Role of host factors in the pathogenesis of Guillain-Barré syndrome

THESIS

SUBMITTED TO

BABASAHEB BHIMRAO AMBEDKAR UNIVERSITY

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DECLARATION

I hereby declare that thesis entitled *"**Role of Host factors in the pathogenesis of Guillain-Barré Syndrome"*** is my own research work carried out under the supervision of Dr. D. R. Modi, Professor, Department of Biotechnology, Babasaheb Bhimrao Ambedkar University, Lucknow (India). I have submitted this thesis to Babasaheb Bhimrao Ambedkar University, Lucknow (India), for the award of the degree of Doctor of Philosophy (Ph.D.) in Biotechnology and all the resources quoted in the thesis have been indicated and acknowledged by complete references.

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CERTIFICATE

This is certify that the thesis "***Role of Host factors in the pathogenesis of Guillain-Barré Syndrome*"** submitted by **Nagendra Kumar Kharwar** is an original research work and has not been previously submitted in part or full for the award of any other degree or diploma to this or other university.

The thesis is submitted to Babasaheb Bhimrao Ambedkar University, Lucknow satisfies all the requirements as stipulated in the Doctor of Philosophy (Ph.D.) regulation – 1999 as amended in 2010 and it is fit for submission and evaluation for the award of the degree of Doctor of Philosophy of the University.

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(Nagendra Kumar Kharwar)

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ABBREVIATIONS

α	Alpha
AIDP	Acute inflammatory demyelinating polyneuropathy
AMAN	Acute motor axonal neuropathy
AMSAN	Acute motor sensory axonal neuropathy
β	Beta
bp	Base pair
BSA	Bovine serum albumin
BNB	Blood-nerve barrier
CCDA	Charcoal cefaperazone deoxycholate agar
cDNA	Complementary deoxyribonucleic acid
CFU	Colony forming unit
CMV	Cytomeaglovirus
CNS	Central nervous system
CO_2	Carbon dioxide
dATP	Adenosine triphosphate
DC	Disease control
dCMAP	Compound muscle action potential amplitude after distal stimulation
dCTP	Cytosine triphosphate
DEPC	Di ethyl pyrocarbonate
dGTP	Guanosine triphosphate
DML	Distal motor latency
DNA	Deoxyribonucleic acid
PID	Post inoculation day
DTT	Dithiothreitol
dTTP	Thymosine triphosphate
EAN	Experimental autoimmune neuritis

EBV	Epstein-Barr virus
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme linked immunosorbent assay
FcγR	Fc gamma receptor
x g	Gravity
GalC	Galactocerebroside
GalNAc	N-Acetyl galactosamine
GBS	Guillain-Barré syndrome
gm	Gram
GM1	Monoganglioside
hrs	Hours
HC	Healthy control
HH	Hippurate hydrolysis
hip	Hippurate gene
H_2O_2	Hydrogen peroxide
HLA	Human leukocyte antigens
HRP	Horse radish peroxidase
IFN-γ	Interferon- gamma
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
IHC	Immunohistochemistry
kDa	Kilodalton
L	Liter
LLN	Lower limit of normal
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
M	Molar

MAG	Myelin associated glycoprotein
MCV	Motor conduction velocity
MF syndrome	Miller Fisher syndrome
MHC	Major histocompatibility complex
MRC	Medical Research Council
$MgCl_2$	Magnesium chloride
mg	Milligram
mg /ml	Milligram per milliliter
min	Minutes
mRNA	Messenger ribonucleic acid
MuMLV	Murine Maloney leukemia virus
µl	Microliter
µg	Microgram
µm	Micrometer
µM	Micromole
Na	Sodium
NaCl	Sodium chloride
NaOH	Sodium hydroxide
ng/ ml	nanogram per mililiter
nm	Nanometer
O.D.	Optical density
OMPs	Outer membrane proteins
OND	Other neurological diseases
OS	Oligosaccharide
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
pCMAP	Compound muscle action potential amplitude after proximal stimulation
PCR	Polymerase chain reaction

pg/ ml	picogram per mililiter
PMSF	Phenyl methyl sulphonyl fluoride
PNS	Peripheral nervous system
RNA	Ribonucleic acid
RNase	Ribonuclease
RPM	Rotations per minute
RT	Room temperature
RT-PCR	Reverse transcriptase- polymerase chain reaction
SDS	Sodium dodecyl sulphate
Sec	Seconds
SGPG	Sulfated glucuronyl paragloboside
TBE	Tris- borate –EDTA
TE	Tris-EDTA
TGF	Tumor growth factor
Th	T helper
TNF-α	Tumor necrosis factor- alpha
ULN	Upper limit of normal
UV	Ultra-violet light
WBCs	White blood cells
w/v	Weight by volume
γ	Gamma

Chapter-I

Introduction

Guillain-Barré syndrome (GBS) is an immune-mediated demyelinating polyneuropathy of peripheral nervous system (PNS); most often triggered by an aberrant immune response to an infectious pathogen (Asbury and Cornblath 1990). It is characterized by acute or subacute symmetrical ascending motor weakness, areflexia and mild to moderate sensory abnormalities (Asbury and Cornblath 1990). Since the number of polio cases decline worldwide, GBS has become the most common cause of acute flaccid paralysis with an annual incidence of 0.6-4 cases per 100,000 populations (Kuwabara 2004; Vucic, Kiernan et al. 2009). GBS remains a serious illness for the affected individuals with considerable morbidity (12% unable to walk after one year) and mortality nearing to 5% (Willison, Soumerai et al. 2000; Winer 2001). Clinically and electrophysiologically, it is classified into three major subtypes: acute inflammatory demyelinating polyneuropathy (AIDP) mostly found in Europe and North America, which is associated with prominent lymphocytic infiltration of peripheral nerves and invasion of myelin sheath and Schwann cells by macrophages (Kuwabara 2004). In East Asia (Kuwabara 2004), acute motor axonal neuropathy (AMAN) cases are prominently found with pure motor involvement associated with serum antibodies against gangliosides GM1, GM1b, GD1a, or GalNAc-GD1a and antecedent *Campylobacter jejuni* enteritis. AMAN can proceed to acute motor sensory axonal neuropathy (AMSAN) which involves severe damage of sensory and motor neurons of PNS.

The general concept of GBS pathogenesis is autoimmunity possibly induced by an infectious event, either respiratory or gastrointestinal, 1-3 weeks preceding GBS(Hahn 1998; Tsang 2002; Solomon and Willison 2003).Although, several microbial infections such as Epstein-Barr virus (EBV), *C. jejuni*, cytomegalovirus (CMV), *Mycoplasma pneumoniae*, *Haemophilus influenzae*, and Variacella-zoster virus etc have been

implicated in the development of GBS (Winer 2001). However, not all such infected individuals develop GBS. These observations emphasize to investigate the role of host factors in the development of GBS. Limited studies have been conducted for identifying potential host factors that may impart susceptibility to GBS.

Besides microbial factors, host susceptibility also plays an important role in the development of a disease among exposed individuals. The same may be true for GBS since only 1 in 1000 patients who are exposed to *C. jejuni* infection develops GBS (Magira, Papaioakim et al. 2003). Several studies have suggested that host factors may play an important role in the development and pathogenesis of GBS. Peeping into the role of host factors revealed some interesting facts e.g., (i) the *C. jejuni* strains are found to have GM1 ganglioside like epitopes in diarrhoeal patients but do not develop anti-ganglioside antibodies; (ii) GBS is rarely found among two individuals from the same family or locality; and (iii) it is still of interest to decipher why some people develop specific forms of GBS instead of the fact that *C. jejuni* LPS may mimic ganglioside like epitopes. Noticeably geographic variations and different immunogenetic backgrounds may account for different clinical and serologic manifestations of GBS in different parts of the world.

Immunological studies in GBS patients indicate that cell-mediated immune response to peripheral nerve also plays an important role in the pathogenesis of GBS. The inflammatory infiltrates in GBS are composed of lymphocytes and macrophages contributing to the nerve damage (Sivieri, Ferrarini et al. 1997) by targeting Schwann cells, the principal myelinating glial cells of peripheral nerves (Koski 1997). Activated macrophages and lymphocytes may produce cytokines, chemokines, Toll-like receptors (TLRs), nucleotide oligomerization domain (NOD) like receptors (NLRs) and various adhesion molecules, all of which cause and perpetuate tissue damage. The

inflammation due to cytokines causes recruitment of leukocytes that result in damage of nerve tissues (demyelination). Since the inflammatory cell infiltrates exert most of their effects through immunoregulatory cytokines, it is of central importance to explore cytokine orchestration in the development of GBS. Cytokines are group of soluble factors which could be involved in early breakdown of the blood nerve barrier, up-regulation of endothelial adhesion molecules, leukocyte attraction to nerve tissue, macrophage activation, and myelin damage (Redford, Hall et al. 1995). It isalso a group of polypeptides involved in host defence and repair. The balance between the pro-inflammatory (Th1) and anti-inflammatory (Th2) cytokines is necessary for a successful immune response. It is possible that systemically and locally released cytokines are important in the pathogenesis of GBS. Various evidences suggest that cytokines like TNF-α is critically involved in immune-mediated demyelination and axonal damage of peripheral nerves (Putzu, Figarella-Branger et al. 2000) by affecting myelin protein and glycolipid synthesis whereas TGF-β can down modulate activated glial cells, inflammatory cells and endothelial cells (Lisak, Skundric et al. 1997).

Among host factors, TLR family plays a central role in the initiation of cellular innate immune responses. Activation of TLRs also enhances the transcription of several pro-inflammatory cytokines. Single nucleotide polymorphisms (SNPs) in TLR genes may increase susceptibility to microbial infections, an attenuated immune response towards antigen and down-regulation of cytokine genes occurs due to mutation in the SNP gene. Ina preliminary study, an association of TLR4 gene polymorphism was observed in axonal subtypes of GBS (Nyati, Prasad et al. 2010). In addition to TLRs, NLRs also comprise a large family of pathogen recognition molecules (PRMs) that are characterized by the presence of a conserved NOD (Inohara, Chamaillard et al. 2005) and thought to function in innate and adaptive immunity that determines the balance

between health and disease (Fritz, Le Bourhis et al. 2007). Activation of NLRs by ligand binding initiates a variety of cellular responses for cytokine production and apoptosis (Ogura, Inohara et al. 2001). The mammalian NOD genes encode proteins that have been implicated in the pathogenesis of immune-mediated diseases, including inflammatory bowel disease (IBD), graft-versus-host disease and Crohn's disease(Hugot, Chamaillard et al. 2001; Ogura, Inohara et al. 2001; Brenmoehl, Lang et al. 2007).

The potential involvement of a family of intracellular and cytoplasmic pathogen recognition receptors (PRRs) along with NOD proteins in C. jejuni recognition are reported (Zilbauer, Dorrell et al. 2007).It was reported earlier that a changed NOD-2 (R702W) protein in the nucleotide binding domain alters an arginine residue, suggesting a potential functional effect of the mutation (Molnar, Hofner et al. 2007). SNPs in NLRs genes may increase susceptibility to the microbial infection. Further a frame-shift mutation in NOD2 is reportedly associated with increased riskfor Crohn's disease (Ogura, Inohara et al. 2001) while genetic variation in the NOD1 gene is associated with susceptibility withIBD (Hysiet al, 2005).

Polymorphism of TNF-α with Increased level is reported in case of GBS patients(Prasad, Nyati et al. 2010), while the IFN-γ level is observed to be elevated in early phase of GBS in animal model (Nyati, Prasad et al. 2012). Recently, in context to GBS, we have reported a role of Th1 cytokines with progressive phase and Th2 response with recovery phase (Nyati, Prasad et al. 2011). Polymorphism in cytokines may be directly or indirectly involved in gene expression and might have some possible role in GBS development.

Studies have also demonstrated secretion of the potent neutrophils chemo- attractant interleukin-8 (IL-8) by IECs in response to wild-type (WT) *C. jejuni* (Hickey, Baqar et

al. 1999; Watson and Galan 2005). Carriers of IL-8-251 T allele may have increased susceptibility to multiple sclerosis because of their differences in neuron survival or increased chances of viral persistence compared to carriers of IL-8-251A allele (Kamali-Sarvestani, Nikseresht et al. 2006).

IL-17 (also known as IL-17A) is another cytokine belonging to the "Th17" T helper cell population and is produced by several cell types including activated T cell subsets (CD4+ and CD8+), natural killer cells, macrophage and neutrophils (Korn, Bettelli et al. 2009).IL-17 induces cytokines and chemokines expression and plays an essential role in immune host defences. It is also implicated in osteoarthritis like autoimmune and inflammatory disease(Southam, Heath et al. 2006).

Apart from cytokines, level of ICAM-1 like adhesion molecule is also found to be attenuated as a consequence of endothelial activation among patients with infection, cancer, inflammatory and autoimmune diseases(Meager, Bird et al. 1996).In a vitro-assay, addition of IFN-γ or TNF-α to IL-17 demonstrated a synergistic increase in inflammatory mediator release and a marked increase in ICAM-1 expression (Gabr, Jing et al. 2011).

The host factors have a leading role in immune mediated disease. However, looking at the significance of GBS from Indian perspective, systematic case-control studies bridging the gap between GBS and host factors involvement are lacking. Limited studies are reported where role of host factors (gene expression and their polymorphisms) in GBS are observed.In view of the above studied observations, we designed the present study to evaluate the association of different gene polymorphisms of NLRs (NOD1 & NOD2), TLR-2, cytokine (IL-17), chemokine (IL-8) and adhesion molecule (ICAM-1) with susceptibility to GBS, and the relation of these polymorphisms with their levels.

Chapter-II

Aim and Objectives

2. Aim

Role of host factors in the pathogenesis of Guillain-Barré syndrome

Objectives

1. Detection of antecedent infectious agents (e.g. *Campylobacter jejuni*, Epstein-Barr virus and Cytomegalovirus) in patients with Guillain-Barré syndrome (GBS).

2. To study the gene polymorphisms of TLRs, NLRs, cytokines and adhesion molecules and find out the risk for the development of GBS.

3. To determine the expression of TLR-2, IL-8, IL-17 and ICAM-1 in blood from patients with GBS.

4. To examine the correlation between the levels of TLR2, cytokine (IL-17), adhesion molecule (ICAM-1), IL-8 and their gene polymorphisms.

Chapter-III

Review of Literature

3.1. Background

The first and specific incident of Guillain Barre Syndromé (GBS) was reported by Jean Baptiste Octave Landry de Thezillatin 1859 (Landry 1859). Landry de Thezillat published a report of 10 patients had ascending paralysis. Until 1876 "Landry's ascending paralysis" was the only term used for this illness. During the World War I, Jean-Alexander Barré, Georges Charles Guillain with Andre Strohl researched on this life threatening disease. They identified the illness to be associated with the peripheral nerves and described motor weakness, areflexia and "albuminocytological dissociation" in the cerebrospinal fluid (CSF) of two French soldiers. In1916, they published the very first comprehensive detailed classic paper on this syndrome. In 1927 two doctors worked on this classic paper, the recognized disease was later named Guillain-Barré syndrome.

3.2. Epidemiology

The incidence of GBS ranges from 0.6 to 4 cases per 100,000 populations each year (Kuwabara 2004; Vucic, Kiernan et al. 2009). However in Asia, the incidence is 1.2 to 1.9 cases per 100,000. Males are more frequently affected than females (1.25:1) (Kuwabara 2004; Vucic, Kiernan et al. 2009). It occurs in all age groups, though it is rare in infancy but the incidence appears to increase with age. Some studies have suggested a possible bimodal distribution of cases with peaks in young adults and elderly (Hughes and Rees 1997). In developed countries, AIDP appears to affect an older population. Meanwhile in northern China, AMAN has been found predominantly among children and young adults (McKhann, Cornblath et al. 1993). There is no consistent geographical variations reported and most studies have failed to identify seasonal variation in GBS. However, summer time peaks do occur in China and

perhaps in Spain, Mexico and Korea (Ramos-Alvarez, Bessudo et al. 1969; Valenciano, Najera et al. 1971; McKhann, Cornblath et al. 1993).

3.3. Antecedent events

3.3.1. Infections

GBS is the prototype of a post-infectious illness; occurrence of viral respiratory disease preceding GBS has been known for a century and diarrheal illness preceding GBS was recognized several decades ago. About two-thirds of the patients report an antecedent acute infectious illness, most commonly a respiratory tract infection or gastroenteritis, one to three weeks before the onset of GBS (Vucic, Kiernan et al. 2009). Since the symptoms of the infection have resolved by the time neuropathic symptoms begin, the pathogen that caused the illness remains unidentified. *Campylobacter jejuni*, has become recognised as the most frequent antecedent pathogen for GBS.Those pathogens for which there is convincing and statistically valid evidence of an association with GBS are mentioned below:

3.3.1.1. *Campylobacter jejuni*

C. jejuni is the leading causative agent of gastroenteritis worldwide (Young, Davis et al. 2007; Miljkovic-Selimovic, Ng et al. 2010). It is a Gram-negative spiral bacterium with tapering ends, belonging to the genus *Campylobacter*; 1.5-6.0 µm long and 0.2-0.5µm wide. *Campylobacter* enteritis is considered to be a food borne disease with infections being derived from a range of food and water based environmental sources. *C. jejuni* is considered to be a commensal organism of chickens (Young, Davis et al. 2007). Although the experimental infection of chickens with *C. jejuni* can lead to diarrhea (Young, Davis et al. 2007), sometimes develops severe paralysis resembling neuropathy(Li, Xue et al. 1996). The link between *C. jejuni* infection and development

of GBS was first reported in 1982 in a 45 year old man who developed GBS with irreversible neurological damage two weeks after *C. jejuni* associated gastroenteritis (Rhodes and Tattersfield 1982). Shortly thereafter numerous reports described patients who developed GBS following *C. jejuni* infection (Molnar, Mertsola et al. 1982; Constant, Bentley et al. 1983; Pryor, Freiman et al. 1984).

3.3.1.1.1. Biological evidence of *Campylobacter* in the pathogenesis of GBS

Several epidemiological studies have firmly established *C. jejuni* as a triggering agent of GBS. Kuroki, et. al. and isolated *C. jejuni* from 30% of GBS patients(Kuroki, Saida et al. 1993), whereas Rees, et. al.had isolation rate of 8%(Rees, Soudain et al. 1995). In a similar study *Campylobacter* was recovered from 4 (44.9%) of 9 GBS patients with diarrhea(Ropper 1988). In a prospective study carried out at SGPGIMS, Lucknow, *C. jejuni* and *C. upsaliensis* was detected in one patient each of AIDP and AMAN type respectively(Prasad, Pradhan et al. 2001). *C. upsaliensis* was also recovered from a US patient with AMAN (Ho, Hsieh et al. 1997). Overall, the isolation rate of *Campylobacter* from the stool of GBS patients ranges from 8-50% (Nachamkin, Allos et al. 1998).

Our centre had shown that *C. jejuni* (26.0%) was the most common preceding infection among GBS patients (Sinha, Prasad et al. 2007). Different antigens with different end points for positivity are used and the seropositivity of *C. jejuni* infection in GBS patients ranges from 24-76%, the highest being reported from China for AMAN patients (Kaldor and Speed 1984; Kuroki, Saida et al. 1993; Ho, Mishu et al. 1995).

3.3.1.1.2. *C. jejuni* infection and GBS subtypes

Many electrodiagnostic and pathological studies have revealed that *C. jejuni* infection is significantly associated with primary axonal dysfunction, but its relationship between

C. jejuni infection and development of a particular GBS subtype is still a matter of debate.In China and Japan, studies have demonstrated an association of *C. jejuni* infection primarily with AMAN type; the distributions of axonal vs. AIDP are as follows: 76% vs. 42% from China (Ho, Mishu et al. 1995); 70 % vs. 15% from Japan (Ogawara, Kuwabara et al. 2000). On the contrary, a large study conducted in America and Europe on 229 GBS patients demonstrated that 56% of AIDP patients with predominantly demyelinating neurophysiology had positive serology for *C. jejuni* infection (Hadden, Gregson et al. 2001). However, more studies are needed to resolve this issue and development of an animal model for *C. jejuni* associated GBS will greatly help in demonstrating that whether, it is linked to only axonal subtype or *C. jejuni* infection also elicits AIDP.

