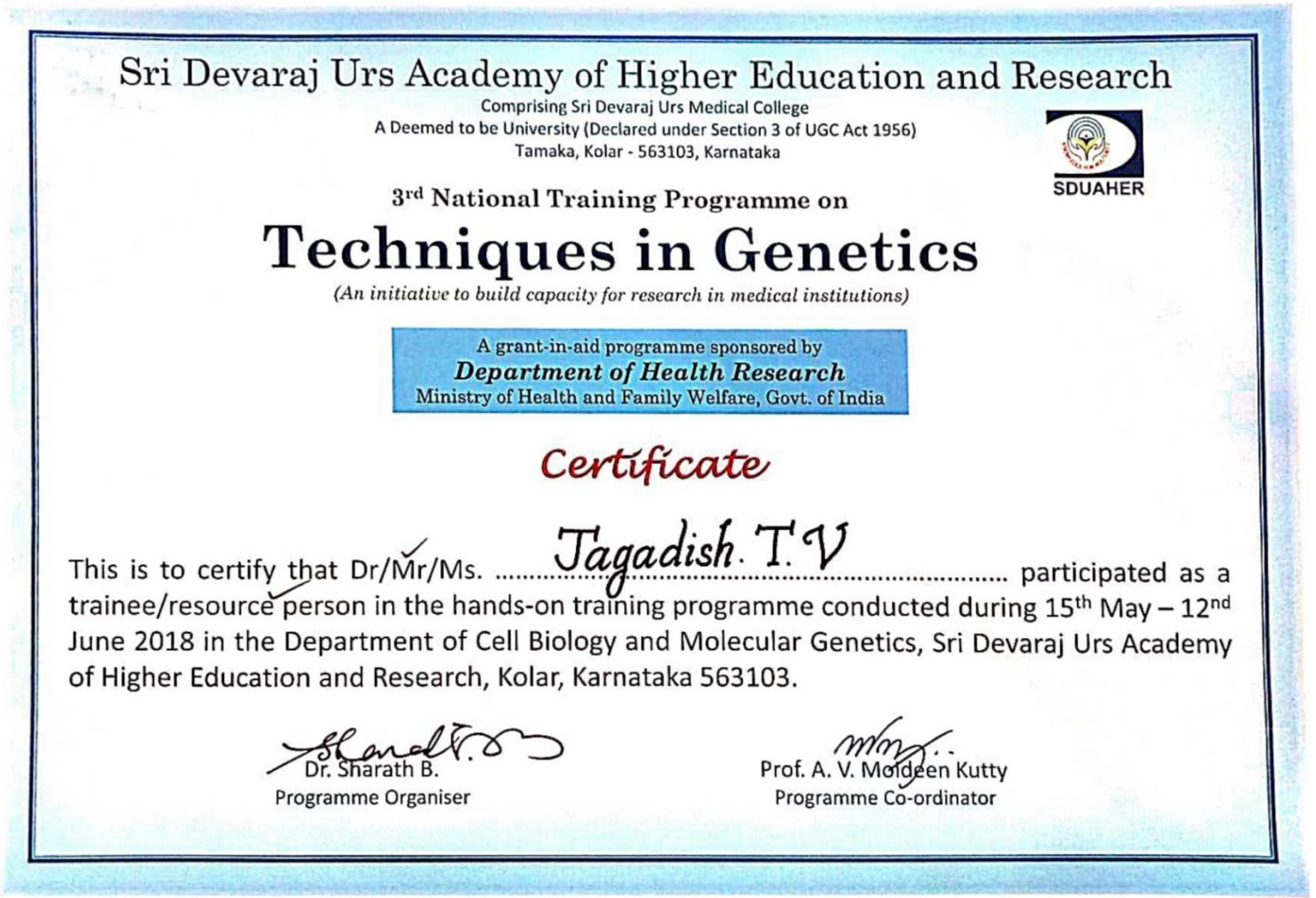
Naveen Arora
(Naveen Kumar Arora)
Professor in-charge, CIIE
Dean, School of Earth & Environmental Sciences
BBA University

Training Program



Sri Devaraj Urs Academy of Higher Education and Research

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Ministry of Health and Family Welfare, Govt. of India

Certificate

This is to certify that Dr/Mr/Ms. Jagadish. T.V participated as a trainee/resource person in the hands-on training programme conducted during 15th May – 12nd June 2018 in the Department of Cell Biology and Molecular Genetics, Sri Devaraj Urs Academy of Higher Education and Research, Kolar, Karnataka 563103.

Dr. Sharath B.
Programme Organiser

Prof. A. V. Moideen Kutty
Programme Co-ordinator

Appendix II

(1) **Central Ethics Committee Certificate**

(2) **Proforma: CSOM patients and Healthy controls**

(3) **Patient/Healthy Information sheet and Consent forms**

CENTRAL ETHICS COMMITTEE
Sri Devaraj Urs Academy of Higher Education & Research
POST BOX NO.62, TAMAKA, KOLAR-563 101, KARNATAKA, INDIA
Ph:08152-210604, 210605, 243003, 243009, ,ext. 438. E-mail: co.rd@sduu.ac.in
SDUAHER
DCGI Registration NO. ECR/425/Ins/KA/2013/RR-16

No. SDUAHER/KLR/CEC/0\ /2017-18 **Date06-10-2017**

Central Ethics Committee, SDUAHER, Kolar

To:

Jagadish T. V.
Ph.D. Scholar.
Department of Cell Biology and Molecular Genetics
SDUAHER
Tamaka, Kolar.

Subject: Ethical clearance for the Ph. D Synopsis

The Central ethics Committee of Sri Devaraj Urs Academy of Higher Education and Research, Kolar has examined Ph.D Synopsis titled "**Studies on Genetic and Immunological Factors in Chronic Otitis Media with Special Emphasis on Toll-Like Receptor 4 Pathway**" and the detailed work plan of the project.

The central ethics committee has unanimously decided to approve the project and grant permission to investigator to carry out the research work. The interim and final report has to be submitted to the ethics committee after completion of the project for the issue of Ethical clearance certificate. Principal investigator should maintain the records of the Project and consent form for not less than 5 year from the date of completion or termination of the project.

Member Secretary
(Dr. TN Suresh)

MEMBER SECRETARY
CENTRAL ETHICS COMMITTEE
SRI DEVARAJ URS ACADEMY OF
HIGHER EDUCATION & RESEARCH
TAMAKA, KOLAR-563 101

Chairman
(Dr. Kiran Katoch)

Chairman
Central Ethics Committee
Sri Devaraj Urs Academy of
Higher Education and Research,
Tamaka Kolar-563101.

Proforma: CSOM

PROFORMA	**Chronic Suppurative Otitis Media**
Name:	Age (y):
IP/HP No:	Phone No:
Address:	Gender Male /Female

Otologic history						
ENT Examination	**Right**			**Left**		
Pinna						
Pre-auricular						
Post-auricular						
EAC						
TM						
Duration of disease						
Affected Ear	Unilateral/Bilateral					
Type of ear discharge	Purulent	Mucoid	Bloody	Clear watery fluid	Foul smelly	
Other symptoms	Diminished hearing	URIs	Itching	Tinnitus	Pain	Vertigo
Site of perforation	Anterior	Posterior	Central	Inferior	Superior	
Hearing loss on Pure tone audiometry						
Normal	Mild conductive		Severe conductive		Sensoneural hearing loss	
Nature of a disease	Cholesteatoma		No Cholesteatoma			

Proforma: Control

Name	
Age (y)	
Gender	□M □F
Contact number	
Address	
Sampling details	
Amount of blood collected	
Date of collection	
Time of sampling	

PART I: PATIENT INFORMATION SHEET

Name of the project: Studies on Genetic and Immunological Factors in Chronic Suppurative Otitis Media with Special Emphasis on Toll-Like Receptor 4 Pathway

Name of the PI: Jagadish T.V.

Name of Organization: Sri Devaraj Urs Academy of Higher Education and Research, Tamaka, Kolar.

Chronic Suppurative Otitis Media is a disease of the middle ear occurring due to infection. Pus formation, inability to hear properly, and feeling of fullness in the ear are the main symptoms of this disease. This disease is commonly occurring in children and may continue into adulthood. The purpose of this study is to analyze the role of *TLR4* gene pathway in the development of Chronic Suppurative Otitis Media. This pathway has been shown to be linked to the disease in experimental animals as well as in twin studies in humans. The results from this study will tell us whether changes in defects in the immune system are responsible for chronic otitis media. It may also help in studying the genetic basis of this chronic middle ear infection

Participant selection: Patients of both sexes, suffering from Chronic Suppurative Otitis Media in the age group 18-60 years, who are willing to participate in the study and give their Informed Written Consent, will be included in the study.

Voluntary Participation: Your participation in this study is entirely voluntary. There is no compulsion to participate in this study. You will be in no way affected if you do not wish to participation in the study. You are required to sign only if you voluntarily agree/permit to participate in this study. Further you are at a liberty to withdraw from the study at any time. We assure that your withdrawal will not affect your treatment by the concerned physician in any way.

Procedure: We will be collecting a small volume of blood (3 ml) from your arm using sterile and disposable needle and syringe. The sample will be used to determine the genetic make-up by a method called DNA sequencing also, the sample will be used for certain properties of innate immunity. Clinical and family history will also be recorded and it is necessary.

Duration: The study will continue for about 3 years.

Risks: No drug will be tested on you. Three ml of blood will be collected using sterile and disposable needle and syringe. Standard of care for the treatment of Otitis Media

will be given to patient (yourself) irrespective of your decision to enroll or not in the project.

Benefits: This study will be useful to understand the relationship between immunological and genetic make-up in Chronic Otitis Media patients and compared with healthy individuals not suffering from the disease nor having any H/O the disease. The results gathered from this study will be beneficial in management of the disease. Participation in this study does not involve any cost for you.

Storage of samples: The samples collected from you will be stored for future research projects on Otitis Media at the University in anonymized way. In such an event, ethics clearance will be obtained.

Confidentiality: All information collected from you will be strictly confidential & will not be disclosed to anyone except if it is required by the law. This information collected will be used only for research. This information will not reveal your identity.

Sharing the Results: The results obtained from this study will be published in scientific / Medical Journals/ Medical conferences.

For any information you are free to contact investigator. This study has been approved by the Institutional Ethics Committee & has been started only after their formal approval. The sample collected will be stored in the institute and I request you to permit us to store and use this sample for any future study.

This document will be stored in the safe locker & a copy given to you for information.

For any further clarification you are free to contact the following researchers:

Dr. Prasad K.C.
Professor
Dept. of ENT
SDUAHER,
Tamaka, Kolar.
Mobile: 9448528729.

Jagadish T. V.
Ph.D. Scholar
Dept. of CBMG
SDUAHER,
Tamaka, Kolar.
Mobile: 8892698143

PART II: INFORMED WRITTEN CONSENT FORM FOR CASE GROUP

Name of Participant (Patient/Volunteer): _____

- This research study has been explained to me; I have been given the chance to discuss it and ask questions. All of my questions have been answered to my satisfaction.
- I understand that this research project involves my. (In case of patient only)
- I have read each page of Patient Information Sheet or it has been read to me.
- I agree to allow access to my health information as explained in the patient information sheet. (In case of patient only)
- I agree to allow collection of my 3ml blood sample and health data for the research purposes explained in the Patient Information Sheet.
- I understand that all the information collected will be kept confidentially.
- I voluntarily consent to take part in this research study.

Participant's (Parent/guardian) signature or thumb impression

Date: _____

If the person providing the consent is illiterate:

A literate witness must sign (if possible, this person should be selected by the person providing the consent).

I have witnessed the accurate reading of the consent form to the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

1) Name of witness_____

Signature of witness _____ Date: _____

2) Name of witness _____

Signature of witness _____ Date: _____

A copy of this ICF has been provided to the participant.

Name of person taking the consent _____

ಭಾಗ 1: ಪ್ರಕರಣ ಗುಂಪಿನ ರೋಗಿಯ ಮಾಹಿತಿ ಪತ್ರ

ಅಧ್ಯನದ ಶೀರ್ಷಿಕೆ: ದೀರ್ಘಕಾಲದ ಕಿವಿಯ ಉರಿಯೂತದ ಟೋಲ್ ಗ್ರಾಹಕ ೪ ಪ್ರತಿಕ್ರಿಯೆಯ ಮೇಲೆ ತಳಿಶಾಸ್ತ್ರ ಮತ್ತು ಪ್ರತಿರಕ್ಷಾ ಶಾಸ್ತ್ರ ಅಂಶಗಳ ಅಧ್ಯಯನ

ನಿಯೋಜಕರ ಹೆಸರು: ಜಗದೀಶ ಟಿ. ವಿ.

ಸಂಸ್ಥೆಯ ಹೆಸರು: ಶ್ರೀ ದೇವರಾಜ್ ಅರಸ್ ಅಕಾಡೆಮಿ ಆಫ್ ಉನ್ನತ ಶಿಕ್ಷಣ ಮತ್ತು ಸಂಶೋಧನಾ ಸಮಸ್ಥೆ, ತಮಾಕಾ, ಕೋಲಾರ

ದೀರ್ಘಕಾಲದ ಕಿವಿಯ ಉರಿಯೂತದ ಮಾಧ್ಯಮವು ಕಿವಿಯಮಧ್ಯ ಭಾಗದ ಸೋಂಕನ್ನು ಒಳಗೊಂಡಿದೆ. ಕೀವುರಚನೆ, ಸರಿಯಾಗಿ ಕೇಳಿಸಿಕೊಳ್ಳಲಾಗದ ಮತ್ತು ಕಿವಿಯ ಉಬ್ಬುವಿಕೆಗಳು ಈ ರೋಗದ ಮುಖ್ಯ ಲಕ್ಷಣಗಳು. ಈ ರೋಗ ಸಾಮಾನ್ಯವಾಗಿ ಮಕ್ಕಳಲ್ಲಿ ಕಂಡು ಬರುತ್ತದೆ ಮತ್ತು ಪ್ರೌಢಾವಸ್ಥೆಗೆ ಮುಂದುವರಿಯಬಹುದು.ಈ ಅಧ್ಯಯನದ ಉದ್ದೇಶ ರಕ್ತದ ಮಾದರಿಗಳನ್ನುಬಳಸಿಕೊಂಡು ದೀರ್ಘಕಾಲದ ಕಿವಿಯ ಉರಿಯೂತ ಮಾಧ್ಯಮದ ಅಭಿವೃದ್ಧಿಯಲ್ಲಿ TLR4 ತಳಿಯ ಪ್ರತಿಕ್ರಿಯಾದ ಪಾತ್ರವನ್ನು ಕಂಡುಕೊಳ್ಳುತ್ತೇವೆ. ಪ್ರಾಯೋಗಿಕ ಪ್ರಾಣಿಗಳಲ್ಲಿನ ರೋಗದೊಂದಿಗೆ ಹಾಗೂ ಮಾನವರಲ್ಲಿ ಅವಳಿ ಅಧ್ಯಯನದಲ್ಲಿ ಈ ಮಾರ್ಗವನ್ನು ಸಂಪರ್ಕಿಸಲಾಗಿದೆ ಎಂದು ತೋರಿಸಲಾಗಿದೆ. ಈ ಅಧ್ಯಯನದಿಂದ ಪ್ರತಿರಕ್ಷಣಾ ವ್ಯವಸ್ಥೆಯಲ್ಲಿನ ಬದಲಾವಣೆಯು ದೀರ್ಘಕಾಲದ ಕಿವಿಯ ಉರಿಯೂತದ ಕಾರಕ ಅಂಶಗಳು ಎಂದು ನಮಗೆ ಕಾಣಿಸುತ್ತದೆ. ಈ ದೀರ್ಘಕಾಲೀನ ಮಧ್ಯಮ ಕಿವಿ ಸೋಂಕಿನ ಆನುವಂಶಿಕ ಆಧಾರವನ್ನು ಅಧ್ಯಯನ ಮಾಡುವುದರಲ್ಲೂ ಇದು ನೆರವಾಗಬಹುದು.

ಭಾಗವಹಿಸುವವರ ಆಯ್ಕೆ: ನಾವು 18-60 ವರ್ಷ ವಯಸ್ಸಿನವರಲ್ಲಿ ದೀರ್ಘಕಾಲದ ಕಿವಿಯ ಉರಿಯೂತ ಮಾಧ್ಯಮದಿಂದ ಬಳಲುತ್ತಿರುವ ಎರಡೂ ಲಿಂಗಗಳ ರೋಗಿಗಳು, ಅಧ್ಯಯನದಲ್ಲಿ ಪಾಲ್ಗೊಳ್ಳಲು ಮತ್ತು ತಮ್ಮ ಮಾಹಿತಿಯುಕ್ತ ಬರಹ ಸಮ್ಮತಿಯನ್ನು ನೀಡಲು ಸಿದ್ಧರಿದ್ದಾರೆ ಮತ್ತು ಅವರು ತಮ್ಮ ತಿಳುವಳಿಕೆಯುಳ್ಳ ಸಮ್ಮತಿಯನ್ನು ಪಡೆದುಕೊಂಡ ನಂತರ ಸೇರಿಸಲಾಗುವುದು.

ಸ್ವಯಂಪ್ರೇರಿತ ಭಾಗವಹಿಸುವಿಕೆ: ಈ ಅಧ್ಯಯನದಲ್ಲಿ ನಿಮ್ಮ ಭಾಗವಹಿಸುವಿಕೆಯು ಸಂಪೂರ್ಣವಾಗಿ ಸ್ವಯಂಪ್ರೇರಿತವಾಗಿದೆ. ಈ ಅಧ್ಯಯನದಲ್ಲಿ ಭಾಗವಹಿಸಲು ಯಾವುದೇ ಒತ್ತಾಯವಿಲ್ಲ. ನೀವು ಅಧ್ಯಯನದಲ್ಲಿ ಭಾಗವಹಿಸಲು ಬಯಸದಿದ್ದರೆ ನೀವು ಯಾವುದೇ ರೀತಿಯಲ್ಲಿ ಪರಿಣಾಮ ಬೀರುವುದಿಲ್ಲ. ಈ ಅಧ್ಯಯನದಲ್ಲಿ ಭಾಗವಹಿಸಲು ನೀವು ಸ್ವಯಂಪ್ರೇರಣೆಯಿಂದ ಒಪ್ಪಿದರೆ/ಅನುಮತಿ ನೀಡಿದರೆ ಮಾತ್ರ ನೀವು ಸಹಿ ಮಾಡಬೇಕಾಗುತ್ತದೆ. ಇದಲ್ಲದೆ ನೀವು ಯಾವುದೇ ಸಮಯದಲ್ಲಿ ಅಧ್ಯಯನದಿಂದ ಹಿಂದೆ ಸರಿಯುವ ಸ್ವಾತಂತ್ರ್ಯದಲ್ಲಿದ್ದೀರಿ. ನಿಮ್ಮ ವಾಪಸಾತಿಯು ಸಂಬಂಧಪಟ್ಟ ವೈದ್ಯರಿಂದ ನಿಮ್ಮ ಚಿಕಿತ್ಸೆಯ ಮೇಲೆ ಯಾವುದೇ ರೀತಿಯಲ್ಲಿ ಪರಿಣಾಮ ಬೀರುವುದಿಲ್ಲ ಎಂದು ನಾವು ಭರವಸೆ ನೀಡುತ್ತೇವೆ.