3.3.1.1.3. Risk of GBS after *Campylobacter* infection

C. jejuni infections are quite common in general population, the risk of developing GBS is quite low: only 1 in 1000 patients who are exposed to *Campylobacter* infection develops GBS (Magira, Papaioakim et al. 2003; van Doorn, Ruts et al. 2008) usually associated with O:19 serotype of *C. jejuni* (Nachamkin, Allos et al. 1998).

3.3.1.2. Cytomeglovirus (CMV)

CMV infection is experienced clinically as upper-respiratory-tract infection, pneumonia, or nonspecific flu-like illness. It is also the most common cause of congenital and perinatal infections throughout the world. In general population, 40-100% individuals are seropositive for CMV (Griffiths 2004). Infection with CMV is the most common antecedent viral infection and is identified in 10-15% of GBS patients by the presence of CMV specific IgG antibodies (Dowling and Cook 1981; Visser, van der Meche et al. 1996; Jacobs, Rothbarth et al. 1998). CMV is particularly common in young female GBS patients, and the clinical picture is notable for prominent

involvement of the sensory and cranial nerves(Visser, van der Meche et al. 1996).In a study from Belgium this association was as high as 22% (Boucquey, Sindic et al. 1991). In a recent study CMV DNA was detected in the cerebrospinal fluid of GBS patients, thus providing a close association between CMV infection and GBS (Steininger, Popow-Kraupp et al. 2004). Visser, et. al. (1996) found that patients with demyelinating GBS who had recent CMV infection showed severe sensory deficits(Visser, van der Meche et al. 1996). The patients with CMV related GBS are found to be significantly younger, have a more severe initial course of disease, and often develop cranial nerve involvement and severe sensory loss.The exact pathogenic mechanism operating behind this group is not clear, however, it is believed that cross-reacting antibodies to GM2 ganglioside are involved in the pathogenesis but specificity of such antibodies and their significance for the pathogenesis of GBS remains unknown.

3.3.1.3. *Mycoplasma pneumoniae*

In recently, the evidence of *M. pneumoniae* infection in GBS patients is reported only 5%-6% (Jacobs, Rothbarth et al. 1998; Ogawara, Kuwabara et al. 2000). While the association is based on discrete case reports or only few case control studies, the features of GBS after *M. pneumoniae* infection are still undefined. Anti-GalC antibodies have been demonstrated in the sera of such GBS patients but their pathophysiologic role has yet to be determined.

3.3.1.4. Epstein-Barr virus

Only limited number of cases control studies have provided evidence of a recent EBV infection in 8-10% GBS patients (Jacobs, Rothbarth et al. 1998; Hadden, Gregson et al. 2001).

3.3.1.5. Other infections

There are some other anecdotal antecedent infections reported in GBS patients are as follows: *H. influenzae*(Mori, Kuwabara et al. 2000) and HIV-1 (Berger, Difini et al. 1987).

3.3.2. Anecdotal associations

Individual case reports including surgery, epidural anaesthesia, renal transplantation, bone marrow transplantation, systemic lupus erythematosus, sarcoidosis, lymphoma and snakebite are available. Antimotility drugs, penicillins and oral contraceptives have been shown to trigger GBS (Seneviratne 2000). However, no cause effect relationship between the drugs and GBS could be established (Awong, et. al. 1996).

3.3.3. GBS and Vaccine

There are numerous anecdotal case reports or small case series have linked GBS to vaccinations on the grounds of a mere temporal association, but no causal relation has been established and potentially confounding coincidental infections were not ruled out.

3.4. Clinical spectrum

Until very recently, the eponym GBS was defined as a single clinical entity; acute inflammatory demyelinating polyneuropathy (AIDP), identified by the early lymphocytic infiltrates in spinal roots and peripheral nerves, and the subsequent macrophage-mediated segmental stripping of myelin may lead to intense inflammation and demyelination.However, recent studies have shown that GBS is a diverse disorder and can be divided into several electrophysiological and pathologic types. The two patterns of the predominantly motor involvement can be distinguished: acute motor sensory axonal neuropathy (AMSAN), initially described by Feasby et al., is

characterized by Wallerian like degeneration of sensory and motor fibres with little demyelination or lymphocytic infiltration (Feasby, Gilbert et al. 1986); the second pattern, acute motor axonal neuropathy (AMAN), has a nearly pure motor axonal involvement with rapid onset of muscle weakess and absent reflexes (Vucic, Kiernan et al. 2009). A related disorder Fishers' syndrome is characterized by acute onset of ataxia, areflexia and opthalmoplegia(Vucic, Kiernan et al. 2009). AIDP is more frequent pattern in North America and Europe (Nachamkin, Allos et al. 1998) while the axonal patterns, in which axons are the primary target of the immune system attack, are common in China (Wu, Liu et al. 1997), Japan (Sobue, Li et al. 1997), Mexico (Hafer-Macko, Hsieh et al. 1996) and other regions of the world. Griffin, et. al.proposed a physiological and pathological classification of GBS which is as follows(Griffin, Li et al. 1995).

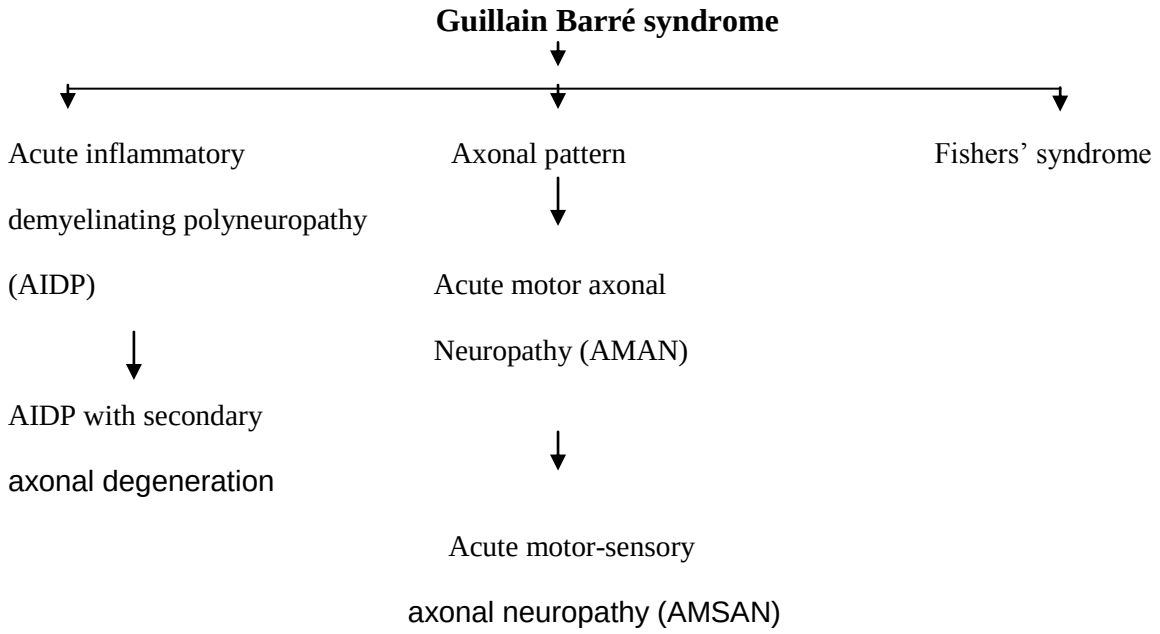


Figure1. GBS and its subtypes

3.5. Detection methods of *C. jejuni,* EBV, CMV *and M. Pneumoniae* infection in GBS

5.5.1 Detection methods of *C. jejuni* in GBS

3.5.1.1. Culture

Culture is still being used to detect *C. jejuni* infection in GBS patients. Sensitivity of culture has been found to be very low in various studies (Sinha, Prasad et al. 2004; Nyati, Prasad et al. 2010). Culture provides a definitive evidence of *C. jejuni* infection in GBS patients, however, due to short median excretion period (16 days) of *C. jejuni* in stool and 1-3 weeks lag time between episode of diarrhea and development of GBS, culture underestimates the infection in these patients. However, culture is insensitive for the detection of bacteria in patients treated with antibiotics, or in patients having mild/subclinical infection or in patients with late reactive complications such as arthritis and GBS or long-lasting intestinal distress (Linton, Lawson et al. 1997). Delayed hospital admission and intake of antibiotics by the patient may also account for low culture positivity.Further, culture of stool samples for *Campylobacter* has been done only in GBS cases with severe diarrhea, thereby missing cases in which the infection may be mild or sub-clinical(Tsang 2002).

3.5.1.2. Enzyme-linked immunosorbent assay (ELISA)

Serology is mainly used to detect the presence of antibodies against *C. jejuni* infection in patient's serum.Serum IgG and IgM levels rise in response to infection and remain elevated for 3-4 weeks before declining to baseline levels (Blaser and Duncan, 1984), but serum IgG levels rise during the first few weeks of infection and then fall rapidly(Kaldor and Speed 1984). Serology for *C. jejuni* uses ELISA with a crude antigenic extract prepared from geographically-prevalent *C. jejuni* strains. Several drawbacks are associated with serology also: there is no consensus on the choice of

antigens; most often a crude antigenic extract and single serum sample is used yielding low specificity, especially in endemic and hyperendemic countries due to high titers of antibodies in the resident population (Tsang 2002; Nyati, Prasad et al. 2012). Furthermore, testing of paired sera and demonstration of significant increase and decrease in antibody titre may be required which is difficult and depends upon the time of sample collection.Moreover, the antibody detection assays can vary considerably between different laboratories in terms of their performance (Koga, Yuki et al. 2001).

3.5.1.3. Polymerase chain reaction (PCR)

When infection has been treated with antibiotics, *Campylobacter* may not be detected by culture, but sufficient bacterial DNA may remain in stool for PCR detection to be successful. PCR has earlier been used in a few studies to detect *Campylobacter* species in stool from patients with gastroenteritis (Linton, Lawson et al. 1997; Yamazaki-Matsune, Taguchi et al. 2007)and from chicken feces (Keramas, Bang et al. 2004)but very few studies are available where this method has been applied in patients with GBS(Sinha, Prasad et al. 2004; Nyati, Prasad et al. 2012). In a recent study real-time polymerase chain reaction (RT-PCR) was used to detect *C. jejuni* in fecal samples from a French cohort of patients with GBS (Sivadon-Tardy, Orlikowski et al. 2010). A multiplex PCR assay suitable for mass screening to detect *Campylobacter* directly from chicken feces has been developed (Bang, Scheutz et al. 2001). Although PCR is a highly specific and sensitive method, its sensitivity varies among the laboratories and PCR cannot exclude the diagnosis of infection (Honavar, Tharakan et al. 1991). Recently, we have tried to detect the association of *C. jejuni* in GBS patients by PCR (19.0% - 22.5%), but its sensitivity was found to be low (Sinha, Prasad et al. 2004; Nyati, Prasad et al. 2012).

3.5.2. Detection method of CMV, EBV and *M. Pneumoniae* in GBS

3.5.2.1. IgG avidity Elisa test

The IgG avidity test was developed to help discriminate between past and recently acquired infection. Following antigenic challenge the IgG antibodies produced initially bind weakly to the antigen (low avidity). As the immune response develops there is maturation of IgG antibody response and the avidity increases progressively over weeks or months (high avidity).The test based on the measurement of the avidity of toxoplasma specific IgG antibodies, was developed (Hedman, Lappalainen et al. 1989). The CMV-specific IgG signal (optical density) of the set washedwith urea buffer is then divided by the CMV-specific IgG signal ofthe set washed with non-urea buffer, thus providing the avidityindex (AI). AI values ≤ 0.50 indicate low avidity, values of 0.51-0.59 indicate intermediate avidity, and values ≥ 0.60 indicate highavidity. The sensitivity of IgG avidity test was found to be low (Levett, Sonnenberg et al. 2005)but a good method to detect these infections in GBS sample.

3.6. Host Factors

It is now clear that development of GBS often preceded by an infectious illness; however, not every individual infected by the above mentioned agents develop GBS. For example only 1 in 1000 patients exposed to *C. jejuni* infection develops GBS (van Doorn, Ruts et al. 2008). This strongly suggests that host susceptibility plays an important role in the development of GBS after infection. It has been hypothesized that there may be disease susceptibility genes that are responsible for predisposing certain individuals who develop GBS after being infected by different microbial agents.Recently, it has been reported that the host factors like high affinity IgG Fc receptors (FcγR), certainhuman leukocyte antigens (HLA) class II molecules, matrix

metalloproteinase (MMP)-2 and MMP-9 play important role in the development of GBS which are the candidate genes that intimately involved in the immune response during the disease (Nyati, Prasad et al. 2010). It now appears likely that soluble substances other than antibodies may result in nerve damage. Of particular interest are cytokines, molecules with signaling function that coordinate the interplay of immunocompetent cells during an immunoinflammatory response (Hartung, Jung et al. 1992; Hartung 1993). Cytokines are particularly attractive candidates in terms of rapid and evanescent influences on nerve function independent of their immuno-regulatory role (Brinkmeier, Wollinsky et al. 1992).

Cytokines are a group of polypeptides involved in host defense and repair. They function by regulating cellular replication, differentiation or activation. Some cytokines are mainly pro-inflammatory (IFN-γ, TNF-α, IL-1β, IL-2) or are anti-inflammatory (IL-4) and immunoregulatory (TGF-β1, IL-10). The IFN-γ is known as signature cytokine of Th1 immune response while IL-4 is recognized as signature cytokine of Th2 lineage. Pro-inflammatory cytokines are believed to play an important role in the induction of cell mediated autoimmune disease whereas anti-inflammatory cytokines promote primarily antibody mediated autoimmune disease (Zhu, Mix et al. 1998). A growing body of evidence suggests that cytokines are critically involved in immune-mediated demyelination of peripheral nerves (Kieseier, Krivacic et al. 2000). Studies on experimental autoimmune encephalomyelitis (EAE), an animal model for immune mediated inflammation of central nervous system, revealed a differential up-regulation and expression of various cytokines by infiltrating lymphocytes and residential cells. EAN is the only animal model of GBS in which differential up-regulation of cytokines during various phases of EAN have been documented (Zhu, Mix et al. 1998). Recent reports are available on the role of cytokines in EAN also. In *C. jejuni* induced GBS,

cytokines may be produced in the peripheral nerves by infiltrating mononuclear cells and Schwann cells in AIDP and by macrophages in the axonal form of GBS. The further role of cytokines in GBS is still under study. However, they are implicated in the cascade of events leading to demyelination and axonal damage, particularly TNF-α; they may also affect myelin protein and glycolipid synthesis (Lisak, Skundric et al. 1997). Cytokines can be protective also: particularly TGF-β since it can down regulate activated glial cells, inflammatory cells and endothelial cells (Lisak, Skundric et al. 1997). Amongs cytokines, IL-17 is the signature cytokine of the "Th17" T helper cell population and is produced by several cell types including activated T cell subsets (CD4+ and CD8+), natural killer cells, macrophage and neutrophils (Korn T, 2009). IL-17F was discovered as member of IL-17 family (IL-17A-IL-17F) mapped on the same chromosome position at 6p12 and share highest degree of homology with IL-17 (Paradowska-Gorycka, Wojtecka-Lukasik et al. 2010) and Human Th17 cells could involve in disruption of blood brain barrier, stimulating the inflammation, macrophage activation, chemotaxis of neutrophils and myelin damage (Chi L, 2010). IL-17 induces cytokines and chemokine expression and plays an essential role in immune host defences and also implicated in various autoimmune and inflammatory diseases (Kawaguchi M, 2006; Arisawa T, 2008; Jang W, 2008 and Seiderer J, 2008). IL-17 induces T cell activation and infiltration into tissues by upregulating the expression of ICAM-1 and amplifies the immune response by inducing the production of IL-6 and prostaglandin E2 (Albanesi C, 1999).ICAM-1 is a surface glycoprotein, expressed on vascular endothelium, macrophages, and human neuronal cells, activated lymphocytes. It mediates leukocyte circulation and extravasations from the blood into the areas of inflammation and macrophage differentiation (Diamond MS, 1991; Salmaso C, 2002).Moreover, ICAM-1 has long been implicated in the pathogenesis of multiple

sclerosis (MS) and experimental autoimmune encephalomyelitis (EAE). For example, increased expression of ICAM-1 has been shown on endothelial cells, microglia, and astrocytes in active MS or EAE lesions (McMurray et al. 1996 and Sipkins et al. 2000). Increased sCAM levels are found in patients with infection, cancer, inflammatory and autoimmune diseases, as a consequence of endothelial activation. Thus, sCAM concentration reflects the endothelial expression (Sfikakis et al. 1997).

IL-17F (Glu126Gly) polymorphism was significantly associated with an increased disease activity in Korean patients with Behcet's disease (BD) (Jang WC, 2008). In another study, polymorphism in IL-17 (His161Arg) was not associated with Crohn's disease (CD). In addition to this, Serum and synovial fluid levels of IL- 17 were in correlation with disease activity in patients with rheumatoid arthritis (RA) (Fujino S, 2003; Brand S, 2006 and Metawi S, 2011). However, another study, reported augmented mRNA expression for interleukin-17 in blood and CSF mononuclear cells among multiple sclerosis cases (Matusevicius D, 1999). Several studies also revealed predisposition for autoimmune diseases due to IL-17 polymorphisms (Matusevicius D, 1999; Fujino S, 2003; Jang W, 2008 and Seiderer J, 2008). So far, only one study had shown elevated expression of IL-17 in GBS (Li S, 2012). Association of ICAM-1 polymorphism (Gly241Arg) was reported in RA of Mexico population (Hernandez, 2009). However, many studies revealed the enhanced expression of ICAM-1 in autoimmune diseases like MS and EAE etc (McMurray, 1996 Duran E, 1999; Sipkins R, 2000).

Therefore studies are being conducted to look for cytokines IL-17 and adhesion molecule (ICAM-1) in the blood of GBS patients. Available studies also demonstrate a strong association between the cytokine levels and the prognosis in GBS: TNF-α and IL-17are elevated in the sera of GBS patients. Creange et al (2001) have also found

increased TNF-α in the CSF and sera of 10 GBS patients in the acute phase of the illness followed by a decline in the recovery phase.

Apart from cytoines, different classes of pattern recognition receptors (PRRs) have now been identified; with most often in parallel their ligands and the adaptors and proteins involved in subsequent signal transduction. The best known PRRs are the TLRs, which are transmembrane receptors characterized by an extracellular leucine rich repeat domain (LRR), involved in the recognition of microbial PAMPs, and an intracellular Toll and Interleukin-1 receptor (TIR) domain. One of the human Toll homologues, TLR2, has been shown to be involved in LPS signaling (Kirschning, Wesche et al. 1998; Yang, Mark et al. 1998; Wright 1999). In addition to this, TLR2 is activated primarily by peptidoglycan, spirochetal glycolipids, lipoproteins and lipoarabinomannan (Schwandner, Dziarski et al. 1999; Takeuchi, Hoshino et al. 1999; Schroder, Opitz et al. 2000). Activation of TLRs enhances the transcription of several pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α via NF κB (Chow, Young et al. 1999; Kopp and Medzhitov 1999; Akira, Takeda et al. 2001). IL-1β in its turn stimulates expression of IL-8 (Fossiez, Djossou et al. 1996; Zwerina, Redlich et al. 2005). IL-8 chemokine was characterized for its ability in recruitment and activation of neutrophils at inflammatory sites (Baggiolini, Dewald et al. 1994; Sampson 2000). IL-8 also promotes inflammatory processes by attracting some subsets of T lymphocytes to the site of inflammation, inducing cytokine production as well as releasing tissue damaging mediators by neutrophils (Taub, Anver et al. 1996; Baggiolini and Loetscher 2000; Martinez, Sironi et al. 2004).

TLR2 polymorphism at position 753 (Arg753Gln), an exchange of arginine by glutamine was correlated with the incidence of sepsis caused by gram-positive bacteria in human (Lorenz, Mira et al. 2000). Another polymorphism in TLR2 at position 677

(Arg677Trp) was associated with susceptibility to lepromatous leprosy (Kang and Chae 2001). Several studies reported that TLR2 polymorphisms predisposed to autoimmune diseases (Emonts, Hazes et al. 2011; Lee, Lee et al. 2012; Kaiser, Tang et al. 2014). So far, only one study had shown association of TLR4 polymorphism with GBS (Nyati, Prasad et al. 2010). Association of IL-8-251A/T polymorphism was studied in autoimmune inflammatory diseases like MS,systemic lupus erythromatosus (SLE) and rheumatoid arthritis (RA) (Kamali-Sarvestani, Nikseresht et al. 2006; Sanchez, Sabio et al. 2006; Lo, Huang et al. 2008). Taking the importance of TLR2 and IL-8 in genetic susceptibility of various diseases, we hypothesized that host factors might determine the intensity of immune response towards microbial ligands, which might play a pivotal role in GBS development.

Other PRRs are also involved in cytosolic detection of microbes have been morerecently identified. NOD1, NOD2 are such receptors involved in intracellular bacterial detection. They are characterized by a tripartite structure, with a C-terminal LRR domain, a central nucleotide binding and oligomerization domain (NOD or NACHT) domain, and an N-terminal caspase and recruitment domain (CARD).Both NOD1 and NOD2 can be auto-activated upon overexpression (Bertin et al., 1999; Inohara et al., 1999; Ogura et al., 2001). Over-expression leads to auto-oligomerization of these molecules through the NBS region. It is believed that this process potentially exposes the CARD domain that can then interact with the CARD domain of a serine/threonine kinase called RIP2 (also known and RICK or CARDIAK) (Inohara et al., 1998; McCarthy et al., 1998; Thome et al., 1998). This recruitment process leads to the activation of RIP2 by a mechanism referred to as “induced proximity”(Inohara et al., 2000) and functions mainly as a NF-kB pathway activating sensor for bacterial muramyl dipeptide (MDP) found in the cell wall of Gram-positive and Gram-negative

bacteria. The role of NOD has been shown in many autoimmune diaseases like Crohn's disease, IBD pathogenesis etc.Therefore studies are being conducted to look for role of NOD in the blood of GBS patients.

3.7. Immunopathogenesis of GBS

3.7.1. Humoral immune response

Oligoclonal plasma cell responses occur in GBS. Complement-fixing antimyelin antibodies are responsible for injury to the myelin sheath, possible damage to the oligodendrocyte and Schwann cell, Fc-receptor stimulation, chemotaxis and myelin opsonization for phagocytosis. Antibodies to specific peripheral myelin antigens are believed to be central in pathogenesis of the disease, and GBS responds to plasmapheresis. Antibodies to specific gangliosides are responsible for functional effects. Anti-GM1 antibodies, found in approximately 25% of GBS patients have been shown to cause conduction block in some studies (Rees, Soudain et al. 1995), and to affect voltage gated Na^+ channel function *in vitro* (Kaida and Kusunoki, 2010;Takigawa et al., 1995; Wirguin et al., 1995). Anti-GQ1b immunglobulins in the Fisher's syndrome variant of GBS interfere with neuromuscular transmission by blocking acetycholine release (Roberts, Willison et al. 1994) (Buchwald et al., 1995). It is quite possible in GBS that T cells cooperate by opening the blood-nerve barrier to allow circulating auto-antibodies access to myelin antigens which are then responsible for the nerve damage (Spies et al., 1995) along with nonspecific demyelination by cytokines, activated complement, and other inflammatory mediators generated by a type of acute phase response to an infectous agent that further generated cellular immune response against the disease.

3.7.1.1. Mechanisms of antibody-mediated dysfunction and tissue damage

3.7.1.1.1. The role of the blood-nerve-barrier

In GBS, the beginning of the disease process is characterized by destruction or malfunction of blood-nerve barrier (BNB). Pathogenic antibodies need to pass through the perineural and endoneural diffusion barrier. The perineuial barrier is relatively deficient in the region of the nerve roots and possibly absent at distal nerve terminals and nerve roots, and these regions are preferentially affected by an immune attack. (Nyati, Prasad et al. 2011). It has long been recognized that the nerve roots are major sites of pathology in inflammatory demyelinating neuropathies (Hahn et al., 1988; Harvey and Pollard, 1992). The resolution of physiological nerve conduction failure at the nodes of Ranvier leads to rapid recovery in some patients; however axonal degeneration is associated with slow and incomplete recovery in other patients(Nyati, Prasad et al. 2011). There is now direct experimental evidence that changes in BNB permeability play an important role in EAN (Hahn et al., 1985; Powell et al., 1983).

3.7.1.1.2. Activation of complement

The binding of antibodies to specific neural epitopes exposed on the outer Schwann-cell surface membrane could produce nerve damage through complement-mediated meachnism. Antibodies can activate complement through classical pathway, but it has been reported that P0 myelin antigen itself can initiate the complement cascade in the absence of antibodies (Kaida and Kusunoki, 2010; Hartung et al., 1992; Koski, 1990). This would result in the formation of low-molecular weight inflammatory mediators such as C3a, C5a, which can open BNB and are chemotactic, the opsonin C3b, and the terminal complement complex (TCC). Activation products of complements (C3a, C5a, C3c, C5b-9) have been detected in serum or plasma and CSF of GBS patients. C3d was

localized to myelin sheaths in sural nerve biopsies from GBS patients, and C5b-9 deposits were observed in one case on myelin sheaths (Hartung et al., 1987; Hays et al., 1988; Koski et al., 1987; Sanders et al., 1986). In EAN, TCC was localized by immunocytochemistry on Schwann cells and along myelin sheaths but was visualized only transiently prior to demyelination (Stoll et al., Ann Neurol 1991).

3.7.1.1.3. Role of complement inhibitors in GBS

Clinical data indicate that complement activation followed by membrane attack complex (MAC) formation is an important mechanism for neuronal and glial injury in GBS. In GBS patients, deposits of complement components along myelinated fibers, C9neo antigen at sites of active myelin breakdown, and MAC on Schwann cell membranes have been reported.Several serine proteases activate classical and alternative pathways of the complement system and a synthetic serine protease inhibitor, nafamostat mesilate (NM: 6-amidino-2-naphthyl-*p*-guanidino-benzoate dimethanesulfonate) which has been used clinicallyin Japan for more than 20 years with no serious adverse effects, has anti-complement activity. Because NM efficiently inhibits the early classical pathway components C1r and C1s, it was shown to inhibit C3 fragment deposition at the higher rate of 0.8 mg/kg/h for 7 days in an animal experiment. NM inhibits C3/C5 convertase in the classical and alternative pathways of the complement system. Preserved Na+ channel clusters associated with restricted deposits of activated C3 fragments were present, suggesting that NM inhibits complement activation steps beyond the C3 convertase step leading to MAC formation. In other words, NM inhibited C3 fragment deposition followed by MAC formation, thereby preventing axonal injury (Phongsisay, Susuki et al. 2008).

3.7.1.1.4. Antiganglioside antibodies

The identification and characterization of neural antigens in demyelinating diseases has yet not been achieved. One area in which considerable progress has been made is in the relationship between anti-glycolipid antibodies and neuropathy. However, still the evidence is based on clinical-serological observations rather than experimental studies. The first breakthrough in the identification of nerve antigens arose when it was recognized that acquired polyneuropathies occurred in association with predominantly late onset monoclonal gammapathies (Ponsford, et. al. 2000). It was hypothesized that the monoclonal paraprotein may have anti-neural activity, and myelin associated glycoprotein (MAG) was the first such antigen to be identified. It was believed that the antigen specificities of the paraproteins were directed to carbohydrate determinants present on different glycolipid distributed in neural tissue, in addition to glycolipid such as MAG. Immunoglobulin M (IgM) paraproteinaemic neuropathy with reactivity against MAG and the cross-reactive glycolipids sulfated glucuronyl paragloboside (SGPG) and sulfated glucuronyl lactosaminyl paragloboside (SGLPG) was the first clinical-serological association to be studied in detail (Latov, 1994). Later on, chronic motor neuropathies were identified in association with polyclonal or monoclonal IgM antibodies against Gal and GalNAc bearing glycolipids: these are now known to be present in ~50% of cases of multi-focal neuropathy with conduction block (Kornberg and Pestronk, 1995). Recently, the research impetus on the role of antibodies directed at glycolipid epitopes has been focused on acquired inflammatory neuropathy.

Since the first report on anti-ganglioside antibodies in GBS (Illyas, et. al. 1988), they have been identified in large group of patients and their association has been established with different clinical phenotypes of GBS. In about half of patients with GBS, serum antibodies to various gangliosides have been found in human peripheral

nerves, including LM1, GM1, GM1b, GM2, GD1a, GalNAc-GD1a, GD1b, GD2, GD3, GT1a, and GQ1b (van Doorn, Ruts et al. 2008). Antibodies to GM1, GM1b, GD1a, and GalNAc-GD1a areassociated with the pure motor or axonal variants of GBS, whereas antibodies to GD3, GT1a, and GQ1b are related to ophthalmoplegia and MFS (van Doorn, Ruts et al. 2008). Although there is a relation between the presence of these antibodies and the clinical symptoms and severity of GBS, the pathological significance of some of these antibodies has yet to be established.

3.7.2. Cellular immune response

3.7.2.1. Evidence from immunopathology

Histopathological studies on nerve biopsy and autopsy material, studies in the animal model EAN, and more recently, immunological studies in GBS patients indicate that cell-mediated immune responses to peripheral nerve also play an important role in the pathogenesis of GBS. Demyelination and mononuclear cellular infiltration is the pathological hallmark of classical GBS (Arnason ans soliven 1993; Asbury et al., 1969; Brechenmacher et al., 1987). Lymphocytes and macrophages accumulate in a focal and perivenular distribution throughout the peripheral nervous system from root to motor terminals (Hall et al., 1992). Infiltrating lymphocytes exhibit characteristic features of proliferative transformation (Arnason and soliven 1993). One immunocytochemical study revealed a predominance of $CD4^+$ T-helper/inducer cells over $CD8^+$ suppressor/ cytotoxic T cells and the presence of some B cells (Cornblath 1990). Macrophages are the major cell type located in close proximity to myelin. They strip off myelin lamellae and phagocytose myelin debris (Hall et al., 1992; Hughes et al., 1992). Macrophages and activated T cells express major histocompatibility complex (MHC) class II antigens. There is also up-regulation of MHC class I and II on Schwann cells and

endothelial cells in the inflammatory lesion of GBS (Atkinson et al., 1993; Mancardi 1988; Mitchell t al., 1991; Pollard 1987; Scarpini et al., 1990).

3.7.2.2. Role of T-cells and mechanisms of T-cells-mediated nerve damage

Observation in the EAN formed the basis for the implication of T-cell-mediated immune responses in the pathogenesis of GBS (Arnason and Soliven. 1993; Hartung et al., 1988; Hartung et al., 1993). In the rat, the decisive role of T lymphocytes in initiating nerve damage was proven by demonstrating that the transfer of autoreactive P2, P0, or P2/P0 peptide specific T-cell lines can induce the clinical, electrophysiological, and morphological features of classical EAN in naïve recipient rats. Predominantly demyelinative changes occur with low cell doses while the addition of higher cell numbers produce axonal damage and marked endoneurial edema (Hartung and., 1990; Heininger et al., 1986; Linington et al., 1984). Ealrier studies indicated the presence of actively proliferating lymphocytes in the blood based on results of the ^{3}H-thymidine incorporation assay (Arnason and Soliven. 1993; Hartung and Toyka, 1990; Iqbal et al., 1981; Korn-Lubetzki and Abramsky 1986).

T-cell activation has been implicated to the pathogenesis of GBS. $CD4^{+}$ helper/inducer cells of the Th2 arm that synthsize IL-4 and IL-5 may cause B-cell proliferation and transformation into plasma cells that manufacture antibodies against peripheral myelin components. $CD4^{+}$ T-cells of the Th1 phenotype could damage myelin by secreting proinflammatory and myelinotoxic cytokines such as IFN-γ, IL-2, TNF-α and TNF-β, and operate by recruiting macrophages to exert nerve damage. Activated $CD8^{+}$ cells may be directly cytotoxic to Schwann cells. The pathogenic sequence of T-cell-mediated responses to nerve antigens comprises a number of distinct steps: homing to and crossing of the BNB, endoneurial activation, clonal expansion, release of injurious molecules and recruitment of other inflammatory effector cells. Breakdown of the BNB

is one of the earliest morphologically events in lesion development (Hahn et al., 1985; Hartung et al., 1988; Olsson 1990; Powell et al., 1983). The intracellular adhesion molecule ICAM-1 is critically involved in T-cell trafficking to peripheral nerve (Hartung Ann Neurol 1993). Histology showed a marked reduction of inflammatory infiltrates and perivascular demyelination in rats so treated (Archelos et al., 1993; Stoll et al., 1993). Another adhesion molecule, E-selectin, which is expressed exclusively on activated endothelial cells, circulates in increased amounts in the blood of acute-phase GBS patients (Hartung et al., 1994; Oka et al., 1994). Activated T cells first breaching the BNB pave the way for antibodies with specificity for nerve antigens which is supported by experimental evidence from animal model EAN (Hahn et al., 1993).

3.7.2.3. The role of macrophages

Studies in EAN also established the decesive role of macrophages in immune-mediated nerve damage, which are essential in the effector phase of the disease (Hartung et al., 1988; Hartung and Toyka 1990; Heininger et al., 1988). Macrophages feature prominently in the nerve lesion of GBS. Mechanisms that are operative include phagocytosis and the release of pro-inflammatory cytokines such as IL-6, TNF-α and IL-1 and other highly active mediators (Baron et al., 1993; Cammer et al., 1986). The presence of IFN-γ-positive cells in nerve roots correlates with the number of macrophages during the course of EAN. The functional role of IFN-γ which markedly augmented disease severity in actively induced EAN while application of anti-IFN-γ antibodies neutralizes the endogenously produced cytokine suppressed the disease (Strigard et al., 1989; Hartung et al., 1990). In EAN, TNF-α appears in nerve lesions around the time of first clinical symptoms. As animal recovered, TNF-α immunoreactivity was no longer detected. Neutralization of endogenously generated TNF-α ameliorates the experimental disease (Stoll et al., 1993). Furthermore, direct

injection of TNF-α into sciatic nerve of mice produced predominantly axonal damage (Said and Hotebeyrie-Joskowicz 1992). On the other hand, macrophages are pivotal in initiating the repair phase once the acute inflammatory response has subsided since they clear myelin debris of the nerves and release of mitogenic stimuli causing Schwann cell proliferation (Baichwal et al., 1988; Baichwal and DeVries 1989; Stoll and Hartung 1992).

3.7.3. Acute inflammatory demyelinating polyradiculoneuropathy (AIDP)

AIDP is viewed as a reactive, self-limited, autoimmune disease and patients with AIDP present with flaccid paralysis, areflexia and usually some sensory loss. Electrophysiological features reflect demyelination in both motor and sensory nerves (Nachamkin, Allos et al. 1998). It is the most prevalent form of sporadic GBS in Europe and North America (Nachamkin, Allos et al. 1998) accounting for 85-90% of GBS cases. It is viewed as an autoimmune disorder, triggered in most cases by an antecedent viral or bacterial infection. AIDP refers to salient pathological findings: the early lymphocytic infiltration in the spinal roots and peripheral nerves followed by macrophage mediated segmental stripping of the myelin (Hahn 1998). AIDP is presumed to be a T-cell mediated disorder due to presence of lymphocytic inflammation and many markers of T-cell activation like soluble IL-2 receptor and gamma interferon have been detected in such patients (Bansil, Mithen et al. 1991). However, many studies have established the importance of antibody mediated nerve fiber damage in AIDP: 1) demonstration of demyelinating antibodies in sera (Sumner, Said et al. 1982; Sawant-Mane, Estep et al. 1994), 2)beneficial effects of plasmapheresis (1997), 3) the presence of anti-myelin (Koski 1990) and anti-glycoconjugate antibodies. In conjunction with lymphocytic infiltration, complement

activation products (C3d and C5-9) have also been demonstrated on the outermost surface of the Schwann cell (abaxonal Schwann cell plasmalemma) suggesting that Schwann cell surface as the target of the immune attack (Hafer-Macko, Hsieh et al. 1996). The nature of the antigen on the abaxonal Schwann cell plasmalemma is still not known but it is likely to be a glycolipid (Ilyas, Willison et al. 1988).

In severe cases of AIDP, inflammatory demyelination is accompanied by axonal loss caused possibly by intense inflammation, oedema and swelling of nerves (Feasby, Hahn et al. 1993; Berciano, Figols et al. 1997). Immune mechanisms involved in AIDP have shown in the Figure2A.

3.7.4. Acute motor axonal neuropathy (AMAN)

AMAN is thought to be triggered in several cases by an enteric *C. jejuni* infection.The concept of axonal variant forms of GBS was further supported by case reports of sporadic acute, purely motoraxonal neuropathies, now termed AMAN.GBS was used synonymously with AIDP clinically until Feasby and colleagues presented electrophysiological picture consistent with early axonal degeneration of motor and sensory fibers in seven GBS patients: rapid fall in compound muscle action potential (CMAP) without the evidence of demyelination (Feasby, Gilbert et al. 1986). Further studies in northern China on the GBS cases occurring as summer epidemics during 1991 & 1992 largely helped in establishing the clinical features of AMAN characterized by severe pure motor involvement and good recovery. However, the Chinese cases should be distinguished from the cases described by Feasby, et. al. which had also sensory involvement. Axonal forms of GBS are reported predominantly from China and Japan (Feasby, Gilbert et al. 1986).

The earliest changes consist of lengthening of nodes of Ranvier and distortion of paranodal myelin pathologically, (Chowdhury and Arora 2001)but immunopathologically, these changes correlate with the binding of IgG and activated complement components on nodes of Ranvier (Hafer-Macko, Hsieh et al. 1996). Subsequently after this, perhaps due to C5a and other chemo-attractants, macrophages are recruited to the nodes of Ranvier from where they penetrate the overlying basal lamina of Schwann cell and enter the periaxonal space of the internode (Chowdhury and Arora 2001). The intrusion of the macrophages opens the periaxonal space thus allowing the antibody and complement to enter. Subsequently macrophages dissect the axon from adaxonal Schwann cell plasmalemma, which eventually causes degeneration of Schwann cell cytoplasm. The whole process is depicted in the Figure 2B. Finally many fibres undergo Wallerian like degeneration (Chowdhury and Arora 2001). The presence of macrophages in the periaxonal space indicates the presence of an important epitope in the axolemma or periaxonal space (Griffin, Li et al. 1995).

3.7.5. Acute motor sensory axonal neuropathy

As mentioned before, in 1986, Feasby, et al. published observations on seven patients who had a very acute and severe illness with motor as well as sensory dysfunction(Feasby, Gilbert et al. 1986).The Wallerian like degeneration of sensory and motor fibres, with little demyelination or lymphocytic infiltrationwas observed in necropsy studies. Macrophages were also shown to be present in periaxonal and intra-axonal spaces (Griffin, Li et al. 1995). Thus, it appears that AMAN and AMSAN have similar pathology and both syndromes can follow *C. jejuni* infection. Therefore, it is assumed that AMAN and AMSAN are part of the spectrum of a single type of immune attack on axon and AMSAN could be regarded as a severe manifestation of the same immunopathological process (Griffin, Li et al. 1995).