ವಿಧಾನ: ನಿಮ್ಮ ಕೈಯಿಂದ ಬರಡಾದ ಮತ್ತು ಬಿಸಾಡಬಹುದಾದ ಸೂಜಿ ಮತ್ತು ಸಿರಿಂಜ್ ಬಳಸಲಾಗುತ್ತದೆ ಬಳಸಿ ೩ ಮಿಲೀ ರಕ್ತವನ್ನು ಸಂಗ್ರಹಿಸುತ್ತೇವೆ.. ಮಾದರಿಯನ್ನು ಡಿ.ಎನ್.ಎ ಅನುಕ್ರಮ ಎಂಬ ವಿಧಾನದಿಂದ ಆನುವಂಶಿಕ ರೂಪ ನಿರ್ಧರಿಸಲು ಮತ್ತು ಪ್ರತಿರಕ್ಷಾ ಗುಣಲಕ್ಷಣಗಳನ್ನು ಕಂಡುಹಿಡಿಯಲು ಬಳಸಲಾಗುತ್ತದೆ. ಕ್ಲಿನಿಕಲ್ ಮತ್ತು ಕುಟುಂಬದ ಇತಿಹಾಸವು ಸಹ ಸಂಗ್ರಹಿಸುತ್ತೇವೆ ಮತ್ತು ಅಗತ್ಯವಾಗುತ್ತದೆ.

ಅವಧಿ: ಸಂಶೋಧನೆಯು ಒಟ್ಟು ೩ ವರ್ಷ ನಡೆಯುತ್ತದೆ

ಅಪಾಯಗಳು: ನಿಮ್ಮ ಮೇಲೆ ಯಾವುದೇ ಔಷಧವನ್ನು ಪರೀಕ್ಷಿಸಲಾಗುವುದಿಲ್ಲ. ಬರಡಾದ ಮತ್ತು ಬಿಸಾಡಬಹುದಾದ ಸೂಜಿ ಮತ್ತು ಸಿರಿಂಜ್ ಬಳಸಿ ಮೂರು ಮಿಲಿ ರಕ್ತವನ್ನು ಸಂಗ್ರಹಿಸಲಾಗುತ್ತದೆ. ಓಟಿಟಿಸ್ ಮೀಡಿಯಾ ಚಿಕಿತ್ಸೆಗಾಗಿ ಕಾಳಜಿಯ ಗುಣಮಟ್ಟವನ್ನು ರೋಗಿಗೆ (ನೀವೇ) ನೀಡಲಾಗುವುದು, ಯೋಜನೆಗೆ ಸೇರ್ಪಡೆಗೊಳ್ಳುವ ಅಥವಾ ಮಾಡದಿರುವ ನಿಮ್ಮ ನಿರ್ಧಾರವನ್ನು ಲೆಕ್ಕಿಸದೆ.

ಪ್ರಯೋಜನಗಳು: ದೀರ್ಘಕಾಲದ ಓಟಿಟಿಸ್ ಮಾಧ್ಯಮ ರೋಗಿಗಳಲ್ಲಿ ರೋಗನಿರೋಧಕ ಮತ್ತು ಆನುವಂಶಿಕ ರಚನೆಯ ನಡುವಿನ ಸಂಬಂಧವನ್ನು ಅರ್ಥಮಾಡಿಕೊಳ್ಳಲು ಈ ಅಧ್ಯಯನವು ಉಪಯುಕ್ತವಾಗಿದೆ ಮತ್ತು ರೋಗದಿಂದ ಬಳಲುತ್ತಿರುವ ಅಥವಾ ಯಾವುದೇ ಊ/ಔ ರೋಗವನ್ನು ಹೊಂದಿರದ ಆರೋಗ್ಯವಂತ ವ್ಯಕ್ತಿಗಳಿಗೆ ಹೋಲಿಸಿದರೆ. ಈ ಅಧ್ಯಯನದಿಂದ ಸಂಗ್ರಹಿಸಿದ ಫಲಿತಾಂಶಗಳು ರೋಗದ ನಿರ್ವಹಣೆಗೆ ಪ್ರಯೋಜನಕಾರಿಯಾಗುತ್ತವೆ. ಈ ಅಧ್ಯಯನದಲ್ಲಿ ಭಾಗವಹಿಸುವಿಕೆಯು ನಿಮಗೆ ಯಾವುದೇ ವೆಚ್ಚವನ್ನು ಒಳಗೊಂಡಿರುವುದಿಲ್ಲ.

ಮಾದರಿಗಳ ಶೇಖರಣೆ: ಭವಿಷ್ಯದ ಸಂಶೋಧನಾ ಯೋಜನೆಗಳಿಗಾಗಿ ನಿಮ್ಮ ರಕ್ತದ ಮಾದರಿಯಿಂದ ತಯಾರಿಸಲಾದ ವಂಶವಾಹಿ ವಸ್ತುಗಳನ್ನು ಸಂಗ್ರಹಿಸಬಹುದು. ಅಂತಹ ಸಂದರ್ಭದಲ್ಲಿ, ನೈತಿಕ ಸಮ್ಮತಿ ಅನುಮತಿ ಪಡಿಯಲಾಗುಹುದು.

ಫಲಿತಾಂಶಗಳ ಹಂಚಿಕೆ: ಈ ಅಧ್ಯಯನದಿಂದ ಪಡೆದ ಫಲಿತಾಂಶಗಳು ವೈಜ್ಞಾನಿಕ ಅಥವಾ ವೈದ್ಯಕೀಯ ನಿಯತಕಾಲಿಕಗಳಲ್ಲಿ ಅಥವಾ ವೈದ್ಯಕೀಯ ಸಮ್ಮೇಳನಗಳಲ್ಲಿ ಪ್ರಕಟವಾಗುತ್ತವೆ. ಈ ಪ್ರಕ್ರಿಯೆಯಲ್ಲಿ ನಾವು ಯಾವುದೇ ಸಮಯದಲ್ಲಿ ನಿಮ್ಮನ್ನು ಒತ್ತಾಯಿಸುವುದಿಲ್ಲ; ಸಹ ಅಧ್ಯಯನದ ನಿಮ್ಮ ಸಹಕಾರವನ್ನು ನಾವು ಬಹಳವಾಗಿ ಶ್ಲಾಘಿಸುತ್ತೇವೆ. ಅಧ್ಯಯನದಲ್ಲಿ ಪಾಲ್ಗೊಳ್ಳಲು ನಿಮ್ಮ ಒಪ್ಪಿಗೆಯನ್ನು ಪಡೆಯಲು ನಾವು ಬಯಸುತ್ತೇವೆ.

ಯಾವುದೇ ಮಾಹಿತಿಗಾಗಿ ನೀವು ತನಿಖೆದಾರರನ್ನು ಸಂಪರ್ಕಿಸಲು ಮುಕ್ತವಾಗಿರುತ್ತೀರಿ. ಈ ಅಧ್ಯಯನವು ಸಾಂಸ್ಥಿಕ ನೈತಿಕ ಸಮಿತಿಯಿಂದ ಅನುಮೋದಿಸಲ್ಪಟ್ಟಿದೆ ಮತ್ತು ಅವರ ಔಪಚಾರಿಕ ಅನುಮೋದನೆಯ ನಂತರ ಮಾತ್ರ ಪ್ರಾರಂಭಿಸಲ್ಪಟ್ಟಿದೆ. ಸಂಗ್ರಹಿಸಿದ ಮಾದರಿಯನ್ನು ಇನ್ಸ್ಟಿಟ್ಯೂಟ್ನಲ್ಲಿ ಸಂಗ್ರಹಿಸಲಾಗುವುದು ಮತ್ತು ಭವಿಷ್ಯದ ಅಧ್ಯಯನಕ್ಕಾಗಿ ಈ ಮಾದರಿಯನ್ನು ಸಂಗ್ರಹಿಸಲು ಮತ್ತು ಬಳಸಲು ನಮಗೆ ಅನುಮತಿಸಲು ನಾನು ವಿನಂತಿಸುತ್ತೇನೆ.

ಈ ಡಾಕ್ಯುಮೆಂಟನ್ನು ಕೋಶ ಜೀವಶಾಸ್ತ್ರ ಮತ್ತು ಆಣ್ವಿಕ ತಳಿಶಾಸ್ತ್ರ ಇಲಾಖೆಯ ಸುರಕ್ಷಿತ ಲಾಕರ್ನಲ್ಲಿ ಮತ್ತು ನಿಮಗೆ ಮಾಹಿತಿಗಾಗಿ ನೀಡಲಾದ ನಕಲಿನಲ್ಲಿ ಸಂಗ್ರಹಿಸಲಾಗುತ್ತದೆ.ಮತ್ತಷ್ಟು ಸ್ಪಷ್ಟೀಕರಣಕ್ಕಾಗಿ ನೀವು ನನ್ನನ್ನು ಸಂಪರ್ಕಿಸಲು ಮುಕ್ತವಾಗಿರುತ್ತೀರಿ.