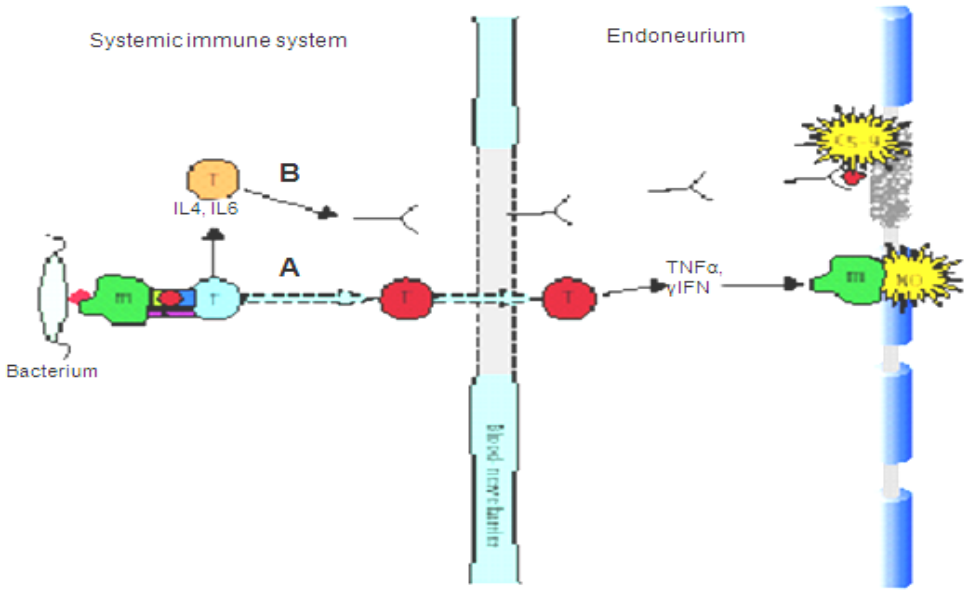


Figure 2A. Immunopathogenesis of AIDP: A bacterial protein epitope is presented by a macrophage to T cell, which penetrates the endothelium, recognizes a cross reactive antigen, **A**- relase cytokines that activate endoneurial macrophages. These release enzymes and nitric oxide (NO) radical and so ultimately invade compact myelin. **B**- activated T cell releases cytokines, help B cells to produce antibodies that cross damaged blood nerve barrier, engage unidentified epitopes on abaxonal Schwann cell surface, fix complement, damage Schawnn cell and produce vesicular dissolution of myelin

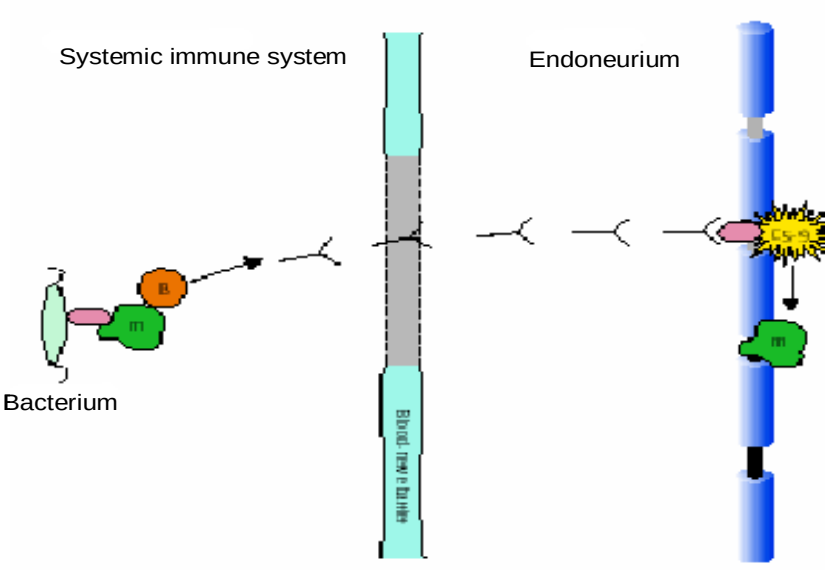


Figure 2B. Immunopathogenesis of AMAN and AMSAN: A bacterial ganglioside-like epitope stimulates B cells to induce antibodies that opsonize cross reactive axolemmal antigens, fix complement and target macrophages to invade the periaxonal space, block conduction or cause axonal degeneration.

3.7.6 Miller Fisher syndrome

Another common variant form of GBS-the Miller Fisher syndrome (MFS) has distinct pathological and immunological characterstics. The syndrome consists of ataxia, ophthalmoplegia (problems controlling eye movements), and areflexia (loss of neurological reflexes).The MFS pattern is triggered by certain *C jejuni* strains that give rise to a characteristic pattern of antibodies to GQ1b ganglioside (Chiba, Kusunoki et al. 1992; Willison, Veitch et al. 1993). IgG antibodies to GQ1b are seen in 96% of MFS cases and parallel the disease course. The antibodies recognise epitopes that are expressed specificallyin the nodal regions of oculomotor nerves, but also in dorsal-root ganglion cells and cerebellar neurons (Chiba, Kusunoki et al. 1992; Kornberg, Pestronk et al. 1996). This pattern corresponds with the clinical features of ophthalmoplegia, ataxia, and areflexia. Anti-GQ1b-containing serum from MFS patients interfered with neuromuscular transmission in a mouse phrenicnerve/diaphragm preparation, probably by blocking the release of acetylcholine from motor-nerve terminals(Roberts, Gelperin et al. 1994). The effect seemed specific, and may offer an explanation for the motor weakness seen in patients with MFS. Antibodies to GQ1b cross-reacted with epitopes contained in the liposaccharide of MFS-associated *C jejuni* strains, again suggesting the possibility of molecular mimicry(Jacobs, Endtz et al. 1995).

3.8. Structural mimicry of gangliosides by microorganisms

As previously stated, autoimmune factors are strongly favored as mediating GBS; however, the precise mechanisms by which this occurs remains unknown. Microbial infections in a susceptible host resulting in an idiosyncratic immune response which cross-reacts with nerve constituents still remains the most reasonable working hypothesis on which current research is based. Anti-ganglioside antibodies were first found in 5/26 (19%) patients with GBS in a study conducted by(Ilyas, Willison et al.

1988). Since then, the presence of anti-ganglioside antibodies with a variety of specificities has been described in GBS patient's sera. The wide range of gangliosides to which antibodies have been reported in GBS patients include: GM1, AsialoGM1, GM1b, GD1a, GD1b, GD3, GT1a, GT1b, GQ1b, LM1, GalC and sulfated glucuronyl paragloboside (SGPG). Much of the literature on anti-ganglioside antibodies in GBS patients correlates the specificity of anti-ganglioside antibody profile with the clinical pattern of the disease and presence of the preceding infection (van Doorn, Ruts et al. 2008). The type of ganglioside mimicry in *C. jejuni* seems to determine the specificity of the antiganglioside antibodies and the associated variant of GBS. *C. jejuni* isolates from patients with pure motor or axonal GBS frequently express a GM1-like and GD1a-like lipo-oligosaccharides (LOS) (van Doorn, Ruts et al. 2008) that mimic the carbohydrates of gangliosides.Several concepts exist on this issue; firstly, anti-GM1 antibodies are irrelevant to the development of GBS and merely exist in GBS serum as secondary events. They are either linked to the disease through preceding infection or as a result of secondary immune response to nerve injury but are independent of its pathogenesis (Press, Mata et al. 2001). The most common antibodies are GM1 antibodies; secondly, cross-reactivity between anti-ganglioside antibodies may exist that have not been fully elucidated. For example, some anti-GM1 may be monospecific whereas others may cross react with other gangliosides; thirdly, related gangliosides epitopes may exist in both myelin and axolemma membranes in varying concentrations and configurations that can lead to preferential binding of antibody under different circumstances in different individuals. Further, this may change during the disease. For example, at the nodes of Ranvier axolemmal GM1 may be cryptically hidden during the early course of the disease but may become exposed for antibody binding due to paranodal demyelination induced by anti GM1 (or other antibody) binding to GM1 at

the paranodal site. Thus an illness that started as AIDP could then evolve into AMAN or AIDP with secondary axonal damage.

3.9. Animal models of GBS

The basis for the different outcomes of *C. jejuni*infection in humans versus chickens is not well understood.This is partly due to the lack of a good smallanimalmodel that reproduces the human disease. Such amodel would enable detailed investigations to be made ofthe basic mechanisms of *C. jejuni* pathogenesis. Ferretscolonized with pathogenic *C. jejuni* isolates can exhibitsymptoms of disease that are seen in humans, includingdiarrhoea and inflammation(Fox, Ackerman et al. 1987), but the high cost andlack of suitable reagents and knockout technology tostudy the host factors that are involved in disease diminishthe attractiveness of this model. Various murine modelshave been tried, but the results have been inconsistent; most do not replicate human disease by producing clinicalsymptoms, although inflammation and other pathologicalindicators have been observed (Yrios and Balish 1986; Fox, Rogers et al. 2004). Experimental autoimmune neuritis (EAN), a T-cell mediated disease in Lewis rats, is considered to be *in vivo* model of GBS (Vriesendorp, Dmytrenko et al. 1993). Injection of Lewis rats with proteins and peptides derived from myelin of the PNS induces a primarily T-cell mediated disease with pathologic features of GBS i.e. demyelination. However, this model does not mimic the development of GBS following *C. jejuni* infection.

As a natural host and important food source for humans, the chicken is a good model for studying the basic aspects of host immunopathogenesis and may be potentially a good target for anti-*Campylobacter* strategies that could ultimately protect human populations. Based on a case of GBS in a human following exposure to paralyzed chickens with pathology similar to that of human AMAN, Li, et. al.developed an

animal model of AMAN by feeding chickens a *C. jejuni* strain isolated from a patient with AMAN(Li, Xue et al. 1996). Numerous chickens, thus infected, developed paralysis and pathological examination of their nerves showed Wallerian-like degeneration similar to human form of the disease. Chickens can be induced to develop GBS like paralytic neuropathy following experimental inoculation of *C. jejuni* (Li, Xue et al. 1996). Therefore this model can be very useful for studying the immunopathogenic mechanism underlying the development of GBS following *C. jejuni* infection. However, further studies are needed on this probable animal model since despite extensive experiments other groups could not reproduce the findings of Li, et. al.(Li, Xue et al. 1996). In a recent study, it is suggested that natural colonization with a GBS-associated *C. jejuni* strain is able to induce specific crossreactive anti-LOS/ganglioside antibodies in chickens (Ang, Dijkstra et al. 2010). In addition to natural and experimental models of animal infection, human intestinal epithelial cell lines, such as the INT 407 cell line, have also been used in *C. jejuni* studies (Borrmann, Berndt et al. 2007).

3.10 Diagnosis

GBS is known as syndrome rather than a disease because it is not apparent that a specific disease-causing agent is involved. Several disorders have symptoms similar to those found in GBS. Collectively, the signs and symptoms form a certain pattern that helps to differentiate Miller-Fisher syndrome from other disorders (Matthews et al. 1991). Nerve conduction studies (NCS) and CSF analysis are important to confirm the diagnosis of GBS. NCSand electromyography (EMG) are important to establish the diagnosis of GBS, and different neurophysiological diagnostic criteria have been proposed (McKhann, Griffin et al. 1988).

3.10.1 Nerve conduction studies (NCS)

NCS supports a suspected clinical diagnosis of GBS, identify the GBS subtype and help to exclude mimic disorders. NCS rely on abnormalities in motor nerves to identify features of demyelination (Table 1), with sensory nerve conduction studies helping to differentiate different forms of axonal GBS, that is, AMAN from AMSAN. The diagnostic yield of NCS is increased by studying at least three sensory and four motor nerves in addition to F-waves and H-reflexes (Brown, Feasby et al. 1993). The typical findings on NCS include the presence of a partial motor conduction block, abnormal temporal dispersion of motor responses, prolonged distal motor and F-wave latencies and reductions in maximum motor conduction velocity. Although in over85% of patients NCS reveal demyelination consistent with the AIDP form of GBS, in up to 13% of cases the initial NCS are normal; in these cases, retesting in 1 to 2 weeks might be required to confirm the diagnosis (van der Meche and Schmitz 1992; Ho, Mishu et al. 1995).

Table1: Neurophysiological criteria of GBS (van Koningsveld, Rico et al. 2001)

GBS subtype	Distal CMAP amplitude (mV)	Conduction block	Temporal dispersion	Motor conduction velocity (m/s)	Distal motor latency (ms)	F-wave latency (ms)
AIDP	Normal or reduced	* Proximal:distal ratio of CMAP amplitudes	> 30% Increase in proximal negative peak CMAP duration	< 70% Lower limit of normal	> 150% Upper limit of normal	> 120% Upper limit of normal
AMAN	Absent or reduced					
AMSAN	Absent or reduced					

3.10.2 Cerebrospinal fluid examination

Besides to NCS and EMG, CSF analysis may confirm a diagnosis of GBS. In 80% of GBS patients, a raised CSF protein concentration is present; with the mononuclear cell count being either normal (albuminocytologic dissociation) or < 50 cells/mm (Asbury and Cornblath 1990). The CSF is normal in the first week of the illness (Hayes et al. 2002).

3.11. Treatment of GBS

The evolution and severity of the neuropathy is variable; it can happen with alarming speed so that intubation and mechanical ventilation may be necessary 24–48 h from onset of symptoms. Therefore, the treatment of GBS is subdivided into techniques for managing severly paralysed patients requiring intensive care unit and ventilatory support, and specific therapy designed to lessen or reversing the nerve damage. Immunomodulating treatments such as plasma exchange and intravenous immunoglobulin (IVIg) are indicated for patients who are unable to walk independently (Kuwabara 2004). The Hughes functional grading scale (Table 2) is widely used to evaluate clinical disability and the functional endpoint. Despite medicaltreatment, GBS often remains a severe disease; 3-10% of patients die and 20% are still unable to walk after 6 months (van Doorn, Ruts et al. 2008). In addition, many patients have pain and fatigue that can persist for months or years.

Table 2 Hughes functional grading scale for Guillain-Barré syndrome

Score	Description
0	Healthy
1	Minor symptoms or signs, able to run
2	Able to walk 5m independently
3	Able to walk 5m with a walker or support
4	Bed- or chair-bound
5	Requiring assisted ventilation
6	Death

3.11.1. Plasma exchange

Plasma exchange or plasmapheresis has been considered as most effective and a gold standard of treatment for GBS for almost 20 years (Vucic, Kiernan et al. 2009). Plasmapheresis removes potentially pathogenic molecules from the blood such as antibodies, complement, cytokines, inflammatory mediators etc. (Thornton and Ballow 1993). Plasma exchange may also indirectly influence cellualar immune response. Comparison of serum titers of peripheral myelin-directed antibodies before and after plasma exchange clearly demonstrated a decline following this procedure, and relapses following plasma exchange in a few patients were associated with rising titers of antimyelin antibodies (Vriesendorp, Dmytrenko et al. 1993).In a North American and French study, 200-250 ml/kg body weight of plasma was exchanged over 7-14 days. Improvement in the patient's health was observed more rapidly, needed less assisted ventilation, spent less time in intensive care units, and less time in the hospital(Kuwabara 2004). Plasmapheresis was beneficial within 4 weeks of symptom onset and the benefits were greatest when treatment was given early (1997). The usual regime is to exchange 4 to 6 plasma volumes over 2 weeks (1997; Vucic, Kiernan et al. 2009). However, plasma exchange did not affect the percentage of patients with severe motor disability. Around 25% cases have reported some kind of relapse approximately 1-2 week after plasma exchange, which is thought to result from antibody rebound and increased levels of peripheral myelin-directed antibodies and persistant active disease (Vriesendorp, Dmytrenko et al. 1993). Plasmapheresis also appears to be feasible, efficacious, and safe in children with GBS (Epstein and Sladky 1990).

3.11.2. Intravenous immunoglobulin (IVIg)

Intravenous Ig is also suggested as apromising therapy for GBS (Hughes and Rees 1997; Kuwabara 2004). The most frequently used IVIg regime is 0.4 g/ kg/ day for 5

days consecutively (Kuwabara 2004; Vucic, Kiernan et al. 2009). The precise mechanism of action of IVIg is not fully understood, but experimental studies suggest that it exerts multiple effects on the induction, proliferation, and effector phases of the immune responses (Stangel, Hartung et al. 1998). IVIg suppresses antibody-dependent cellular toxicity (Pashov, Bellon et al. 1997), decreases natural killer cell function (Tenser, Hay et al. 1993), inhibits autoantibody production (Kondo, Ozawa et al. 1991), neutralizes circulating pathogenic antibodies (Berchtold, McMillan et al. 1989), and interferes with complement activation (Basta and Dalakas 1994). Such mechanisms are dependent on the presence of intact Ig molecules, which has a half-life of approximately 3 weeks. Thus, the observed long-term immunomodulatory effect of IVIg may be due to interference with the amplification phase of the immune response, which involves the proliferation of T-lymphocytes. Our recent study in accordance with earlier published data suggested that IVIgs used for the treatment of GBS suppresses the levels of pro-inflammatory cytokines such as TNF-α and IL-1β during recovery, however remained relatively high in untreated patients (Sharief, Ingram et al. 1999; Nyati, Prasad et al. 2011). Several comparative studies suggested superiority of IVIg over plasma exchange (van der Meche and Schmitz 1992). However, back pain meningeal reaction, fever, trachycardia, and headache during or within course of comleting the infusion are known side effects of IVIg (Thornton and Ballow 1993).

3.11.3. Corticosteroids

Unlike plasmapheresis and IVIg, corticosteroids are largely ineffective in GBS (Hughes, Swan et al. 2007). Six trials, studying 587 participants, have failed to demonstrate improvement in disability after 4 weeks of treatment with steroids (Hughes, Newsom-Davis et al. 1978; Singh and Gupta 1996), and have reported less improvement in GBS patients treated with oral steroids compared to placebo (Hughes,

Newsom-Davis et al. 1978; Singh and Gupta 1996). In contrast, GBS patients treated with a combination of intravenous methylpredisone and IVIg tended to improve more rapidly compared to IVIg treatment alone (van Koningsveld, Schmitz et al. 2004), thereby suggesting a possible role of intravenous steroids in GBS. The lack of benefit of oral corticosteroids in GBS is surprising, but may be explained partly by inhibition of macrophages responsible for the clearance of myelin debris (Hughes and Cornblath 2005; Vucic, Kiernan et al. 2009).

3.11.4. Other treatments

Cerebrospinal fluid filtration is a new, potentially effective treatment for patients with GBS (Kuwabara 2004). In a recent study conducted in 37 patients with GBS who were unable to walk unassisted, functional improvement was assessed at 28 days after randomization to CSF infiltration or plasma exchange are equally efficacious (Wollinsky et al., 2002). However this treatment needs further confirmation.

A therapeutic benefit from interferon-β (IFN-β) has been suggested because IFN- β inhibits in vitro lymphocyte adhesion to recombinant vascular adhesion molecule-1(Schaller, Radziwill et al. 2001). In another study on EAN, two new cyclo-oxygenase-2 inhibitors were observed to inhibit clinical and histopathological features of the disease, suggesting that these are useful as additional therapeutic agents in GBS (Miyamoto, Oka et al. 2002).

3.12. Vaccination

Several anectodal case reports and epidemiological studies have reported GBS following vaccinations against several pathogens. Such vaccines include rabies, oral polio, influenza, measles, measles/mumps/rubella, tetanus toxoid and Hepatitis B (Hahn 1998). Concerns about vaccine-induced GBS were first raised following the

1976-77 influenza vaccination season, when a significant increased risk of GBS was reported within 6-8 weeks of receiving the "swine flu vaccine (Schonberger, Bregman et al. 1979; Langmuir, Bregman et al. 1984). Further, GBS was reported after immunization with the hepatitis vaccine and the meningococcal conjugate vaccine (Souayah, Nasar et al. 2007; Vucic, Kiernan et al. 2009). Rabies vaccine prepared from the infected brain tissues of adult animals had an increased risk of inducing GBS due to the contamination with myelin antigens (Hemachudha, Griffin et al. 1988).Two case-control surveys of approximately 200 GBS patients from southeast England, which included individuals immunised with influenza, typhoid, cholera, and diphtheria-tetanus-pertussis vaccines, did not show any significant association between occurrence of GBS and a previous immunisation(Hughes and Rees 1997). However, the causal association between all the other mentionedvaccinations with GBS has not been established and potentially confounding coincident infections cannot be ruled out.