ಹೆಚ್ಚಿನ ಸ್ಪಷ್ಟೀಕರಣಕ್ಕಾಗಿ ನೀವು ಈ ಕೆಳಗಿನ ಸಂಶೋಧಕರನ್ನು ಸಂಪರ್ಕಿಸಲು ಮುಕ್ತರಾಗಿದ್ದೀರಿ.

ಡಾ.ಪ್ರಸಾದ್ ಕೆ.ಸಿ
ಪ್ರೊಫೆಸರ್
ಇ. ಏನ್. ಟಿ. ಇಲಾಖೆ
ಟಮಕ, ಕೋಲಾರ. ಮೊಬೈಲ್:
೯೪೪೮೫೨೮೯೯.

ಜಗದೀಶ್ ಟಿ. ವಿ..
ಸಂಶೋಧನಾ ವಿದ್ಯಾರ್ಥಿ
ಕೋಶ ಜೀವಶಾಸ್ತ್ರ ಮತ್ತು ಆಣ್ವಿಕ ತಳಿಶಾಸ್ತ್ರ ಇಲಾಖೆ
ಟಮಕ, ಕೋಲಾರ. ಮೊಬೈಲ್:
೮೮೯೨೬೯೦೪೩

ಭಾಗ 2: ಮಾಹಿತಿಯುಕ್ತ ಪ್ರಕರಣ ಗುಂಪಿನ ಬರಹ ಸಮ್ಮತಿ ಪತ್ರ

ಪಾಲ್ಗೊಳ್ಳುವವರ ಹೆಸರು (ರೋಗಿಯ / ಸ್ವಯಂಸೇವಕ): _____

- ಈ ಸಂಶೋಧನಾ ಅಧ್ಯಯನವನ್ನು ನನಗೆ ವಿವರಿಸಲಾಗಿದೆ; ಇದನ್ನು ಚರ್ಚಿಸಲು ಮತ್ತು ಪ್ರಶ್ನೆಗಳನ್ನು ಕೇಳಲು ನನಗೆ ಅವಕಾಶ ನೀಡಲಾಗಿದೆ. ನನ್ನ ಎಲ್ಲಾ ಪ್ರಶ್ನೆಗಳಿಗೆ ನನ್ನ ತೃಪ್ತಿಗೆ ಉತ್ತರಿಸಲಾಗಿದೆ.
- ಈ ಸಂಶೋಧನಾ ಯೋಜನೆಯು ಒಳಗೊಂಡಿರುತ್ತದೆ ಎಂದು ನಾನು ಅರ್ಥಮಾಡಿಕೊಂಡಿದ್ದೇನೆ. (ರೋಗಿಗೆ ಮಾತ್ರ)
- ನಾನು ರೋಗಿಯ ಮಾಹಿತಿ ಪತ್ರ ಪ್ರತಿ ಪುಟವನ್ನು ಓದಿದ್ದೇನೆ ಅಥವಾ ನನಗೆ ಓದುತ್ತಿದ್ದೇನೆ.
- ರೋಗಿಯ ಮಾಹಿತಿ ಹಾಳೆಯಲ್ಲಿ ವಿವರಿಸಿದಂತೆ ನನ್ನ ಆರೋಗ್ಯ ಮಾಹಿತಿಯನ್ನು ಪ್ರವೇಶಿಸಲು ನಾನು ಒಪ್ಪುತ್ತೇನೆ. (ರೋಗಿಗೆ ಮಾತ್ರ)
- ನನ್ನ 3 ಮಿಲಿ ರಕ್ತದ ಮಾದರಿ ಮತ್ತು ಆರೋಗ್ಯದ ಮಾಹಿತಿ ಸಂಗ್ರಹಣೆಗಾಗಿ ಸಂಶೋಧನಾ ಉದ್ದೇಶಗಳಿಗಾಗಿ ರೋಗಿಯ ಮಾಹಿತಿ ಪತ್ರದಲ್ಲಿ ವಿವರಿಸಲು ಅನುಮತಿಸಲು ನಾನು ಒಪ್ಪುತ್ತೇನೆ.
- ಸಂಗ್ರಹಿಸಿದ ಎಲ್ಲಾ ಮಾಹಿತಿಯನ್ನು ಗೌಪ್ಯವಾಗಿ ಇರಿಸಲಾಗುವುದು ಎಂದು ನಾನು ಅರ್ಥಮಾಡಿಕೊಂಡಿದ್ದೇನೆ.
- ನಾನು ಈ ಸಂಶೋಧನಾ ಅಧ್ಯಯನದಲ್ಲಿ ಪಾಲ್ಗೊಳ್ಳಲು ಸ್ವಯಂಪ್ರೇರಣೆಯಿಂದ ಸಮ್ಮತಿಸುತ್ತೇನೆ.

ಪಾಲ್ಗೊಳ್ಳುವವರ ಹೆಸರು (ಪೋಷಕರು) ಸಹಿ ಅಥವಾ ಹೆಬ್ಬೆರಳು ಗುರುತು

ದಿನಾಂಕ: _____

ಅನಕ್ಷರಸ್ಥರಿಗೆ: ಸಾಕ್ಷಿದಾರರು ಸಹಿ ಮಾಡಬೇಕು (ಪಾಲ್ಗೊಂಡವರು ಆಯ್ಕೆ ಮಾಡಬೇಕು ಮತ್ತು ಸಂಶೋಧನಾ ತಂಡದ ಯಾವುದೇ ಸಂಪರ್ಕ ಇರಬಾರದು).

ಭಾಗವಹಿಸುವ ಅನಕ್ಷರಸ್ಥರು ತಮ್ಮ ಹೆಬ್ಬೆಟ್ಟಿನ ಗುರುತು ಮಾಡಬೇಕು. ನಾನು ಸಮರ್ಥ ಸ್ಪರ್ಧಿ ಸಮ್ಮತಿ ನಮೂನೆಗಳ ಮಾಹಿತಿಯನ್ನು ನಿಖರವಾದ ತಿಳಿದಿರುತ್ತೇನೆ. ವೈಯಕ್ತಿಕ ಪ್ರಶ್ನೆಗಳನ್ನು ಕೇಳಲು ಅವಕಾಶ ಹೊಂದಿರುತ್ತೇನೆ. ನಾನು ವೈಯಕ್ತಿಕ ಮುಕ್ತವಾಗಿ ಒಪ್ಪಿಗೆ ನೀಡಿದ್ದಾರೆ ಎಂದು ಖಚಿತ ಪಡಿಸಿರುತ್ತೇನೆ.

1) ಸಾಕ್ಷಿಯ ಹೆಸರು: _____ 2) ಸಾಕ್ಷಿಯ ಹೆಸರು: :

ಸಾಕ್ಷಿಯ ಸಹಿ: _____ ಸಾಕ್ಷಿಯ ಸಹಿ: _____

ದಿನಾಂಕ: _____ ದಿನಾಂಕ: _____

ಈ ಐಸಿಎಫ್ ಒಂದು ಪ್ರತಿಯನ್ನು ಹೊಂದಿರುತ್ತೇನೆ.

ಒಪ್ಪಿಗೆ ತೆಗೆದುಕೊಳ್ಳುವ ವ್ಯಕ್ತಿಯ ಹೆಸರು

ಒಪ್ಪಿಗೆ ತೆಗೆದುಕೊಳ್ಳುವ ವ್ಯಕ್ತಿ ಸಹಿ _____ದಿನಾಂಕ: _____

PART I: INFORMATION SHEET FOR CONTROL GROUP

Name of the project: Studies on Genetic and Immunological Factors in Chronic suppurative Otitis Media with Special Emphasis on Toll-Like Receptor 4 Pathway

Name of the PI: Jagadish T. V.

Name of Organization: Sri Devaraj Urs Academy of Higher Education and Research, Tamaka, Kolar.

Chronic suppurative Otitis Media is a disease of the middle ear occurring due to infection. Pus formation, inability to hear properly, and feeling of fullness in the ear are the main symptoms of this disease. This disease is commonly occurs in children and may continue into adulthood. The purpose of this study is to analyze the role of *TLR4* gene pathway in the development of Chronic Otitis Media. This pathway has been shown to be linked to the disease in experimental animals as well as in twin studies in humans. The results from this study will tell us whether changes in defects in the immune system are responsible for chronic otitis media. It may also help in studying the genetic basis of this chronic middle ear infection

Participant selection: Normal healthy individuals in the age group of 18 -60 years will be included after obtaining their informed consent for the purpose of comparison in the study.

Voluntary Participation: Your participation in this study is entirely voluntary. There is no compulsion to participate in this study. You will be in no way affected if you do not wish to participation in the study. You are required to sign only if you voluntarily agree/permit your participate in this study. Further you are at a liberty to withdraw from the study at any time. We assure that your withdrawal will not affect your treatment by the concerned physician in any way.

Procedure: We will be collecting a small volume of blood (3 ml) from your arm using sterile and disposable needle and syringe. The sample will be used determine the genetic make-up by a method called DNA sequencing also, the sample will be used for certain properties of innate immunity. Clinical and family history will also be recorded and it is necessary.

Duration: The study will continue for about 3 years.

Risks: No drug will be tested on you. Three ml of blood will be collected using sterile and disposable needle and syringe

Benefits: This study will be useful to understand the relationship between immunological and genetic make-up in healthy individuals not suffering from the disease nor having any H/O the disease. The results gathered from this study will be beneficial in management of the disease. Participation in this study does not involve any cost for you.

Storage of samples: The samples collected from you will be stored for future research projects on Otitis Media at the University in anonymized way. In such an event, ethics clearance will be obtained.

Confidentiality: All information collected from you be strictly confidential & will not be disclosed to anyone except if it is required by the law. This information collected will be used only for research. This information will not reveal your identity.

Sharing the Results: The results obtained from this study will be published in scientific / Medical Journals/ Medical conferences.

For any information you is free to contact investigator. This study has been approved by the Institutional Ethics Committee & has been started only after their formal approval. The sample collected will be stored in the institute and I request you to permit us to store and use this sample for any future study.

This document will be stored in the safe locker & a copy given to you for information.

For any further clarification you are free to contact the following researchers:

Dr. Prasad K.C.
Professor
Dept. of ENT
SDUAHER
Tamaka, Kolar.
Mobile: 9448528729.

Jagadish T V
Ph.D. Scholar
Dept. of CBMG
SDUAHER,
Tamaka, Kolar.
Mobile: 8892698143

PART II: INFORMED WRITTEN CONSENT FOR CONTROL GROUP

Name of Participant (Control /Volunteer): _____

- This research study has been explained to me; I have been given the chance to discuss it and ask questions. All of my questions have been answered to my satisfaction.
- I understand that this research project involves my blood sample.
- I have read each page of Information Sheet or it has been read to me.
- I agree to allow access health information as explained in the information sheet.
- I agree to allow collection of my 3 ml blood sample and health data for the research purposes explained in the Information Sheet.
- I understand that all the information collected will be kept confidentially.
- I voluntarily consent to take part in this research study.

Participant's signature or thumb impression

Date: _____

If the person providing the consent is illiterate:

A literate witness must sign (if possible, this person should be selected by the person providing the consent).

I have witnessed the accurate reading of the consent form to the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

1) Name of witness_____

Signature of witness _____ Date: _____

2) Name of witness _____

Signature of witness _____ Date: _____

A copy of this ICF has been provided to the participant.

Name of person taking the consent _____

ಭಾಗ ೧: ಸಾಮಾನ್ಯ ಗುಂಪಿನ ರೋಗಿಯ ಮಾಹಿತಿ ಪತ್ರ

ಅಧ್ಯನದ ಶೀರ್ಷಿಕೆ: ದೀರ್ಘಕಾಲದ ಕಿವಿಯ ಉರಿಯೂತದ ಟೋಲ್ ಗ್ರಾಹಕ ೪ ಪ್ರತಿಕ್ರಿಯೆಯ ಮೇಲೆ ತಳಿಶಾಸ್ತ್ರ ಮತ್ತು ಪ್ರತಿರಕ್ಷಾ ಶಾಸ್ತ್ರ ಅಂಶಗಳ ಅಧ್ಯಯನ

ನಿಯೋಜಕರ ಹೆಸರು: ಜಗದೀಶ ಟಿ. ವಿ.

ಸಂಸ್ಥೆಯ ಹೆಸರು: ಶ್ರೀ ದೇವರಾಜ್ ಅರಸ್ ಅಕಾಡೆಮಿ ಆಫ್ ಉನ್ನತ ಶಿಕ್ಷಣ ಮತ್ತು ಸಂಶೋಧನಾ ಸಮಸ್ಥೆ, ತಮಾಕಾ, ಕೋಲಾರ

ದೀರ್ಘಕಾಲದ ಕಿವಿಯ ಉರಿಯೂತದ ಮಾಧ್ಯಮವು ಕಿವಿಯಮಧ್ಯ ಭಾಗದ ಸೋಂಕನ್ನು ಒಳಗೊಂಡಿದೆ. ಕೀವುರಚನೆ, ಕೇಳಿಸಿಕೊಳ್ಳಲು ಅಸಾಮರ್ಥ್ಯ ಮತ್ತು ಕಿವಿಯ ಉಬ್ಬುವಿಕೆಗಳು ಈ ರೋಗದ ಮುಖ್ಯ ಲಕ್ಷಣಗಳು. ಈ ರೋಗ ಸಾಮಾನ್ಯವಾಗಿ ಮಕ್ಕಳಲ್ಲಿ ಕಂಡು ಬರುತ್ತದೆ ಮತ್ತು ಪ್ರೌಢಾವಸ್ಥೆಗೆ ಮುಂದುವರಿಯಬಹುದು.ಈ ಅಧ್ಯಯನದ ಉದ್ದೇಶ ರಕ್ತದ ಮಾದರಿಗಳನ್ನುಬಳಸಿಕೊಂಡು ದೀರ್ಘಕಾಲದ ಕಿವಿಯ ಉರಿಯೂತ ಮಾಧ್ಯಮದ ಅಭಿವೃದ್ಧಿಯಲ್ಲಿ **TLR4** ತಳಿಯ ಪ್ರತಿಕ್ರಿಯಾದ ಪಾತ್ರವನ್ನು ಕಂಡುಕೊಳ್ಳುತ್ತೇವೆ. ಪ್ರಾಯೋಗಿಕ ಪ್ರಾಣಿಗಳಲ್ಲಿನ ರೋಗದೊಂದಿಗೆ ಹಾಗೂ ಮಾನವರಲ್ಲಿ ಅವಳಿ ಅಧ್ಯಯನದಲ್ಲಿ ಈ ಮಾರ್ಗವನ್ನು ಸಂಪರ್ಕಿಸಲಾಗಿದೆ ಎಂದು ತೋರಿಸಲಾಗಿದೆ. ಈ ಅಧ್ಯಯನದಿಂದ ಪ್ರತಿರಕ್ಷಣಾ ವ್ಯವಸ್ಥೆಯಲ್ಲಿನ ಬದಲಾವಣೆಯು ದೀರ್ಘಕಾಲದ ಕಿವಿಯ ಉರಿಯೂತದ ಕಾರಕ ಅಂಶಗಳು ಎಂದು ನಮಗೆ ಕಾಣಿಸುತ್ತದೆ. ಈ ದೀರ್ಘಕಾಲೀನ ಮಧ್ಯಮ ಕಿವಿ ಸೋಂಕಿನ ಆನುವಂಶಿಕ ಆಧಾರವನ್ನು ಅಧ್ಯಯನ ಮಾಡುವುದರಲ್ಲೂ ಇದು ನೆರವಾಗಬಹುದು.

ಭಾಗವಹಿಸುವವರ ಆಯ್ಕೆ: ಈ ಅಧ್ಯಯನದ ಹೋಲಿಕೆಯ ಉದ್ದೇಶಕ್ಕಾಗಿ ತಿಳುವಳಿಕೆಯುಳ್ಳ ಮಾಹಿತಿಯುಕ್ತ ಬರಹ ಸಮ್ಮತಿಯ ಒಪ್ಪಿಗೆ ಪಡೆದ ನಂತರ 18-20 ವರ್ಷ ವಯಸ್ಸಿನ ಸಾಮಾನ್ಯ ಆರೋಗ್ಯವಂತ ವ್ಯಕ್ತಿಗಳನ್ನು ಅಧ್ಯಯನದಲ್ಲಿ ಸೇರಿಸಿಕೊಳ್ಳಲಾಗುವೂದು.

ಸ್ವಯಂಪ್ರೇರಿತ ಭಾಗವಹಿಸುವಿಕೆ: ಈ ಅಧ್ಯಯನದಲ್ಲಿ ನಿಮ್ಮ ಭಾಗವಹಿಸುವಿಕೆಯು ಸಂಪೂರ್ಣವಾಗಿ ಸ್ವಯಂಪ್ರೇರಿತವಾಗಿದೆ. ಈ ಅಧ್ಯಯನದಲ್ಲಿ ಭಾಗವಹಿಸಲು ಯಾವುದೇ ಒತ್ತಾಯವಿಲ್ಲ. ನೀವು ಅಧ್ಯಯನದಲ್ಲಿ ಭಾಗವಹಿಸಲು ಬಯಸದಿದ್ದರೆ ನೀವು ಯಾವುದೇ ರೀತಿಯಲ್ಲಿ ಪರಿಣಾಮ ಬೀರುವುದಿಲ್ಲ. ಈ ಅಧ್ಯಯನದಲ್ಲಿ ಭಾಗವಹಿಸಲು ನೀವು ಸ್ವಯಂಪ್ರೇರಣೆಯಿಂದ ಒಪ್ಪಿದರೆ/ಅನುಮತಿ ನೀಡಿದರೆ ಮಾತ್ರ ನೀವು ಸಹಿ ಮಾಡಬೇಕಾಗುತ್ತದೆ. ಇದಲ್ಲದೆ ನೀವು ಯಾವುದೇ ಸಮಯದಲ್ಲಿ ಅಧ್ಯಯನದಿಂದ ಹಿಂದೆ ಸರಿಯುವ ಸ್ವಾತಂತ್ರ್ಯದಲ್ಲಿದ್ದೀರಿ. ನಿಮ್ಮ ವಾಪಸಾತಿಯು ಸಂಬಂಧಪಟ್ಟ ವೈದ್ಯರಿಂದ ನಿಮ್ಮ ಚಿಕಿತ್ಸೆಯ ಮೇಲೆ ಯಾವುದೇ ರೀತಿಯಲ್ಲಿ ಪರಿಣಾಮ ಬೀರುವುದಿಲ್ಲ ಎಂದು ನಾವು ಭರವಸೆ ನೀಡುತ್ತೇವೆ.