Chapter-IV

Materials & Methods

4.1. Study subjects

4.1.1. Patients

A total of 105 consecutive GBS patients admitted between 2011 and 2013 in the Neurology ward of Sanjay Gandhi Postgraduate Institute of Medical Sciences, an 800 bedded tertiary care centre in northern India, were included in our study. All these patients fulfilled the diagnostic criteria of GBS as described by Asbury and Cornblath (1990).Severity of disease was scored at the time of each blood sampling using the following scale: (A) able to walk >5 meters without assistance; (B) able to walk >5 meters with assistance; (C) bedridden or chairbound; (D) requiring assisted ventilation for at least part of the day (Hughes et al., 1978).

4.1.2. Controls

Healthy controls included 100 age and sex matched volunteers without any history of apparent infectious illness within the last 4 weeks.

4.2. Samples

The following samples were collected from patients and controls: Blood (8 ml)

i) 2.0 ml in EDTA vial for DNA analysis and stored at -20ºC

ii) 2.0 ml in plain vial to separate sera for ELISA and stored at -80ºC

iii) 1.0 ml in heparin vial for RNA isolation and stored at -80 ºC

iv) 3.0 ml in heparin vial for PBMC isolation and stored at -80 ºC

The first blood sample was collected from patients within 48 hours of admission. None of the study subjects were receiving steroid treatment at least 4 weeks prior to the sample collection.

4.3. Diagnosis of GBS

Patients of GBS were selected on the basis of criteria described by Asbury and Cornblath (1990). The features required for the diagnosis were as under:

A. **Progressive motor weakness of more than one limb**: The degree can range from minimal weakness of the legs, with or without mild ataxia, no total paralysis of the muscles of all four extremities and the trunk, bulbar and facial paralysis, and external ophthalmoplegia.

   Areflexia (loss of tendon jerks): Universal areflexia is the rule, though distal areflexia with definite hyporeflexia of the biceps and knee jerks can suffice if other features are consistent.

B. **Features strongly supportive of diagnosis**

   Cranial features (ranked in order of importance)

   - Progression: Symptoms and signs of motor weakness develop rapidly but cease to progress by 4 weeks into the illness. Approximately 50% will reach the nadir by two weeks, 80% by three weeks and more than 90% by 4 weeks.
   - Relative symmetry: Symmetry is seldom absolute, but uaually, if one limb is affected, the opposite is as well.
   - Mild sensory symptoms or signs.
   - Cranial nerve involvement: Facial weakness occurs in approximately 50% and is frequently bilateral. Other cranial nerves may be involved, particularly those innervating the tounge and muscles of deglutition, and sometimes the extra-ocular motor nerves. On occasion (less than 5%),

the neuroptahy may begin in the nerves to the extraocular muscles or other cranial nerves.

- Recovery: It usually begins 2 to 4 weeks after progression stops. Recovery may be delayed for months. Most patients recover functionally.
- Autonomic dysfunction: Tachycardia and other arrhythmias, postural hypotension, hypertension and vasomotor symptoms, when present support the diagnosis. These findings may fluctuate. Care must be exercised to exclude other basis for these symptoms, such as pulmonary embolism.
- Absence of fever at the onset of neuritic symptoms.

Variants (not ranked)

- Fever at onset of neuritic symptoms
- Severe sensory loss with pain.
- Progression beyond four weeks. Occasionally a patients' disease may continue to progress for many weeks longer than four or the patient will have a minor relapse.
- Cessation of progression without recovery or with major permanent residual deficit remaining.
- Sphincter function: Usually the sphincters are not affected, but transient bladder paralysis may occur during the evolution of symptoms.
- Central nervous system (CNS) involvement: ordinarily, GBS is thought of as a disease of PNS. Evidence of CNS involvement is controversial.

In occasional patients such findings as severe ataxia interpretable as cerebellar in origin, dysarthria, extensor plantor responses, and ill defined sensory leveles are demonstrable and these need not exclude the diagnosis if other features are typical.

C. Cerebrospinal fluid features strongly supportive of diagnosis

- CSF proteins: After the first week of symptoms, CSF protein is elevated or has been shown to rise on serial lumbar punctures.
- CSF cells: Counts of 10 or fewer mononuclear leukocytes/mm^3 in CSF.

Variants

- No rise in CSF protein from 1 to 10 weeks after the onset of symptoms (rare).
- Counts of 11 to 50 mononuclear leukocytes/mm^3 in CSF.

D. **Electrodiagnostic features strongly supportive of the diagnosis**

Slow nerve conduction or block at some point during the illness. Conduction velocity usually less than 60% of normal, but the process is patchy and not all nerves are affected. Distal latencies may be increased to as much as three times normal. F-wave responses were studied as an indication of slowing over proximal portions of nerve trunks and roots.

Determination of muscle power grading

Weakness of muscles of both upper and lower limbs was determined by Medical Research Council (MRC) scale and graded from 0-5. MRC used scale of 0-5 were as follows:

M0 No contraction

M1 Flicker contraction

M2 Muscle contraction with active motion with gravity eliminated

M3 Full range of motion against gravity

M4 Full range of motion against gravity with some resistance

M5 Full range of motion against gravity with maximum resistance for that muscle

4.4 Detection of *Cytomegalovirus,* Epstein Barr Virus and *M. Pneumoniae* Infection in GBS patients and Controls

Detection analysis by IgG avidity ELISAs for CMV, EBV and *M. Pneumoniae*infection in patient serum was performed using commercially available kits as per manufacturer's instructions (Disease Diagnostica Senese, Italy). ELISAs were performed in triplicates independently for each sample. The results (expressed as % avidity) reflect the extent of antigen antibody complex dissociation caused by the denaturing agentbased on the standard provided with the kits.

4.4.1. Molecular detection of C. Jejuni

4.4.1.1. Sero-dignosis of Campylobacter infection

In-house Enzyme-linked immunosorbent assay (ELISA) for Campylobacter antibodies was performed for the detection of anti-IgM and anti-IgG antibodies against *C.Jejuni*. Detergent insoluble outermembrane protein from *C. jejuni* strains isolated from GBS-patients (i.e. geographically prevalent strains) were used as antigen. Cultures were grown in liquid media (Brucella broth). Bacteria were pelleted by centrifuging at 10,000g for 10 minutes followed by three washings of the pellet with PBS. Pellet was then suspended in 10mM Tris HCL (pH 8.0) containing phenyl methyl sulphonyl

fluoride (PMSF) and disintegrated by sonicatin (four time 30 sec). Sonicated cultures were spin at 1,500g for 20 min at 4°C to remove the cell debris. Supernatantat containing cell membrane was subjected to ultracentrifugation at 48,200g for 1 hrs at 4°C. Supernatant was discarded and pellet was dissolved in 150µl distilled water, followed by addition of 1.2ml of 1.67% sodium-lauroyl-sarcosine solution in 11mM Tris-HCL (pH 7.6). The solution was mixed well and extraction was done for 20 min at room temperature. The sample was then ultracentrifuged at 46,000g for 90 min at 20°C. The pellet comprising of outer membrane proteins was finally dissolved in 500µl distilled water containing PMSF.

Protein estimation

Resultant protein was quantified by Lowry's method.

Principle: The alkaine copper sulphate reacts with peptides to give a violet coloured complex (absorption maxima at 500 nm) after reduction of the folin reagent by the alkaline copper protein (Lowry et al., 1951).

Reagent: Solution A: 2% alkaline sodium carbonates solution in 0.1M NaOH.

Solution B: 0.5% copper sulphate in 1% sodium potassium tartrate

Solution C: alkaline copper solution: mixture of solutions A and B (49:1): folin & Ciocalteau's phenol reagent

Procedure: For protein estimation, all standards (BSA) and unknown samples were prepared in water to a final volume of 1.2 ml, then 3 ml solution C was added. The reaction mixture was mixed by vortexing and allowed to stand for 15 min at room temperayure; 300µl of 1X Folin reagent was added and mixed rapidly by vortexing. Tubes were allowed to stand for another 30 min. the OD was taken at 500 nm and a standard curve was drawn.

4.4.1.2. In-house ELISA for campylobacter antibodies

ELISA was carried out in 96 well nunc Immuno Maxisorb polystyrene plate (Nunc, Wiesbaden, Federal Republic of Germany {FEG}). Checker board titration with positive and negative controls was carried was carried out for deciding optimal antigen concentration, sera dilution and conjugate dilution. After standardization, ELISA plates were coated with 10 µg/ml OMP in carbonate- bicarbonate buffer (pH 9.8) for 1 hrs at 37^{o}C followed by overnight incubation at 4^{0}Cin humidified chamber. Next day plates were washed in phosphate buffer saline (PBS, pH7.4) once and unspecific binding sites were blocked with 3% bovine serum albumin (BSA) at 37^{0}C for 1 hour. The plates were then washed with PBST (PBS-0.05% Tween) thrice and 100 microliter of diluted sera (1:500) for IgM and 1: 1000 for IgG was added in duplicates. Plates were incubated for 1 hrs at 37 degree. Plates were then washed thrice with PBST (PBS-0.05% Tween-20). Horse raddish peroxidase (HRP) congjuatged anti-IgM (1:6000 R and anti-IgG 1:1000 antibodies obtained from Sigma (ST Louise USA) was added and the plates were incubated for 1 hrs at 370. Plates were again washed thrice with PBST, finally 100 microlitter tetrmethilline benzidine (TMB) was added to each well for 20 minutes. Reaction was stopped with 1N H_2SO_4 and OD was recorded at 450nm.

4.4.1.2.1 Defining cut off value

More than the mean SI of all controls + 2 standard deviations were taken as a cut off value for the diagnosis of *C. jejuni* infection in GBS.

C. jejuni infection was defined when at least one of the following criteria was fulfilled.

i. Stool culture and/ or PCR positive (Sinha et al. 2004

ii. Presece of IgG and IgM antibodies in ELISA (Rees et al, 1995b)

iii. High titer IgG or IgM antibody with history of diarrhea within last four weeks (Rees et al, 1995b)

4.5. Polymorphisms

4.5.1 Isolation of DNA from blood

The samples collected in EDTA vials were subjected for DNA isolation by salting out method. In brief, 500 µl of EDTA blood was taken in 1.5 ml micro centrifuge tube. To each tube 1 ml lysis buffer was added. The contents were mixed gently by inversion for 1 minute. The tube was spun in a refrigerated centrifuge at 11,000 rpm for 5 minutes, and the supernatant was discarded. The pellet was re-suspended in 200 µl lysis buffer, mixed and then centrifuged. The pellet was dissolved in 200 µl of double distilled water and then mixed thoroughly with the tip. It was then centrifuged at 14,000 rpm for 1 minute. The supernatant was discarded; 80 µl of proteinase K buffer and 10 µl of 10% SDS was added to the pellet. Frothing was done in the solution with the help of micro tip. Then 100 µl of NaCl (5M, cold) was added to the above solution followed by tapping to mix the whole solution properly; 200 µl of water was added to the above solution. Then 400 µl of Phenol: Chloroform (4:1) was added. The tubes were inverted to mix until the contents turned milky and then centrifuged at 12,000 rpm for 10 minutes. The aqueous layer was taken out in a fresh tube. In this 1 ml chilled absolute was added. Mixing was done by gently inverting the tubes. The tubes were centrifuged at 1400 rpm for 4 minutes. The supernatant was discarded and the excess fluid was completely be drained off. The pallet was washed in 70% ethanol and centrifuged. The excess liquid was drained off and the pellet was air dried and then finally dissolved in 50 µl of TE buffer (10mM Tris, 1mM EDTA, ph 8.5). The tubes were kept at 56 ºC overnight to dissolve the DNA.

4.5.1.1. Quantification of nucleic acids

DNA concentration was determined from the optical density (OD) of the samples using the following formula,

DNA concentration (µg/ml) = OD 260×DF×50

where, OD 260 is the absorbance of the diluted samples at 260 nm and DF is the dilution factor. The purity of DNA was determined by calculating the ratio of OD 260 to OD 280.

4.5.2.1. Genotyping

Single nucleotide polymorphisms (SNPs) were detected by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis. DNA samples of 100 ng/µl concentration were used for SNP detection; primersequences for TLR-2 (Arg677Trp & Arg753Gln), IL-17 (Glu126Gly&His161Arg), ICAM-1 (Gly241Arg), IL-8(-251T > A), NOD1 (Glu266Lys) &NOD2 (Arg702Trp&Gly908Arg) amplifications are shown in Table 3. PCR amplifications were performed in a 25 µL volume containing 10 X assay buffer, 200 mM each of dATP, dCTP, dGTP, dTTP, 0.1 mM of each primer, 1.0 U of Taq DNA polymerase (Bangalore Genei, Bengaluru, India). PCR protocols were as follows: initial denaturation for 10 min at 95 ^{0}C followed by 35 PCR cycles of denaturation for 30 s at 94 ^{0}C, annealing for 30 s at different temperature (Table 1), extension for 30 s at 72 ^{0}C with final extension of 5 min at 72 ^{0}C. Amplification products (20 µl) were digested with 1U of respective restriction enzymes, Fermentas, Burlington, Canada (Table 4) at 37 ^{0}C overnight, electrophoresed on 3.5% agarose (Sigma–Aldrich, St Louis, MO, USA) gel, visualized under UV illumination and stained with ethidium bromide.

Table 3: Primer sequences used for PCR-RFLP

Gene	Primer sequences (5'-3')	Product size (bp)	Annealing temperature (°C)	Reference
IL-17 Glu126Gly	F- GTGTAGGAACTTGGGCTGCATCAAT R- AGCTGGGAATGCAAACAAAC	470 bp	64	Gorycka et al., 2010
IL-17 His161Arg	F- GTGTAGGAACTTGGGCTGCATCAAT R- AGCTGGGAATGCAAACAAAC	470 bp	64	Gorycka et al., 2010
ICAM1 Gly241Arg	F- CCGTGGTCTGTTCCCTGTAC R- GAAGGAGTCGTTGCCATAGG	110 bp	58.5	Hernandez et al., 2009
TLR2 (Arg677Trp)	GGGACTTCATTCCTGGCAAGT GGCCACTCCAGGTAGGTCTT	340 bp	58	Lorenz E et al., 2000
TLR2 Arg753Gln	GCCTACTGGGTGGAGAACCT GGCCACTCCAGGTAGGTCTT	340 bp	58	Lorenz E et al., 2000
IL-8(-251T > A)	CATGATAGCATCTGTAATTAACTG CTCATCTTTTCATTATGTCAG AG	349 bp	58.5	Vendramin et al., 2011
NOD1 Glu266Lys	AAGTGACAGGCTGTGCTGC CTTCCCACTGAGCAGGTTG	232 bp	58.5	Kara et al., 2010
NOD2 Arg702Trp	GGCGCCCCTGGAATTC CCTCACCCGGTGCAGC	185 bp	64	Cantó et al., 2007
NOD2 Gly908Arg	CCCAGCTCCTCCCTCTTTC AAGTCTGTAATGTAAACGCCAC	163 bp	64	Cantó et al., 2007

Table 4: Band patterns and restriction enzymes used in PCR-RFLP

Band pattern of digested PCR product			
Genotype	**Wild**	**Mutant**	**Restriction enzymes**
IL-17 Glu126Gly	470	75 and 395	AvaII
IL-17 His161Arg	52, 130 + 288	52 + 418 bp	Nla III
ICAM1 Gly241Arg	110 bp	90+20 bp	BsrGI
TLR2 (Arg677Trp)	227bp	302 + 38 bp	AciI
TLR2 Arg753Gln	227 bp	265 + 75 bp	AciI
IL-8(-251T >A)	349 bp	202 + 147 bp	MunI
NOD1 Glu266Lys	232 bp	170 bp	Eco881
NOD2 Arg702Trp	54 & 76 bp	35 + 130 bp	MspI
NOD2 Gly908Arg	163	27 + 136 bp	HhaI

4.5.2.2. Statistical analysis

The SPSS 16.0 statistical package (Chicago, IL, USA) was used for data management and analysis. Power of the study was calculated using Quanto software version 1.0 (http://hydra.usc.edu/gxe) to achieve 80% of the statistical power for Odds Ratio (OR) $\geq$ 2.0 at significance level (α) <0.05. Logistic regression analysis was applied to estimate association with GBS susceptibility after adjusting for age and gender and considered significant if the *p* values were <0.05. Hardy-Weinberg equilibrium was checked in controls by goodness of fit χ^2 test. For comparisons between the groups of study populations $\chi 2$ test was used.

4.6. Expression of IL-17, IL-8, ICAM-1, and TLR-2 by mRNA analysis

4.6.1. RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR)

Semi quantitative analysis of expression of different cytokines at transcript level was done by RT-PCR densitometry method. Cytokine RT-PCR was carried out on RNA extracted from the blood using Qiagen RNeasy kit (Qiagen Inc., CA, USA) according

to manufacturer's instructions. cDNA was synthesized by RevertAid^{TM} H minus first strand cDNA synthesis kit (Fermantas Life Sciences, USA) taking 100 ng of RNA for each sample. After reverse transcription, primer specific amplification of IL-17, IL-8, ICAM-1, and TLR-2 was performed (Table 5); β-actin was used as housekeeping gene. PCR products were examined by 1.8% agarose gel electrophoresis and photographed under ultra-violet illumination. Band intensities were quantified by densitometry scanning software ImageQuant TL (GE-Pharmacia Biotech, USA). To normalize mRNA levels, density of IL-17, IL-8, ICAM-1, TLR-2 and β-actin bands from the same samples were scanned, and data were calculated as the ratios of band intensity values relative to band intensity value of β-actin.

Table 5: Primer sequences used for cytokine specific RT-PCR analysis

Gene	Primer sequences (5'-3')	Reference
GAPDH*	F-GTGGGCGCCCAGGCACCA R-CTCCTTAATGTCACGCACGATTT	Wei et al. 2004
IL-17	F- ATGACTCCTGGGAAGACCTCATTG R- TTAGGCCACATGGTGGACAATCGG	Wei et al. 2004
IL-8	F- ATGACTTCCAAGCTGGCCGTGGCT R- TCTCAGCCCTCTTCATCAAAAACTTCTC	Wei et al. 2004
ICAM-1	F- AATGTCATCCTGCCCCGGGGG R- AGGGCAGTTTGAATAGCAA	Fan et al., 1998
TLR-2	F- AGGCGGACATCCTGAACCT R- GGCCAGCAAATTACCTGTGTG	Wei et al. 2004

*****House keeping gene used as internal control. F, Forward; R, Reverse; bp, base pair

4.6.1.2. Statistical analysis

Data were analyzed with SPSS statistical software, version 17.0 (SPSS Inc., Chicago, IL, USA) and *p* values ≤0.05 were considered significant. Values from densitometry were expressed as mean ± SD of triplicate experiments performed independently for each sample for semi-quantitative RT-PCR.

4.6.2. Enzyme-linked immunosorbent assay (ELISA)

Quantitative analysis by ELISAs for IL-17, IL-8 and ICAM-1 in cell supernatants was performed using commercially available kits as per manufacturer's instructions (R&D systems, Minneapolis, USA). ELISAs were performed in triplicates independently for each sample. The results were expressed as picograms of cytokines/ ml (pg/ ml), based on the standard provided with the kits.

4.6.2.1. Isolation of peripheral blood mononuclear cells

Fresh blood samples were collected from patients and control subjects in heparin vials. The blood was diluted in 1:1 ratio with 1 M phosphate buffered saline (PBS). Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll paque (Sigma Aldrich, MO, USA) density gradient method. In brief, in a 15 ml centrifuge tube 3 ml of Ficoll paque was taken and 10 ml of diluted blood was loaded without disturbing the interface, centrifuged at 1800 rpm for 30 minutes, PBMCs were isolated, washed thrice with PBS. Cell viability was determined by trypan blue staining (GIBCO BRL, Germany) and cells were counted on haemocytometer as per the standard procedure. Cells were finally suspended in RPMI-1640 supplemented with 10% FCS.