ವಿಧಾನ: ನಿಮ್ಮ ಕೈಯಿಂದ ಬರಡಾದ ಮತ್ತು ಬಿಸಾಡಬಹುದಾದ ಸೂಜಿ ಮತ್ತು ಸಿರಿಂಜ್ ಬಳಸಲಾಗುತ್ತದೆ ಬಳಸಿ ೩ ಮಿಲೀ ರಕ್ತವನ್ನು ಸಂಗ್ರಹಿಸುತ್ತೇವೆ.. ಮಾದರಿಯನ್ನು ಡಿ.ಎನ್.ಎ ಅನುಕ್ರಮ ಎಂಬ ವಿಧಾನದಿಂದ ಆನುವಂಶಿಕ ರೂಪ ನಿರ್ಧರಿಸಲು ಮತ್ತು ಪ್ರತಿರಕ್ಷಾ ಗುಣಲಕ್ಷಣಗಳನ್ನು ಕಂಡುಹಿಡಿಯಲು ಬಳಸಲಾಗುತ್ತದೆ. ಕ್ಲಿನಿಕಲ್ ಮತ್ತು ಕುಟುಂಬದ ಇತಿಹಾಸವು ಸಹ ಸಂಗ್ರಹಿಸುತ್ತೇವೆ ಮತ್ತು ಅಗತ್ಯವಾಗುತ್ತದೆ.

ಅವಧಿ: ಸಂಶೋಧನೆಯು ಒಟ್ಟು ೩ ವರ್ಷ ನಡೆಯುತ್ತದೆ

ಅಪಾಯಗಳು: ನಿಮ್ಮ ಮೇಲೆ ಯಾವುದೇ ಔಷಧವನ್ನು ಪರೀಕ್ಷಿಸಲಾಗುವುದಿಲ್ಲ. ಬರಡಾದ ಮತ್ತು ಬಿಸಾಡಬಹುದಾದ ಸೂಜಿ ಮತ್ತು ಸಿರಿಂಜ್ ಬಳಸಿ ಮೂರು ಮಿಲಿ ರಕ್ತವನ್ನು ಸಂಗ್ರಹಿಸಲಾಗುತ್ತದೆ. ಓಟಿಟಿಸ್ ಮೀಡಿಯಾ ಚಿಕಿತ್ಸೆಗಾಗಿ ಕಾಳಜಿಯ ಗುಣಮಟ್ಟವನ್ನು ರೋಗಿಗೆ (ನೀವೇ) ನೀಡಲಾಗುವುದು, ಯೋಜನೆಗೆ ಸೇರ್ಪಡೆಗೊಳ್ಳುವ ಅಥವಾ ಮಾಡದಿರುವ ನಿಮ್ಮ ನಿರ್ಧಾರವನ್ನು ಲೆಕ್ಕಿಸದೆ.

ಪ್ರಯೋಜನಗಳು: ದೀರ್ಘಕಾಲದ ಕಿವಿಯ ಉರಿಯೂತದ ಮಾಧ್ಯಮ ರೋಗಿಗಳಲ್ಲಿ ರೋಗನಿರೋಧಕ ಮತ್ತು ಆನುವಂಶಿಕ ರಚನೆಯ ನಡುವಿನ ಸಂಬಂಧವನ್ನು ಅರ್ಥಮಾಡಿಕೊಳ್ಳಲು ಈ ಅಧ್ಯಯನವು ಉಪಯುಕ್ತವಾಗಿದೆ ಮತ್ತು ರೋಗದಿಂದ ಬಳಲುತ್ತಿರುವ ಅಥವಾ ಯಾವುದೇ ಊ/ಔ ರೋಗವನ್ನು ಹೊಂದಿರದ ಆರೋಗ್ಯವಂತ ವ್ಯಕ್ತಿಗಳಿಗೆ ಹೋಲಿಸಿದರೆ. ಈ ಅಧ್ಯಯನದಿಂದ ಸಂಗ್ರಹಿಸಿದ ಫಲಿತಾಂಶಗಳು ರೋಗದ ನಿರ್ವಹಣೆಗೆ ಪ್ರಯೋಜನಕಾರಿಯಾಗುತ್ತವೆ. ಈ ಅಧ್ಯಯನದಲ್ಲಿ ಭಾಗವಹಿಸುವಿಕೆಯು ನಿಮಗೆ ಯಾವುದೇ ವೆಚ್ಚವನ್ನು ಒಳಗೊಂಡಿರುವುದಿಲ್ಲ.

ಮಾದರಿಗಳ ಶೇಖರಣೆ: ಭವಿಷ್ಯದ ಸಂಶೋಧನಾ ಯೋಜನೆಗಳಿಗಾಗಿ ನಿಮ್ಮ ರಕ್ತದ ಮಾದರಿಯಿಂದ ತಯಾರಿಸಲಾದ ವಂಶವಾಹಿ ವಸ್ತುಗಳನ್ನು ಸಂಗ್ರಹಿಸಬಹುದು. ಅಂತಹ ಸಂದರ್ಭದಲ್ಲಿ, ನೈತಿಕ ಸಮ್ಮತಿ ಅನುಮತಿ ಪಡಿಯಲಾಗುಹುದು.

ಫಲಿತಾಂಶಗಳ ಹಂಚಿಕೆ: ಈ ಅಧ್ಯಯನದಿಂದ ಪಡೆದ ಫಲಿತಾಂಶಗಳು ವೈಜ್ಞಾನಿಕ ಅಥವಾ ವೈದ್ಯಕೀಯ ನಿಯತಕಾಲಿಕಗಳಲ್ಲಿ ಅಥವಾ ವೈದ್ಯಕೀಯ ಸಮ್ಮೇಳನಗಳಲ್ಲಿ ಪ್ರಕಟವಾಗುತ್ತವೆ. ಈ ಪ್ರಕ್ರಿಯೆಯಲ್ಲಿ ನಾವು ಯಾವುದೇ ಸಮಯದಲ್ಲಿ ನಿಮ್ಮನ್ನು ಒತ್ತಾಯಿಸುವುದಿಲ್ಲ; ಸಹ ಅಧ್ಯಯನದ ನಿಮ್ಮ ಸಹಕಾರವನ್ನು ನಾವು ಬಹಳವಾಗಿ ಶ್ಲಾಘಿಸುತ್ತೇವೆ. ಅಧ್ಯಯನದಲ್ಲಿ ಪಾಲ್ಗೊಳ್ಳಲು ನಿಮ್ಮ ಒಪ್ಪಿಗೆಯನ್ನು ಪಡೆಯಲು ನಾವು ಬಯಸುತ್ತೇವೆ.

ಯಾವುದೇ ಮಾಹಿತಿಗಾಗಿ ನೀವು ತನಿಖೆದಾರರನ್ನು ಸಂಪರ್ಕಿಸಲು ಮುಕ್ತವಾಗಿರುತ್ತೀರಿ. ಈ ಅಧ್ಯಯನವು ಸಾಂಸ್ಥಿಕ ನೈತಿಕ ಸಮಿತಿಯಿಂದ ಅನುಮೋದಿಸಲ್ಪಟ್ಟಿದೆ ಮತ್ತು ಅವರ ಔಪಚಾರಿಕ ಅನುಮೋದನೆಯ ನಂತರ ಮಾತ್ರ ಪ್ರಾರಂಭಿಸಲ್ಪಟ್ಟಿದೆ. ಸಂಗ್ರಹಿಸಿದ ಮಾದರಿಯನ್ನು ಇನ್ಸ್ಟಿಟ್ಯೂಟ್ನಲ್ಲಿ ಸಂಗ್ರಹಿಸಲಾಗುವುದು ಮತ್ತು ಭವಿಷ್ಯದ ಅಧ್ಯಯನಕ್ಕಾಗಿ ಈ ಮಾದರಿಯನ್ನು ಸಂಗ್ರಹಿಸಲು ಮತ್ತು ಬಳಸಲು ನಮಗೆ ಅನುಮತಿಸಲು ನಾನು ವಿನಂತಿಸುತ್ತೇನೆ.

ಈ ಡಾಕ್ಯುಮೆಂಟನ್ನು ಕೋಶ ಜೀವಶಾಸ್ತ್ರ ಮತ್ತು ಆಣ್ವಿಕ ತಳಿಶಾಸ್ತ್ರ ಇಲಾಖೆಯ ಸುರಕ್ಷಿತ ಲಾಕರ್ನಲ್ಲಿ ಮತ್ತು ನಿಮಗೆ ಮಾಹಿತಿಗಾಗಿ ನೀಡಲಾದ ನಕಲಿನಲ್ಲಿ ಸಂಗ್ರಹಿಸಲಾಗುತ್ತದೆ.ಮತ್ತಷ್ಟು ಸ್ಪಷ್ಟೀಕರಣಕ್ಕಾಗಿ ನೀವು ನನ್ನನ್ನು ಸಂಪರ್ಕಿಸಲು ಮುಕ್ತವಾಗಿರುತ್ತೀರಿ.

ಹೆಚ್ಚಿನ ಸ್ಪಷ್ಟೀಕರಣಕ್ಕಾಗಿ ನೀವು ಈ ಕೆಳಗಿನ ಸಂಶೋಧಕರನ್ನು ಸಂಪರ್ಕಿಸಲು ಮುಕ್ತರಾಗಿದ್ದೀರಿ.