4.6.2.2. Whole cell lysate preparation

The cells were lysed by gently rocking the suspension at 4°C for 15 minutes. The lysate was centrifuged at 14,000 x g at 4°C in a pre-cooled centrifuge for 15 minutes. Supernatant was transferred immediately to a fresh centrifuge tube and the pellet was discarded. Protein concentration of the cell lysate was determined by performing Bradford assay (1976). Briefly, protein samples and varying concentrations of bovine serum albumin (BSA, 1-10 g) were taken in separate tubes and the volume adjusted to 100 l with PBS (pH 7.2, 0.15 M). A tube without any protein was taken as blank. One

ml of Bradford reagent was added to each tube. The solutions were mixed thoroughly by vortexing and allowed to stand for 2 min at 250C. The absorbance protein solution was recorded at 595 nm. A standard curve was prepared using the absorbance values of the BSA protein solutions, and the concentration of the protein in the unknown samples was determined from the standard curve. These protein samples were boiled for 5 min with Laemmli lysis buffer for SDS-PAGE and subsequent western blot analysis.

4.6.2.3. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Polyacrylamide gel was prepared according to the standard protocol. Briefly, the separating gel solution containing APS and TEMED was poured between the two glass plates to three fourths of the total height of the assembly and allowed to polymerize for 30 minutes. On top of the separating gel, stacking gel solution was poured very slowly so that the level of the separating gel was not disturbed. A 0.75 nm thick 10-well Teflon comb was inserted into the stacking gel solution. The gel solutions were allowed to polymerize at the room temperature for 20-30 minutes, after which the comb was gently removed. The wells were washed with electrophoresis buffer (pH -8.3) to remove unpolymerized acrylamide. Electrophoresis buffer was added to the lower and upper chambers of the apparatus. Gel electrophoresis was carried using a mini-vertical gel apparatus (GE Healthcare, Buckinghamshire, UK). 10-15 l of cell extract containing 30 g of protein mixed with Laemmli lysis buffer was heated for 10 min in a hot water bath at 100o C and loaded into individual wells in the stacking gel. Molecular weight standards (14-96 M.wt, Genei, Bengaluru, India) was also loaded to obtain molecular masses of separated proteins. The electrophoresis chamber was connected to a power pack and electrophoresis was carried out at a constant voltage of 200 volts for 2 hrs i.e., till the bromophenol blue dye front was 0.5 cm above the base of the gel.

4.6.3. Immuno blotting

Immunoblotting blotting was performed by the method of Towbin et al., (1979) for expression of TLR2 protein. The gel with the separated proteins was carefully removed and transferred to transfer buffer. Whatman No.1 filter paper was soaked in transfer buffer and placed over the cathode of blotting apparatus (Mini Trans-Blot cell, Bio-Rad, CA, USA). A nitrocellulose membrane (Sartorius, Germany) was soaked in transfer buffer and placed on the top of the filter papers.The gel was then placed over the membrane and covered with filter paper soaked in transfer buffer. Transfer was carried under "constant current" for 1 hrs at 0.8 mA/sq. cm. of the gel. After transfer for one hour, nitrocellulose membrane was stained using Ponceau S protein stain (Sigma, St Louis, MO, USA) for 1-2 minutes to assess the transfer of proteins. The membrane was rinsed in water to remove excess stain. Molecular weight marker positions were marked using a pencil. The membrane was placed in 5% defated milk in Tris-buffer saline (TBS, pH 7.5) and incubated overnight at 4oC. Following morning, the membrane was washed in TBS-T (TBS containing 0.05% Tween-20) for 5 min with three changes. The membrane was then blotted by transferring into a primary anti-rabbit antibody solution (1:1000) and kept at 40C for 6 hrs. The blotted membrane was washed thrice as described before and then transferred into the secondary antibody solution and probed with anti-goat horseradish peroxidase (HRP) conjugated IgG antibody and kept at room temperature for 1 hr. The membrane was washed thrice with TBS-T. HRP development reagent (ECL detection kit, GE Healthcare, Buckinghamshire, UK) was supplied as two solutions, one containing Lumiglo and the other containing peroxide. These two solutions were mixed in the ratio of 1:40 and the membrane was immersed in the mixture for 1 min, wrapped with saran wrap exposed to X-ray film and developed.

4.6.3.1. Densitometric scanning

Density of the proteins on the autoradiogram was quantified by Bio-Rad model GS-700 imaging densitometer using molecular analyst software, version 1.5 (Bio-Rad, CA, USA). The autoradiograms were scanned in the transmittance mode at a resolution setting of 150 dpi, using a gray filter. The intensities of bands were compared on the basis of adjusted volume (mean optical density × area in square millimeters).

4.6.3.2. Statistical analysis

All data were entered in computer in a structured format. Initial validation and consistency checks were carried out. Values from densitometry were expressed as mean ± SD of triplicate experiments performed independently for each sample for semi-quantitative RT-PCR analysis. Differences in cytokine concentrations between experimental *vs.* control were analyzed using independent't' test. Data were analyzed with SPSS statistical software, version 16.0 (SPSS Inc., Chicago, IL, USA) and p values ≤ 0.05 were considered significant.

ELISA data was expressed as mean ± SD of triplicate experiments performed independently for each sample. One-way anova: Post Hock (Bonferroni) test was performed to determine the expression level of IL-17, ICAM-1 and IL-8 and to compare continuous data (age). Differences in cytokine concentrations between the GBS *vs.* healthy controls were analyzed using one-way analysis of variance (ANOVA) test. The statistical significance of the data of Westernblot was determined using Student's t test with Graph Pad Prism software (San Diego, CA, USA) and triplicate experiments performed independently for each sample.

Chapter-V

Results

5.1. Study Population

The present study was conducted at Sanjay Gandhi Postgraduate Institute of Medical Sciences (SGPGIMS), Lucknow, an 800 bedded tertiary care centre in northern India. One hundred five patients with GBS (male (84), female (21), mean age ± SD= 30.20±10.97 years) admitted to Neurology ward, SGPGIMS, Lucknow. All the cases fulfilling the diagnostic criteria described by Asbury and Cornblath were enrolled into the study (Asbury and Cornblath 1990). Further subtyping of the GBS patients on the basis of clinical and electrophysiological criteria classified 38 and 67 patients into AIDP and axonal subtype of GBS respectively (Table 4). Among the axonal cases, 37 had pure motor involvement (AMAN), while 30 patients had both motor and sensory neuropathy (AMSAN). The GBS patients were admitted on an average 4.5 ± 3.2 days after onset of motor symptoms.

Blood samples were collected for all the GBS patients within 48 hours of admission. Patients with GBS had not received any steroid treatment at least 4 weeks prior to the sample collection. All the clinical details of the patients are summarized in the Table 6 and Figure 3.

Age and sex matched 100 individuals (male (78), female (22), mean age ± SD = 28.12±16.97 years without any history of apparent infectious illnessess within the last four weeks, were included as healthy controls. Therefore, the total numbers of studied individuals were 205 (100 normal healthy controls (HC) and 105 patients).

5.2. Presence of infectious illness preceding four weeks of GBS

All the patients with GBS and with neurological diseases other than GBS were asked for the presence of symptoms and signs of any apparent infectious illness within the last 4 weeks. History of preceding infection was available in 51(48.6%) patients. These

illnesses were as follows: respiratory 21 (20%), diarrhoea with and without abdominal pain 10 (9.5%), both respiratory and diarrhoea 5 (4.8%), fever 14 (13.3%) and chicken pox 1 (1%).

Table 6: Demography of patients with Guillain-Barré syndrome and controls

Characters	**GBS subtypes* (n =105)**			**Healthy controls**
	AMAN	**AMSAN**	**AIDP**	
No. of subjects	37 (35.2%)	30 (28.6%)	38 (36.2%)	100
Male: Female	28: 9	23: 7	33: 5	78: 22
#Age in years (mean±SD)	29.14±13.55	27.35±8.57	34.11±10.78	28.12±16.97
Preceding illness	(n =20)	(n =29)	(n =24)	0
a. Diarrhea	6	2	2	0
b. Respiratory illness	9	5	7	0
c. Diarrhea and Respiratory illness	3	1	1	0
d. Fever	5	6	3	0
e. Chicken Pox	0	1	0	0

- Mean duration of sample collection from patients (n=105) in progressive phase since onset of motor deficits =5.5 ± 1.2 days
- *AIDP, Acute inflammatory demyelinating polyneuropathy; AMAN, acute motor axonal neuropathy; AMSAN, acute motor axonal sensory neuropathy;
- # Mean age of GBS patients, 30.20±10.97

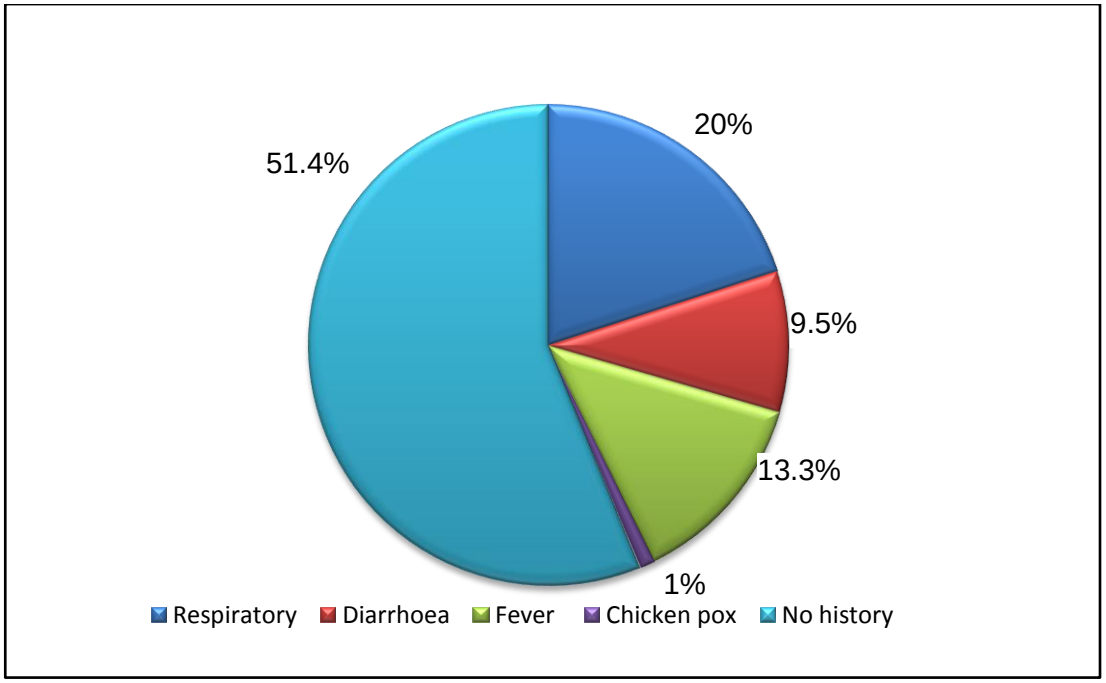


Figure 3: Distribution of preceding infectious illnesses in GBS patients

5.3. Detection of antecedent infectious agents (e.g. *Campylobacter jejuni,* Epstein-Barr virus, Cytomegalovirus and M. pneumoniae) in patients with Guillain-Barré syndrome (GBS)

Serum samples from all the subjects were subjected to IgG avidity for the detection of EBV-VCA & CMV and *M. pneumoniae* using commercial kits. Recent, EBV-VCA infection was detected in 13 (12.4%) of GBS patients. Detection of CMV was found in 15 (14.28%) of GBS patients. However, 10 (9.52%) of GBS patients had *M. pneumoniae* infection and 2 (1.9%) in healthy controls. Sero-dignosis method revealed that 25 (23.8%) of GBS patients had *C. jejuni* infection and 4 (3.8%) healthy controls (Table 7); mixed infections are shown in Table 8.

Table 7: Spectrum of infections in GBS patients and healthy controls

S.No.	Infections	GBS	Healthy control
1.	Epstein-Barr virus	13 (12.4%)	0
2.	Cytpmegalovirus	15 (14.28%)	0
3.	*Mycoplasma pnemoniae*	10 (9.52%)	2 (1.9%)
4.	*Campylobacter jejuni*	25 (23.8%)	4 (3.8%)
5.	Subjects without infection	66 (62.85%)	94 (94%)

Table 8: Mixed infection amongs GBS patients

S.No.	Epstein- Barr virus	Cytomegalo-virus	*M. pnemoniae*	*C. jejuni*	No. of Patients
1	+	+	-	-	1
2	+	-	+	-	1
3	+	-	-	+	3
4	-	+	-	+	3
5	-	-	+	+	1
6	+	-	+	+	1
7	+	+	+	-	1
8	+	+	-	+	1
9	-	+	+	+	1
10	+	+	+	+	3
11	+	-	-	-	2
12	-	+	-	-	6
13	-	-	+	-	3
14	-	-	-	+	12
15	-	-	-	-	66

5.4 Polymorphisms of TLR-2, NLRs (NOD1&NOD2), cytokine (IL-17), IL-8 and adhesion molecules (ICAM-1) polymorphisms in patients with GBS and risk for the development of GBS

5.4.1. Frequency distribution of the TLR2Arg753Gln and Arg677Trp Genotype variants in patients with GBS and control group

Genotypic distribution of TLR2Arg753Gln and Arg677Trp polymorphism demonstrated increased risk for GBS patients with heterozygous genotypes Arg/Gln ($p<0.0001$, OR = 8.17 95% CI = 3.26-20.48) and Arg/Trp ($p<0.0001$, OR= 86.62, 95% CI = 11.63-644.77). Meanwhile, the frequencies of wild homozygous genotypes was observed at 65.7% vs 94% for TLR2Arg753Gln and 53.33% vs 99% for Arg677Trp polymorphisms in a patient and control wise manner. Patients with GBS showed significantly higher frequency in comparison to controls for allele 753Gln (17.14% vs 6%, $p<0.0006$; OR, 3.24; 95% CI, 1.63-6.43) and allele 677Trp (23.34% vs 0.5%, $p<0.0001$; OR, 60.56; 95% CI, 8.26-443.61) (Table 9) (Figure 4 and 5).

Table 9: Influence of TLR-2 polymorphisms on GBS susceptibility

Gene polymorphism	Patients (%)	Controls (%)	*p* value	OR# (95% CI)
TLR2Arg753Gln Genotype				
Arg/ Arg	69 (65.7%)	94 (94%)	≤0.0001*	0.12 (0.04-0.30)
Arg /Gln	36 (34.3%)	6 (6%)	≤0.0001*	8.17 (3.26-20.48)
Gln/Gln	0 (0%)	0 (0%)	-	-
Allele				
Arg	174 (82.86%)	188 (94%)	0.0006*	0.30(0.15-0.61)
Gln	36 (17.14%)	12 (6%)	0.0006*	3.24 (1.63-6.43)
TLR2Arg677Trp Genotype				
Arg / Arg	56 (53.33%)	99 (99%)	≤0.0001*	0.01 (0.001-0.08)
Arg /Trp	49 (46.67%)	1 (1%)	≤0.0001*	86.62 (11.63-644.77)
Trp / Trp	0 (0%)	0 (0%)	-	-
Allele				
Arg	161 (76.66%)	199 (99.5%)	<0.0001*	0.01(0.002-0.12)
Trp	49 (23.34%)	1 (0.5%)	<0.0001*	60.56 (8.26-443.61)

* Statistical significance is attained when $p \leq 0.05$

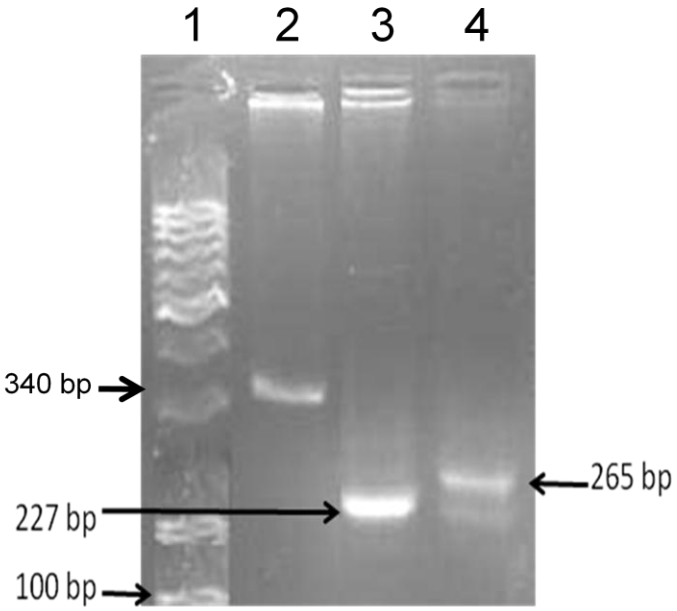
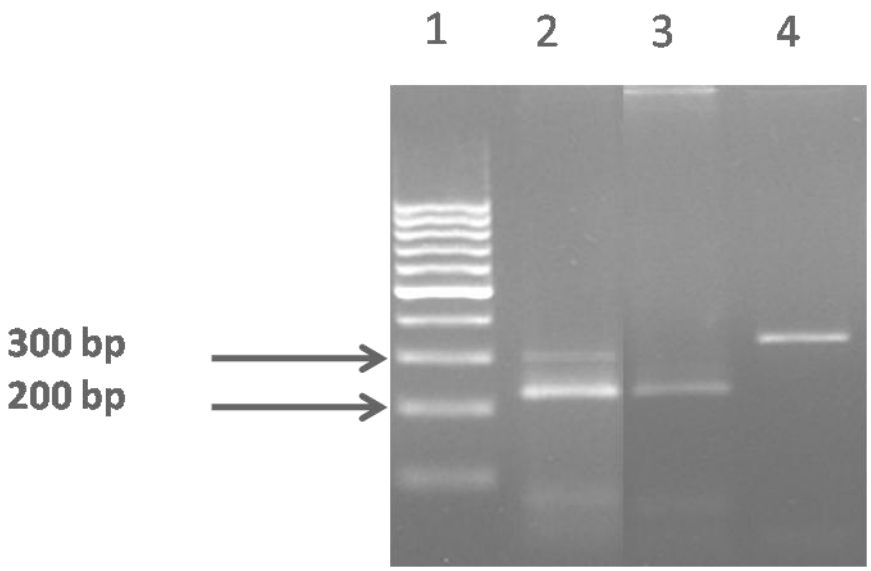


Figure 4: Identification of the TLR-2 Arg 677Trp genotypes. (A) AciI digestion of wild-type sequence shows bands of 340 bp. wild-type sequence shows bands of 227 bp, whereas a heterozygous shows 265, 227&75 bp. Lane1 is 100 bp DNA marker, lane 2 shows uncut pcr product of 340 bp, lane 3 shows wild type genotype of 227 bp and lane 4 represent heterozygous genotype.



TLR2Arg677Trp Genotype

Figure 5: Identification of the TLR-2 Arg 677Trp genotypes. (A) AciI digestion of PCR product shows wild-type sequence shows bands of 227 bp, whereas a heterozygous shows 302, 227&75 bp. Lane1 is 100 bp DNA marker, lane 2 shows heterozygous genotype, lane 3 shows wild type genotype and lane 4 represents uncut pcr product of 340 bp).

5.4.2. NOD1 (Glu266Lys) polymorphism

The genotypic frequencies of both homozygous (Lys/Lys) and heterozygous (Glu/Lys) variants of NOD1 were higher in GBS patients than healthy controls (Table 10 and Figure 6). Further, the homozygous variant showed increased risk with GBS (p=0.013, OR=2.90; 95% CI=1.25-6.70) and heterozygous variant also showed susceptible association with GBS than healthy controls (p=0.057, OR=2.17 95% CI= 0.98- 4.82). The frequency of variant allele (266Lys) was also higher among GBS patients (64.29%) than healthy controls (52.50%) and showed 1.63 fold risk association with GBS (p=0.016, OR=1.63, 95% CI= 1.10-2.42)

Table 10: Influence of NOD1 polymorphism on GBS susceptibility

Gene polymorphism	Patients (%)	Controls (%)	*p* value	OR# (95% CI)
rs2075820 Genotype				
Glu/Glu	12 (11.43%)	24 (24%)	-	Reference
Glu/Lys	51 (48.57%)	47 (47%)	0.057	2.17 (0.98-4.82)
Lys/Lys	42 (40%)	29 (29%)	0.013*	2.90 (1.25-6.70)
Allele				
Glu	75 (35.71%)	95 (47.5%)	-	Reference
Lys	135 (64.29%)	105 (52.5%)	0.016*	1.63 (1.10-2.42)

* Statistical significance is attained when $p \leq 0.05$

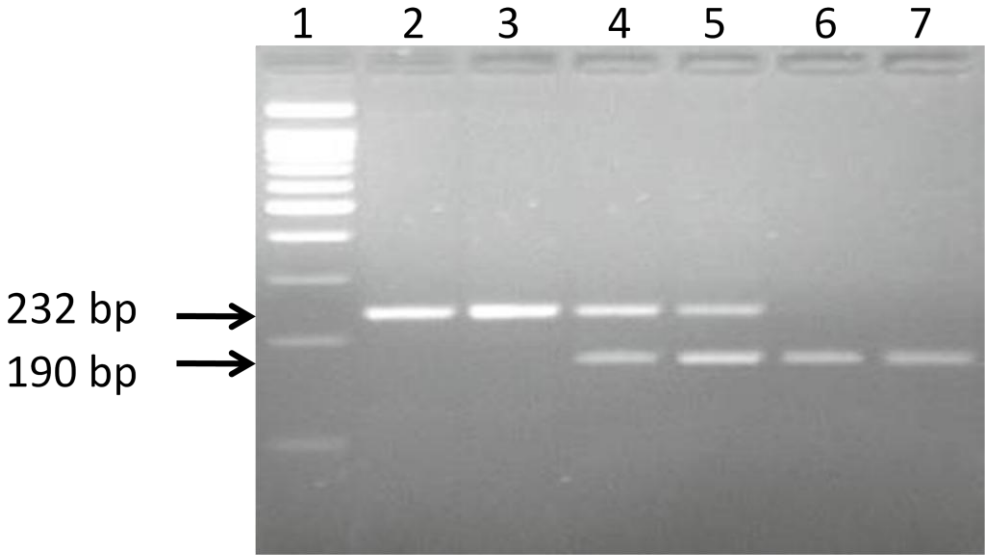


Figure 6: Identification of the NOD1 Glu266Lys genotypes. (A) Eco881 digestion of PCR product shows wild-type Glu/Glu (232 bp) genotype in lanes 2 and 3, mutant homozygous Lys/Lys (190 bp) genotype in lanes 6 and 7, followed by heterozygous Glu/Lys (232&190 bp) genotype in lanes 4 and 5.

5.4.2.1. Association of NOD1 (Glu266Lys) polymorphism with GBS subtypes

We further analysed the data to determine association of NOD1 (Glu266Lys) polymorphisms with GBS subtypes. Both heterozygous and homozygous variants did not show risk association with any of the GBS subtypes (AMAN, AMSAN and AIDP)

when compared with healthy controls (Table 11). However, NOD1 variant allele (266Lys) showed increased susceptibility to AMAN (p=0.001, OR=2.62, 95% CI=1.45-4.73) and AIDP (p= 0.018, OR=1.6, 95% CI=1.22-3.42) subtypes.

Table 11: NOD1 (Glu266Lys) genotype distribution in GBS subtypes and controls

NOD1 Genotype	**GBS Subtypes**			
	AMAN(a) (%)	**AMSAN (b) (%)**	**AIDP (c) (%)**	**Control (d) (%)**
Glu266Lys				
Glu/Glu	2 (5.4)	7 (23.33)	3 (7.89)	24 (24)
Glu/Lys	15 (40.55)	18 (60)	18 (47.37)	47 (47)
Lys/Lys	20 (54.05)	5 (16.67)	17 (44.74)	29 (29)
Allele				
Glu	19 (25.68)	32 (53.33)	24 (31.58)	95 (47.5)
Lys	55 (74.32)	28 (46.67)	52 (68.42)	105 (52.5)

(a and d)		**(b and d)**		**(c and d)**	
***p* value**	**OR 95%CI**	***p* value**	**OR 95% CI**	***p* value**	**OR (95%CI)**
-	References	-	References	-	References
0.091	0.26 (0.06-1.23)	0.594	0.76 (0.28-2.07)	0.096	0.33 (0.09-1.22)
0.008	0.12 (0.03-0.57)	0.417	1.69 (0.48-6.02)	0.024*	0.21 (0.06-0.82)
-	References	-	References	-	References
0.001	2.62 (1.45-4.73)	0.428	0.79 (0.44-1.41)	0.018*	1.60 (1.22-3.42)

* Statistical significance is attained when $p \leq 0.05$

5.4.3. NOD2 (Arg702Trp and Gly908Arg) polymorphisms and GBS

Increased risks for the development of GBS were revealed with heterozygote genotypes of NOD2, Arg702Trp (p=0.001, OR=5.78, 95% CI=2.95-11.31) and Gly908Arg (p=0.010, OR=2.07; 95% CI=1.19-3.62) (Table 12, Figure 7, and Figure 8). NOD2 alleles i.e., 702Trp (p=0.001, OR=4.48, 95% CI=2.44–8.24) & 908Arg (p=0.038, OR=1.61, 95% CI= 1.03-2.53) also showed significant risk association with GBS.

Table 12: Influence of NOD2 polymorphisms on GBS susceptibility

Gene polymorphism	Patients (%)	Controls (%)	*p* value	OR# (95% CI)
NOD2 Arg702Trp Genotype				
Arg/Arg	51 (48.57%)	85 (85%)	--	Reference
Arg/Trp	52 (49.52%)	15 (15%)	0.001	5.78 (2.95-11.31)
Trp/Trp	2 (1.9%)	0 (0%)	0.999	NC
Allele				
Arg	154(73.33%)	185 (92.5%)	--	Reference
Trp	56(26.67%)	15 (7.5%)	0.001	4.48 (2.44-8.24)
NOD2 Gly908Arg Genotype				
Gly / Gly	42(40%)	58 (58%)	--	Reference
Gly / Arg	63(60%)	42 (42%)	0.010	2.07 (1.19-3.62)
Arg / Arg	0(0%)	0 (0%)	--	------
Allele				
Gly	147(70%)	158 (79%)	--	Reference
Arg	63(30%)	42 (21%)	0.038	1.61 (1.03-2.53)

* Statistical significance is attained when $p \leq 0.05$

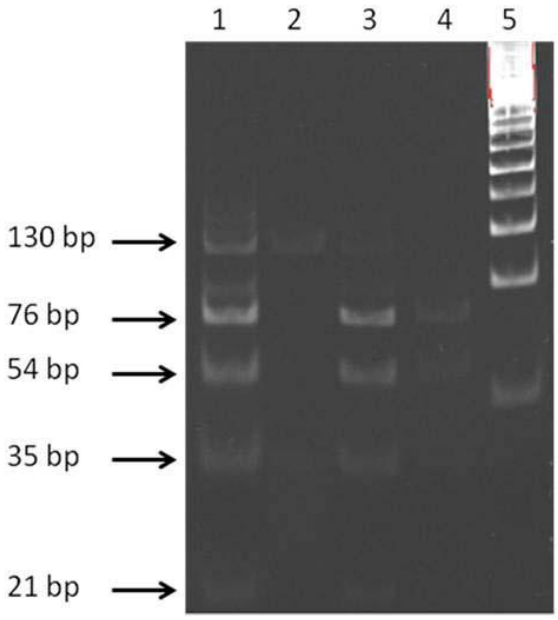


Figure 7: Identification of the NOD2 Arg702Trp genotypes. (A) MspI digestion of PCR product (185 bp) shows wild-type sequence (76, 54, 35 & 21 bp), homozygous (130, 35 & 21 bp), whereas a heterozygous (130, 76, 54, 35 &21 bp). Lane1 shows heterozygous genotype, lane 2 represents homozygous mutant genotype, lane 3, 4 show wild genotype and lane 5 is 50 bp marker.

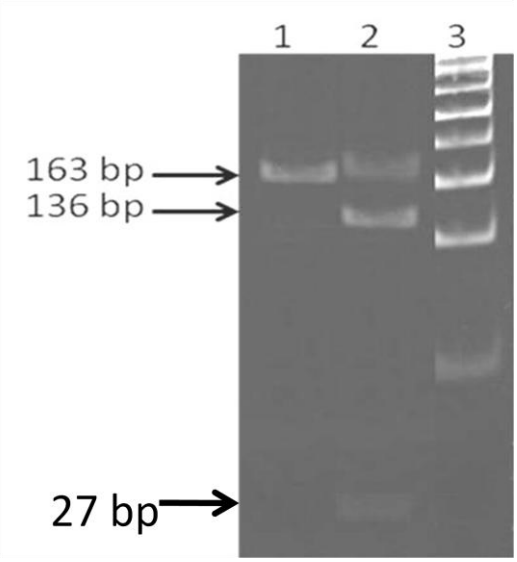


Figure 8: Identification of the NOD2 Gly908Arg genotypes. (A) HhaI digestion of PCR product (163 bp) shows wild-type sequence (163 bp), and heterozygous (163, 136 & 27 bp). Lane1 shows wild genotype, lane 2 represents heterozygous genotype and lane 3 is 50 bp marker.

5.4.3.1. Association of NOD2 polymorphisms (Arg702Trp and Gly908Arg) with GBS subtypes

Significant protective association for AMAN (p=0.001, OR=0.12, 95%CI=0.05-0.28), AMSAN (p=0.029, OR=0.35, 95% CI=0.14–0.90) and AIDP (p=0.001, OR=0.14, 95%CI=0.06-0.33) subtypes were found with NOD2 Arg702Trp heterozygote genotype. On the contrary, predisposing association was observed with NOD2 702Trp allele for AMAN (p=0.001, OR=6.29, 95%CI=3.08-12.84), AMSAN (p=0.039, OR=2.47, 95% CI=1.05-5.82) and AIDP (p=0.001, OR=4.71, 95% CI=2.27-9.75) subtypes. Heterozygote (Gly/Arg) genotype revealed protective association (p=0.003, OR=0.30, 95%CI=0.13-0.66), and variant allele (Arg) of Gly908Arg polymorphism showed risk associations with AIDP subtype (p=0.014, OR=2.07, 95% CI=1.16-3.70) (Table 13).

Table 13: NOD2 Genotype distribution in GBS subtypes and controls

NOD2 Genotypes	GBS Subtypes			
	AMAN(a) (%)	**AMSAN (b) (%)**	**AIDP (c) (%)**	**Control (d) (%)**
Arg/Arg	14 (37.84)	20 (66.67)	17 (44.74)	85 (85)
Arg/Trp	21 (56.76)	10 (33.33)	21 (55.26)	15 (15)
Trp/Trp	2 (5.4)	0	0	0
Allele				
Arg	49 (66.21)	50 (83.33)	55 (72.37)	185 (92.5)
Trp	25 (33.79)	10 (16.67)	21 (27.63)	15 (7.5)
Gly / Gly	15 (40.54)	16 (53.33)	11 (28.95)	58 (58)
Gly / Arg	22 (59.46)	14 (46.67)	27 (71.05)	42 (42)
Arg / Arg	0	0	0	0
Allele				
Gly	52 (70.27)	46 (76.67)	49 (64.47)	158 (79)
Arg	22 (29.73)	14 (23.33)	27 (35.53)	42 (21)

(a and d)		**(b and d)**		**(c and d)**	
***p* value**	**OR 95%CI**	***p* value**	**OR 95%CI**	***p* value**	**OR 95%CI**
-	References	-	References	-	References
0.001	0.12 (.05-.28)	0.029	0.35 (0.14-0.90)	0.001	0.14 (0.06-0.33)
0.999	NC	-	-	-	-
-	References	-	References	-	References
0.001	6.29 (3.08-12.84)	0.039	2.47 (1.05-5.82)	0.001	4.71 (2.27-9.75)
-	References	-	References	-	References
0.071	0.49 (0.23-1.06)	0.651	0.83 (0.37-1.88)	0.003	0.30 (0.13-0.66)
-	-	-	-	-	-
-	References	-	References	-	References
0.131	1.59 (0.87-2.97)	0.700	1.15 (0.58-2.28)	0.014	2.07 (1.16-3.70)

* Statistical significance is attained when $p \leq 0.05$

5.4.3.2. Distribution of NOD2 haplotype frequency among patients with GBS and control subjects

Haplotype analysis was carried out in order to evaluate whether the NOD1 and NOD2 gene polymorphisms have any additive effect on GBS (Table 14). The occurrence of Arg702-Gly908 haplotype comprising of wild genotype (Arg/Gly) among control and patients was 79% and 68.5% respectively. Total four haplotypes were found among patients while three were found among controls. Significant risk with Trp702-Arg908 ($p \leq 0.001$, OR=3.87, 95% CI=2.09-7.18) haplotype and protective association with Arg702-Arg908 (p=0.022, OR=0.40, 95% CI=0.19-0.86) haplotype for GBS were observed.

Table 14: NOD2 Haplotype frequency distribution between patients and control subjects

Haplotype	Patient (N=105)	Control (N=100)	p-value	OR	95% CI
Arg702-Gly908	144 (68.5%)	158 (79.0%)	Reference		
Trp702-Gly908	3 (1.4%)	-	-	-	-
Trp702-Arg908	53 (25.2%)	15 (7.5%)	<0.001	3.87	2.09-7.18
Arg702-Arg908	10 (4.7%)	27 (13.5%)	0.0221	0.40	0.19-0.86

5.4.3.3 Linkage disequilibrium analysis

Standardized linkage disequilibrium (LD) coefficient (D´), conventional measure of LD (r^2) and p values were measured among the GBS cases (Table 15). The LD analysis showed five significant associations in this study. It is evident from the analysis that significant risk was associated with GBS in the presence of mutant alleles. Pair-wise LD showed risk for GBS in the presence of Glu (Glu266Lys)-Lys(Glu266Lys) (D´=0.99, p-value= <2e-16), Glu (Glu266Lys)-Trp (Arg702Trp) (D´=0.99, p-value= 2.89e-07), Glu (Glu266Lys)-Arg (Gly908Arg) (D´=0.99, p-value= 1.92e-07), Trp (Arg702Trp)-Arg (Gly908Arg) (D´=0.99, p-value= <2e-16) and Trp (Arg702Trp)-Arg (Gly908Arg) (D´=0.99, p-value= <2e-16) alleles among the studied individuals.

Table 15: Linkage disequilibrium association among NOD1 Glu266Lys (rs2075820), NOD2 Arg702Trp (rs2066844), NOD2 Gly908Arg (rs2066845) polymorphisms in GBS patients

		Glu (Glu266Lys)	Lys (Glu266Lys)	Arg (Arg702Trp)	Trp (Arg702Trp)	Gly (Gly908Arg)	Arg (Gly908Arg)
Glu (Glu266Lys)	D’	*	0.9995	0.4852	0.9997	0.5657	0.9997
	r^2	*	0.8072	0.3096	0.8112	0.4228	0.785
	p-value	*	<2e-16	1.97e-05	2.89e-07	3.903-09	1.92e-07
Lys (Glu266Lys)	D’	*	*	0.9991	0.5508	0.9992	0.7353
	r^2	*	*	-0.3175	0.3609	-0.3719	-0.4663
	p-value	*	*	5.74e-07	8.82e-05	2.33e-09	1.65e-07
Arg (Arg702Trp)	D’	*	*	*	0.9995	0.8911	0.9995
	r^2	*	*	*	0.5175	0.7609	0.5008
	p-value	*	*	*	1.89e-08	<2e-16	3.17e-08
Trp (Arg702Trp)	D’	*	*	*	*	0.9226	0.9998
	r^2	*	*	*	*	0.5594	0.9676
	p-value	*	*	*	*	6.44e-10	<2e-16
Gly (Gly908Arg)	D’	*	*	*	*	*	0.9994
	r^2	*	*	*	*	*	0.5865
	p-value	*	*	*	*	*	<2e-16
Arg (Gly908Arg)	D’	*	*	*	*	*	*
	r^2	*	*	*	*	*	*
	p-value	*	*	*	*	*	*

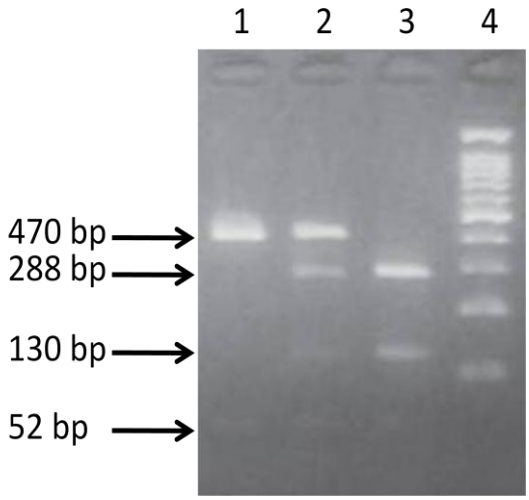
5.4.4. Association of IL-17F (His161Arg and Glu126Gly) polymorphism and GBS

To analyse the association of IL-17F (Glu126Gly and His161Arg) polymorphisms with GBS susceptibility, the genotypic frequency was compared between patients and controls. All the polymorphisms were in agreement with Hardy-Weinberg equilibrium in patients and control subjects. The study showed that only homozygous variant (Gly/Gly) showed increased susceptibility to GBS (53.34% vs. 23%, OR=3.82, 95% CI=2.09- 6.99, p< 0.0001). Further, G allele also showed increased risk with GBS (71.43% vs. 53.5%, p=0.0002; OR=2.17; 95% CI=1.44-3.26). On the other hand, His161Arg gene polymorphism did not show any association with GBS susceptibility (Table 16, Figure 9, and Figure 10).

Table 16: Inflence of IL-17 polymorphim on GBS susceptibility

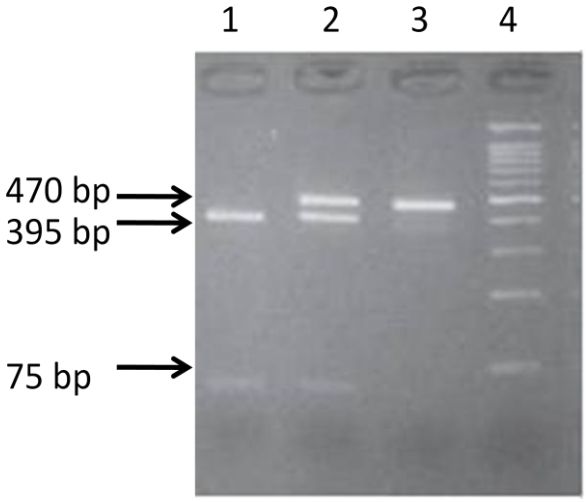
Gene polymorphism	Patients (%) N=105	Controls (%) N=100	*p* value	OR# (95% CI)
IL-17 (Glu126Gly) Genotype				
Glu/Glu	11(10.47%)	16(16%)	--	References
Glu/Gly	38(36.19%)	61(61%)	0.0986	0.36 (0.20-0.63)
Gly/Gly	56(53.34%)	23(23%)	<0.0001	3.82 (2.09-6.99)
Allele				
Glu	60(28.57)	93(46.5)	--	References
Gly	150(71.43)	107(53.5)	0.0002	2.17 (1.44-3.26)
IL-17 (His161Arg) Genotype				
His/His	46(43.8%)	37(37%)	--	References
His/Arg	51(48.5%)	61(61%)	0.0922	0.60 (0.34-1.05)
Arg/Arg	8(7.6%)	2(2%)	0.1018	4.04 (0.83-19.52)
Allele				
His	143(68.09%)	135(67.5%)	--	References
Arg	67(31.91%)	65(32.5%)	0.8848	0.94 (0.53-1.68)

* Statistical significance is attained when $p \leq 0.05$



IL-17 His161Arg

Figure 9: Identification of the IL-17F His161Arg genotypes. NlaII restriction enzyme digestion of exon 3 at position 7488A/G of the IL-17F and PCR product was 470 bp. NlaIII digestion of PCR product shows heterozygous sequence (52, 130, 288, 470 bp), whereas a homozygous mutant genotype (52,130 and 288 bp). M is 100 bp DNA marker, lane 1 shows wild type (7488G/G), lane 2 shows heterozygous for the exon 3 of IL-17F (7488 A/G) and lane 3 represents homozygous mutant of IL-17F (7488A /A).