ಡಾ.ಪ್ರಸಾದ್ ಕೆ.ಸಿ
ಪ್ರೊಫೆಸರ್
ಇ. ಏನ್. ಟಿ. ಇಲಾಖೆ
ಟಮಕ, ಕೋಲಾರ. ಮೊಬೈಲ್:.
೮೪೯೨೬೯೦೪೩

ಜಗದೀಶ್ ಟಿ. ವಿ..
ಸಂಶೋಧನಾ ವಿದ್ಯಾರ್ಥಿ
ಕೋಶ ಜೀವಶಾಸ್ತ್ರ ಮತ್ತು ಆಣ್ವಿಕ ತಳಿಶಾಸ್ತ್ರ ಇಲಾಖೆ
ಟಮಕ, ಕೋಲಾರ. ಮೊಬೈಲ್: ೯೪೪೮೫೨೮೭೯

ಭಾಗ 2: ಮಾಹಿತಿಯುಕ್ತ ಸಾಮಾನ್ಯ ಗುಂಪಿನ ಬರಹ ಸಮ್ಮತಿ ಪತ್ರ

ಪಾಲ್ಗೊಳ್ಳುವವರ ಹೆಸರು (ಸಾಮಾನ್ಯ / ಸ್ವಯಂಸೇವಕ): _____

- ಈ ಸಂಶೋಧನಾ ಅಧ್ಯಯನವನ್ನು ನನಗೆ ವಿವರಿಸಲಾಗಿದೆ; ಇದನ್ನು ಚರ್ಚಿಸಲು ಮತ್ತು ಪ್ರಶ್ನೆಗಳನ್ನು ಕೇಳಲು ನನಗೆ ಅವಕಾಶ ನೀಡಲಾಗಿದೆ. ನನ್ನ ಎಲ್ಲಾ ಪ್ರಶ್ನೆಗಳಿಗೆ ನನ್ನ ತೃಪ್ತಿಗೆ ಉತ್ತರಿಸಲಾಗಿದೆ.
- ಈ ಸಂಶೋಧನಾ ಯೋಜನೆಯು ನನ್ನನ್ನು ಒಳಗೊಂಡಿರುತ್ತದೆ ಎಂದು ನಾನು ಅರ್ಥಮಾಡಿಕೊಂಡಿದ್ದೇನೆ.
- ನಾನು ಸಾಮಾನ್ಯ ಮಾಹಿತಿ ಪತ್ರ ಪ್ರತಿ ಪುಟವನ್ನು ಓದಿದ್ದೇನೆ.
- ಮಾಹಿತಿ ಹಾಳೆಯಲ್ಲಿ ವಿವರಿಸಿದಂತೆ ನನ್ನ ಆರೋಗ್ಯ ಮಾಹಿತಿಯನ್ನು ಪ್ರವೇಶಿಸಲು ನಾನು ಒಪ್ಪುತ್ತೇನೆ.
- ನನ್ನ 3 ಮಿಲಿ ರಕ್ತದ ಮಾದರಿ ಮತ್ತು ಆರೋಗ್ಯದ ಮಾಹಿತಿ ಸಂಗ್ರಹಣೆಗಾಗಿ ಸಂಶೋಧನಾ ಉದ್ದೇಶಗಳಿಗಾಗಿ ಗಿಯ ಮಾಹಿತಿ ಪತ್ರದಲ್ಲಿ ವಿವರಿಸಲು ಅನುಮತಿಸಲು ನಾನು ಒಪ್ಪುತ್ತೇನೆ.
- ಸಂಗ್ರಹಿಸಿದ ಎಲ್ಲಾ ಮಾಹಿತಿಯನ್ನು ಗೌಪ್ಯವಾಗಿ ಇರಿಸಲಾಗುವುದು ಎಂದು ನಾನು ಅರ್ಥಮಾಡಿಕೊಂಡಿದ್ದೇನೆ.
- ನಾನು ಈ ಸಂಶೋಧನಾ ಅಧ್ಯಯನದಲ್ಲಿ ಪಾಲ್ಗೊಳ್ಳಲು ಸ್ವಯಂಪ್ರೇರಣೆಯಿಂದ ಸಮ್ಮತಿಸುತ್ತೇನೆ.

ಪಾಲ್ಗೊಳ್ಳುವವರ ಹೆಸರು ಸಹಿ ಅಥವಾ ಹೆಬ್ಬೆರಳು ಗುರುತು

ದಿನಾಂಕ: _____

ಅನಕ್ಷರಸ್ಥರಿಗೆ :ಸಾಕ್ಷಿದಾರರು ಸಹಿ ಮಾಡಬೇಕು (ಪಾಲ್ಗೊಂಡವರು ಆಯ್ಕೆ ಮಾಡಬೇಕು ಮತ್ತು ಸಂಶೋಧನಾ ತಂಡದ ಯಾವುದೇ ಸಂಪರ್ಕ ಇರಬಾರದು).

ಭಾಗವಹಿಸುವ ಅನಕ್ಷರಸ್ಥರು ತಮ್ಮ ಹೆಬ್ಬೆಟ್ಟಿನ ಗುರುತು ಮಾಡಬೇಕು. ನಾನು ಸಮರ್ಥ ಸ್ಪರ್ಧಿ ಸಮ್ಮತಿ ನಮೂನೆಗಳ ಮಾಹಿತಿಯನ್ನು ನಿಖರವಾದ ತಿಳಿದಿರುತ್ತೇನೆ. ವೈಯಕ್ತಿಕ ಪ್ರಶ್ನೆಗಳನ್ನು ಕೇಳಲು ಅವಕಾಶ ಹೊಂದಿರುತ್ತೇನೆ. ನಾನು ವೈಯಕ್ತಿಕ ಮುಕ್ತವಾಗಿ ಒಪ್ಪಿಗೆನೀಡಿದ್ದಾರೆ ಎಂದು ಖಚಿತ ಪಡಿಸಿರುತ್ತೇನೆ.

2) ಸಾಕ್ಷಿಯ ಹೆಸರು: _____2) ಸಾಕ್ಷಿಯ ಹೆಸರು: _____

ಸಾಕ್ಷಿಯ ಸಹಿ: _____ಸಾಕ್ಷಿಯ ಸಹಿ: _____ ದಿನಾಂಕ: _____ ದಿನಾಂಕ: _____

ಈ ಐಸಿಎಫ್ ಒಂದು ಪ್ರತಿಯನ್ನು ಹೊಂದಿರುತ್ತೇನೆ.ಒಪ್ಪಿಗೆ ತೆಗೆದುಕೊಳ್ಳುವ ವ್ಯಕ್ತಿಯ ಹೆಸರು

ಒಪ್ಪಿಗೆ ತೆಗೆದುಕೊಳ್ಳುವ ವ್ಯಕ್ತಿ ಸಹಿ _____ದಿನಾಂಕ: _____

Appendix III

(1) **Master chart**

Master Chart: CSOM

S. No	Patient ID	Gender	Laterality	Disease duration	TM perforation	*TLR4*	*NFkB*	TNFα	IL-10
						$2^{-(\Delta\Delta Ct)}$		Fold change	
1	OM1	Female	Unilateral	2 years	Anterior	1.69	6.87	1.07	0.90
2	OM2	Female	Bilateral	4 years	Central	7.06	9.65	11.30	0.87
3	OM3	Male	Bilateral	8 years	Central	3.97	3.78	11.54	0.42
4	OM4	Male	Unilateral	1 year	Central	7.10	7.71	3.62	0.42
5	OM5	Male	Bilateral	4 years	Central	0.34	3.41	1.31	0.98
6	OM6	Male	Bilateral	5 years	Central	0.18	1.57	3.19	0.77
7	OM7	Female	Bilateral	2 years	Anterior	1.16	2.89	16.33	0.32
8	OM8	Female	Unilateral	1 year	Central	8.44	11.55	14.48	0.89
9	OM9	Male	Unilateral	2 years	Central	2.71	2.35	0.73	0.34
10	OM10	Female	Bilateral	4.3 years	Anterior	2.45	1.39	0.99	0.84
11	OM11	Female	Bilateral	5 years	Posterior	1.74	1.77	1.83	1.15
12	OM12	Female	Bilateral	10 years	Central	1.45	2.87	0.89	0.74
13	OM13	Female	Unilateral	5 years	Central	4.47	1.34	1.04	0.54
14	OM14	Female	Unilateral	6 years	Central	2.91	2.23	2.41	0.81
15	OM15	Female	Bilateral	2 years	Central	1.92	3.78	1.16	0.81
16	OM16	Female	Bilateral	4 years	Central	3.20	2.48	13.47	0.58
17	OM17	Female	Unilateral	2 years	Central	1.51	0.91	0.84	0.59
18	OM18	Female	Unilateral	6 months	Central	1.52	2.35	0.96	0.74
19	OM19	Female	Unilateral	5 years	Central	1.61	1.32	6.25	0.47
20	OM20	Female	Unilateral	9 months	Central	2.53	2.64	0.22	0.47
21	OM21	Female	Unilateral	1 years	Posterior	4.26	2.89	2.92	0.58
22	OM22	Female	Unilateral	2 years	Central	3.43	9.18	1.33	0.58
23	OM23	Female	Unilateral	10 years	Posterior	7.01	9.52	0.18	0.31
24	OM24	Female	Unilateral	5 years	Central	1.71	3.29	2.51	0.60
25	OM25	Male	Bilateral	3 years	Posterior	2.30	1.17	4.81	0.73
26	OM26	Female	Unilateral	5 years	Posterior	2.10	2.20	3.00	0.86
27	OM27	Female	Bilateral	5 years	Central	2.43	1.47	4.63	0.69
28	OM28	Male	Bilateral	4 years	Central	2.55	17.03	3.84	0.94
29	OM29	Male	Bilateral	2 years	Posterior	2.14	2.16	5.48	0.86
30	OM30	Male	Bilateral	5 years	Central	1.82	2.14	3.93	0.92
31	OM31	Male	Bilateral	6 years	Central	2.20	3.29	5.04	0.87
32	OM32	Male	Bilateral	4 years	Anterior	1.33	1.12	5.43	0.66
33	OM33	Female	unilateral	1 year	Posterior	1.55	1.06	2.12	0.51
34	OM34	Female	Unilateral	2 years	Posterior	1.36	1.25	1.44	0.34
35	OM35	Female	Unilateral	2 years	Central	4.23	1.41	2.25	0.83
36	OM36	Female	Bilateral	6.5 years	Central	1.82	2.50	1.26	0.65
37	OM37	Male	Bilateral	3 years	Central	3.01	6.16	5.13	0.31
38	OM38	Male	Bilateral	3 years	Central	1.26	1.55	2.85	0.19
39	OM39	Female	Bilateral	2 years	Posterior	2.60	1.20	0.45	0.23

40	OM40	Female	Unilateral	6 months	Central	4.11	10.34	1.35	1.07
41	OM41	Male	Unilateral	1 years	Central	3.25	1.51	3.88	0.57
42	OM42	Male	Unilateral	1.5 years	Central	3.36	1.22	0.49	0.34
43	OM43	Male	Unilateral	6 years	Central	2.23	1.83	1.03	0.26
44	OM44	Female	Unilateral	8 months	Central	1.56	2.89	1.19	0.49
45	OM45	Female	Unilateral	2 years	Anterior	1.11	5.86	0.73	0.74
46	OM46	Male	Unilateral	1.5 years	Central	1.18	6.82	1.62	0.85
47	OM47	Male	Unilateral	2 years	Central	1.80	7.16	1.47	0.62
48	OM48	Male	Bilateral	7.5 years	Central	1.73	2.08	3.73	0.37
49	OM49	Male	Bilateral	8 years	Central	6.96	3.70	0.97	0.73
50	OM50	Male	Bilateral	4 years	Anterior	2.23	5.74	0.95	0.94
51	OM51	Female	Bilateral	6 years	Posterior	1.96	8.82	11.45	0.20
52	OM52	Female	Bilateral	2.5 years	Central	5.03	37.27	1.04	0.35
53	OM53	Male	Bilateral	4 years	Posterior	2.57	1.30	1.12	0.54
54	OM54	Female	Bilateral	6 years	Anterior	2.81	2.01	1.02	0.32
55	OM55	Male	Bilateral	6 years	Central	11.24	11.11	0.62	0.33
56	OM56	Male	Bilateral	4 years	Central	2.95	3.12	3.81	0.64
57	OM57	Male	Bilateral	6 years	Central	2.23	1.65	1.82	0.19
58	OM58	Female	Bilateral	7 years	Central	2.38	1.83	0.14	0.53
59	OM59	Male	Bilateral	6.5 years	Central	4.17	7.31	1.14	0.58
60	OM60	Male	Bilateral	4.5 years	Central	4.38	2.66	0.41	0.37
61	OM61	Male	Bilateral	2 years	Central	3.14	2.16	4.77	0.51
62	OM62	Female	Bilateral	7 years	Posterior	1.97	2.79	8.08	0.55
63	OM63	Male	Bilateral	5 years	Central	3.23	2.57	12.29	0.51

Master Chart: Control

S. No	Controls ID	Gender	*TLR4*	*NFkB*	TNFα	IL-10
			$2^{-(\Delta\Delta Ct)}$		Fold change	
1	OC1	Female	1.24	1.64	1.02	0.74
2	OC2	Female	1.04	1.27	0.07	0.90
3	OC3	Male	1.22	1.39	2.33	0.87
4	OC4	Male	1.88	1.29	3.01	0.84
5	OC5	Male	1.55	1.21	7.35	0.76
6	OC6	Male	1.05	2.58	2.17	0.50
7	OC7	Female	1.95	4.47	0.74	0.42
8	OC8	Female	1.62	0.91	4.08	0.68
9	OC9	Male	1.02	1.22	0.15	0.76
10	OC10	Female	1.73	1.46	11.78	0.35
11	OC11	Female	1.48	1.67	2.20	0.72
12	OC12	Female	1.42	1.74	0.35	0.18
13	OC13	Female	3.14	1.08	0.44	0.71
14	OC14	Female	1.92	1.74	3.45	0.81
15	OC15	Female	2.16	1.14	5.05	0.64
16	OC16	Female	2.35	3.12	2.45	0.74
17	OC17	Female	2.17	0.48	0.50	0.86
18	OC18	Female	1.37	0.99	1.19	0.99
19	OC19	Female	1.92	3.16	0.43	0.97
20	OC20	Female	2.01	2.91	4.54	0.92
21	OC21	Female	1.97	1.39	2.88	0.75
22	OC22	Female	3.05	0.86	0.99	0.97
23	OC23	Female	1.84	1.13	1.20	1.00
24	OC24	Female	1.37	0.43	1.11	0.98
25	OC25	Male	1.10	0.68	0.94	0.67
26	OC26	Female	1.61	1.33	10.38	0.87
27	OC27	Female	0.93	0.88	1.48	0.87
28	OC28	Male	1.60	21.11	0.99	0.97
29	OC29	Male	0.83	0.33	2.13	0.86
30	OC30	Male	0.95	2.81	0.82	0.83
31	OC31	Male	0.54	1.39	1.03	0.49
32	OC32	Male	0.86	0.48	0.86	0.98
33	OC33	Female	1.37	0.67	0.40	0.89
34	OC34	Female	4.82	0.89	1.94	0.98
35	OC35	Female	2.55	1.58	0.62	0.95
36	OC36	Female	0.89	0.67	0.87	0.52
37	OC37	Male	1.51	5.17	3.96	0.26
38	OC38	Male	2.06	4.00	1.68	0.28

39	OC39	Female	2.03	4.44	1.94	0.45
40	OC40	Female	2.28	1.80	2.00	0.88
41	OC41	Male	1.41	1.03	1.11	0.62
42	OC42	Male	1.32	1.06	1.03	1.08
43	OC43	Male	1.36	3.36	13.93	0.77
44	OC44	Female	0.99	1.38	4.31	0.62
45	OC45	Female	1.13	1.41	0.60	2.05
46	OC46	Male	0.93	1.38	2.11	0.26
47	OC47	Male	2.03	3.12	1.15	0.54
48	OC48	Male	1.88	1.09	1.69	0.85
49	OC49	Male	7.06	0.33	1.01	0.78
50	OC50	Male	2.25	2.17	2.17	0.95
51	OC51	Female	2.87	1.37	1.13	0.78
52	OC52	Female	1.30	1.35	1.41	0.90
53	OC53	Male	1.36	0.47	2.00	1.88
54	OC54	Female	1.18	1.91	4.71	0.34
55	OC55	Male	8.00	3.05	0.50	0.90
56	OC56	Male	1.89	2.16	1.71	0.62
57	OC57	Male	1.52	1.87	4.81	0.60
58	OC58	Female	2.16	2.27	2.02	0.84
59	OC59	Male	1.55	0.24	4.10	0.71
60	OC60	Male	1.43	0.21	2.96	0.90
61	OC61	Male	1.22	1.19	1.29	0.69
62	OC62	Female	0.88	3.16	1.59	0.63
63	OC63	Male	1.39	1.36	2.66	0.59

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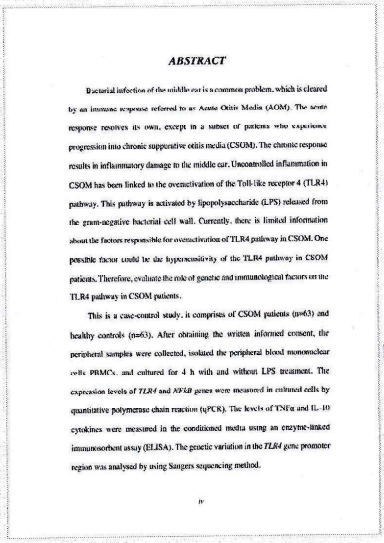

ABSTRACT

Bacterial infection of the middle ear is a common problem, which is cleared by an immune response referred to as Acute Otitis Media (AOM). The acute response resolves its own, except in a subset of patients who experience progression into chronic suppurative otitis media (CSOM). The chronic response results in inflammatory damage to the middle ear. Uncontrolled inflammation in CSOM has been linked to the overactivation of the Toll-like receptor 4 (TLR4) pathway. This pathway is activated by lipopolysaccharide (LPS) released from the gram-negative bacterial cell wall. Currently, there is limited information about the factors responsible for overactivation of TLR4 pathway in CSOM. One possible factor could be the hypersensitivity of the TLR4 pathway in CSOM patients. Therefore, evaluate the role of genetic and immunological factors on the TLR4 pathway in CSOM patients.

This is a case-control study, it comprises of CSOM patients (n=63) and healthy controls (n=63). After obtaining the written informed consent, the peripheral samples were collected, isolated the peripheral blood mononuclear cells PBMCs, and cultured for 4 h with and without LPS treatment. The expression levels of *TLR4* and *NFkB* genes were measured in cultured cells by quantitative polymerase chain reaction (qPCR). The levels of TNFα and IL-10 cytokines were measured in the conditioned media using an enzyme-linked immunosorbent assay (ELISA). The genetic variation in the *TLR4* gene promoter region was analysed by using Sangers sequencing method.

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Recommendations

10. Recommendations

- This study highlights the importance of the TLR4 pathway in the pathogenesis of CSOM.
- Future studies may need to explore the TLR4 pathway for developing drugs that can mitigate the inflammatory damage in CSOM.