IL-17 Glu126Gly

Figure 10: Identification of the IL-17 Glu126Gly genotypes. AvaII restriction enzyme digestion of exon 3 at position 7383A/G of the IL-17F and PCR product was 470 bp. AvaII digestion of PCR product shows wild-type sequence band size 470 bp, whereas a homozygous polymorphic allele shows a single band of 75 and 395 bp and heterozygous shows a band of 75, 395 & 470 bp. M is 100 bp DNA marker, lane 1 represents homozygous mutant of exon 3 (7383 A/A), lane 2 shows heterozygous for the exon 3 of IL-17F (7383A/G), lane 1 shows wild type of IL-17F (7383G/G).

5.4.5. Frequency distribution of the IL-8-251 A/T variant in patients with GBS and healthy controls

To analyze the association of IL-8 polymorphisms with GBS, the genotype frequency was compared between controls and patients with GBS. The logistic regression analysis revealed no significant association of IL-8-251A/T polymorphism (polymorphic and heterozygous) with GBS (p=0.8748 and p=0.0698) (Table 17, and Figure 11).

Table 17: Influence of IL-8-251A/T polymorphism on GBS susceptibility

Gene polymorphism	Patients (%)	Controls (%)	*p* value	OR# (95% CI)
IL-8(-251A/T) Genotype				
A/A	18(17.14%)	29(29%)	0.0476*	0.50 (0.26-0.98)
A / T	60(57.14%)	44(44%)	0.0698	1.69 (0.97-2.94)
T /T	27(25.71%)	27(27%)	0.8748	0.93 (0.50-1.74)
Allele				
A	96(45.71%)	102(51%)	0.322	0.80(0.54-1.19)
T	114(54.28%)	98(49%)	0.322	1.23(0.83-1.82)

* Statistical significance is attained when $p \leq 0.05$

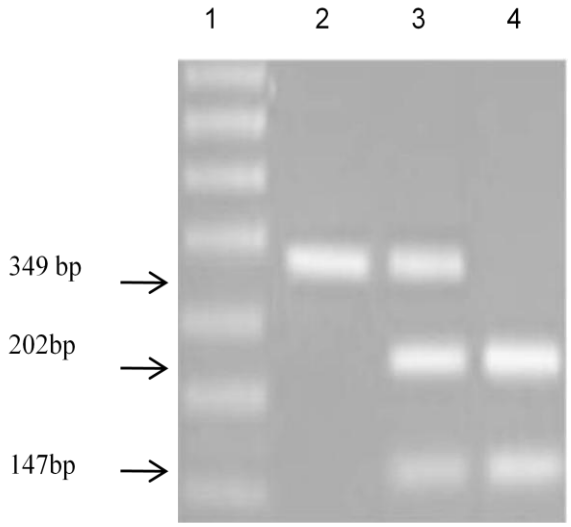


Figure 11: Identification of the IL-8 -251 A/T genotypes. (A) MunI digestion of PCR product (349 bp) shows wild-type sequence (349 bp), homozygous (202 & 147 bp), whereas a heterozygous (349, 202 & 147 bp). Lane 1 is 100 bp marker, lane 2 & lane 3 represent wild type & heterozygous genotype respectively and lane 4 shows homozygous mutant.

5.4.6. Association of ICAM-1 Gly241Arg polymorphism and GBS

To analyse the association of ICAM-1 polymorphisms with GBS, the genotype frequency was compared between controls and patients with GBS. Logistic regression analysis revealed that heterozygous genotype (G/A) of ICAM-1 Gly241Arg polymorphism had association only with GBS ($p<0.0001$; OR= 4.05, 95% CI=2.26-7.26), while not any homozygous polymorphic (AA) genotype was detected either in patients or in controls. Frequency of homozygous wild type was 30.47% in patients and 64% in controls (Table 18, and Figure 12).

Table 18: Influence of ICAM-1 polymorphism on GBS susceptibility

Gene polymorphism	Patients (%)	Controls (%)	*p* value	OR[#] (95% CI)
ICAM-1 (Gly241Arg) Genotype				
Gly/Gly	32(30.47%)	64(64%)	-	References
Gly/Arg	73(69.53%)	36(36%)	<0.0001*	4.05 (2.26-7.26)
Arg/Arg	0	0	-	-
Allele				
Gly	137(65.23%)	164(82%)	-	References
Arg	73(34.77%)	36(18%)	0.0001*	2.42 (1.53-3.84)

* Statistical significance is attained when $p \leq 0.05$

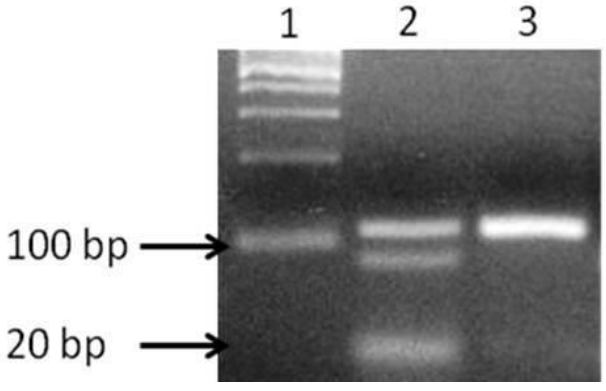


Figure 12: Identification of the ICAM-1(Gly241Arg) genotypes. (A) Bsr GI digestion of PCR product (120 bp) shows wild-type sequence (110 bp) and heterozygous (110, 90 & 20 bp). Lane 1 is 100 bp marker, lane 2 represents heterozygous genotype, and lane 3 represents wild type genotype.

5.5. Expression Study

The expression pattern of Toll like receptors (TLR-2), chemokine (IL-8), Interleukin (IL-17) and adhesion molecule (ICAM-1) were checked at mRNA level and in serum.

5.5.1. Expression of TLR-2 in GBS

5.5.1.1 mRNA level of TLR2 in GBS patients and healthy control

The RT PCR results showed that the mRNA expression of TLR-2 was increased significantly in GBS patients than in controls (Table 19, and Figure 13). However increased trend was observed in heterozygous genotype than wild type but not significant.

Table 19: mRNA expression of TLR 2 (Arg677Trp & Arg753Gln)

Study groups	**Level of TLR2 mean±SE**	***p value***
Control	0.319±0.113	Reference
Patients	1.727±0.412	0.033*
Genotype (Arg677Trp)		
RR	0.79±0.073	Reference
RW	1.52±0.717	0.477
WW	-	-
Genotype (Arg753Gln)		
RR	1.05±0.179	Reference
RQ	1.129±0.618	0.902
QQ	-	-

* Statistical significance is attained when $p \leq 0.05$

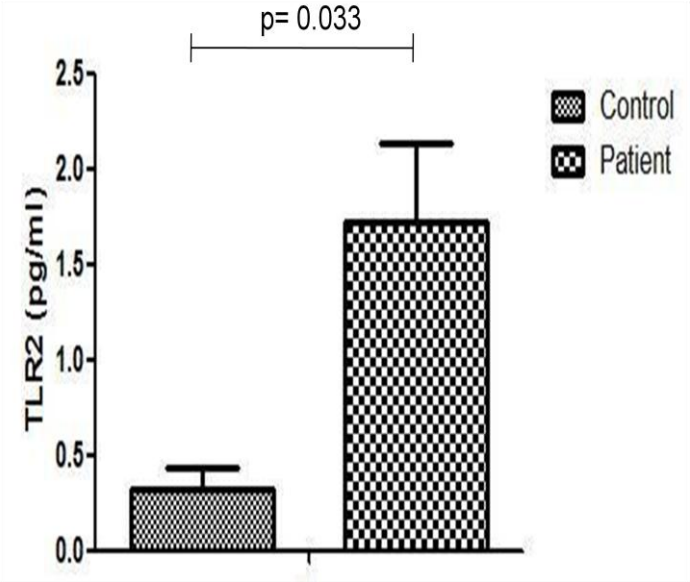


Figure 13: mRNA level of TLR-2 in patients with GBS and in healthy control.

5.5.1.2 Expressions of TLR2 by immunoblotting

The immunoblot experiment performed and an enhanced expression of TLR-2 was observed in GBS patients (fold change-1.112) compared to healthy controls (fold change-1) (figures 14 & 15). However increased expressions were noted in Arg 753Gln heterozygous genotype (fold change-1.04) than wild type (fold change-1) and Arg 677 Trp heterozygous genotype (fold change-1.11) than wild type (fold change-1).

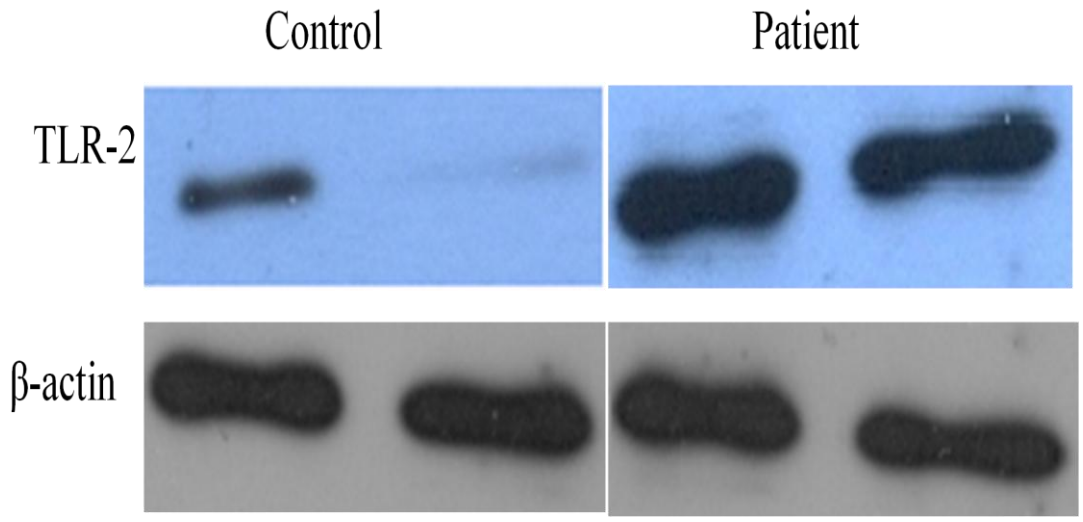


Figure 14: Representation of band pattern in control and patient of TLR2: Densitometric scans from three experiments were normalized to native β-actin levels and showed expression pattern of TLR-2.

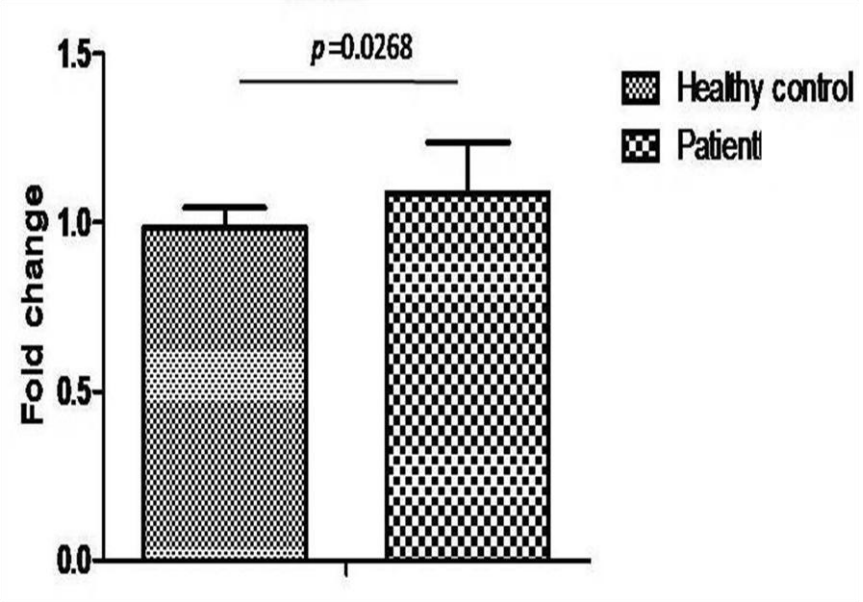


Figure 15: Level of TLR-2 in patients with GBS and in healthy control.

5.5.2. Expression of IL-8 in GBS patients and healthy control

The expression of IL-8 chemokine was checked at mRNA level and in serum of GBS patients and healthy control.

5.5.2.1. mRNA level of IL-8 in GBS patients and healthy control

The RT PCR results showed that the expression of IL-8 was increased significantly in GBS patients than in controls (3.47±0.14 vs 1.27±0.07, p=0.001) (Figure 16). Meanwhile, an increased expression was observed in homozygous variants genotype (3.37±0.09) than other wild (2.97±0.18) type and heterozygous genotypes (3.1±0.11) but the difference was not signicant. Insignificant p-values were observed for mutant (p=0.0504) and heterozygous genotypes (p=0.539) of IL-8 when compared with the wild type genotype.

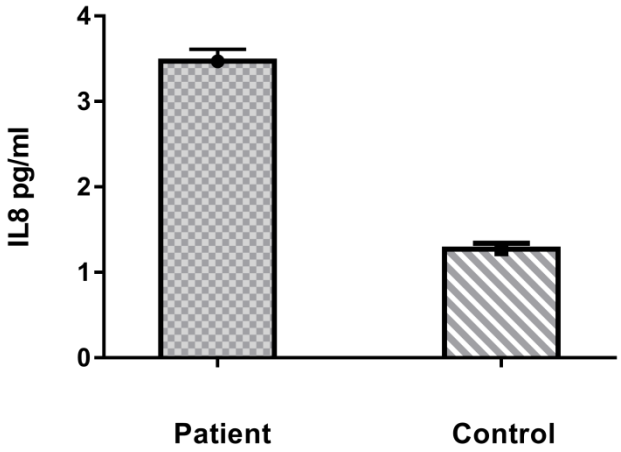


Figure 16: mRNA level of IL-8 in patients with GBS and in healthy control

5.5.2.2 Level of IL-8 in serum of GBS patients and healthy control

Serum level of IL-8 was elevated in GBS Patients. The level of IL-8 was found elevated in sera of GBS Patients than healthy controls (112.55±13.96 vs 34.79±4.37; $p \leq 0.001$) (Figure 17). However an increased expression was noted in homozygous variants genotype than other wild type and heterozygous genotypes but the difference was not signicant (Table 20).

Table 20: Level of IL-8 in serum of patients and healthy control

Study groups	Level of IL-8 (pg/ml) mean±SE	*p value*
Control	34.79±4.373	Reference
Patients	112.55±13.96	0.001*
IL-8 -251 A/T Genotype		
A/A	107.90±9.93	Reference
A / T	105.13±7.63	0.825
T /T	110.5±13.49	0.877

* Statistical significance is attained when $p \leq 0.05$

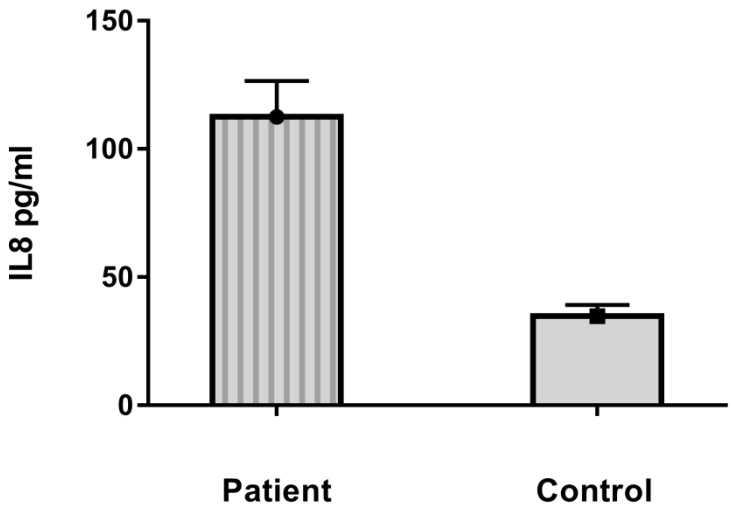


Figure 17: The serum level of IL-8 was observed in GBS patients and healthy control.

5.5.3. Expression of IL-17

The expression of IL-17 chemokine was checked at mRNA level and in serum of GBS patients and healthy control.

5.5.3.1: Elevated mRNA level of IL-17 in GBS patient:

The mRNA level of IL-17 showed increased trend among patients than controls (p=0.066, 3.66±0.79 vs 2.05±0.29) (Figure 18). Moreover, patients with IL-17 polymorphic genotype (G/G) had increased mRNA expression than other genotypes, A/G and A/A (Table 21).

Table 21: mRNA expression of IL-17 in GBS and healthy control

Study groups	**Level of IL-17 mean±SE**	***p value***
Control	3.66±0.79	Reference
Patients	2.05±0.29	0.066
IL-17 (His161Arg) Genotype		
AA	2.74±0.42	Reference
AG	2.68±0.39	0.916
GG	3.17±0.83	0.645

***** Statistical significance is attained when $p \leq 0.05$

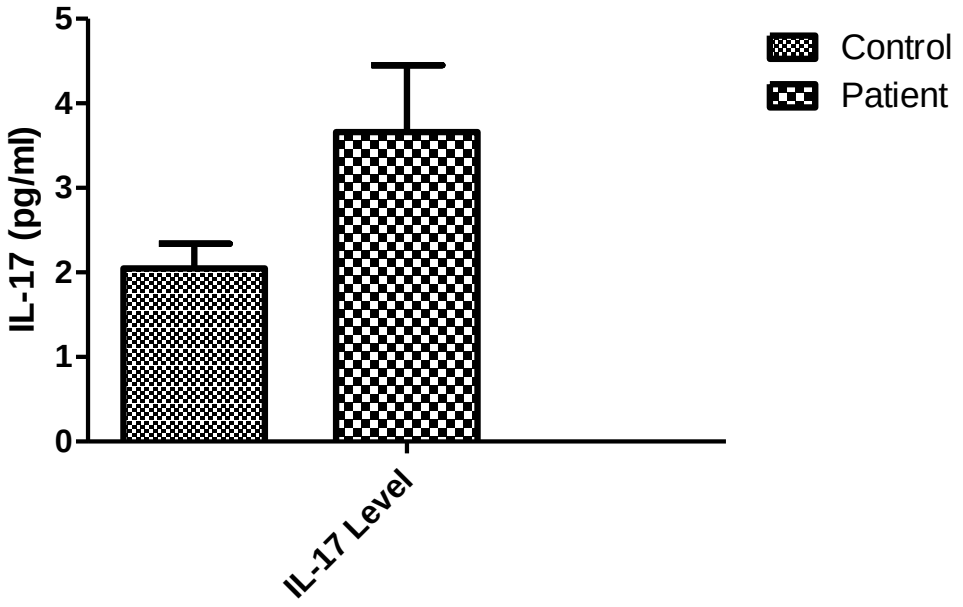


Figure 18: mRNA level of IL-17 in patients with GBS and healthy control.

5.5.3.2: Sera Levels of IL-17 was elevated in GBS Patients.

The level of IL-17 in sera was elevated in GBS patients and the elevation was significantly higher than healthy controls ($P \leq 0.001$, 42.00±5.33 vs 6.0±0.77) (Figure 19). Further, patients with IL-17 genotype (G/G) showed elevated level of expression compared to A/G and A/A genotypes (Table 22).

Table 22: Serum level of IL-17 in GBS patient and control

Study groups	**Level of IL-17 (pg/ml) mean±SE**	***p value***
Control	6.0±0.77	Reference
Patients	42.00±5.33	0.001*
IL-17 (His161Arg) Genotype		
AA	38.01±0.44	Reference
AG	38.06±1.45	0.973
GG	42.18±6.65	0.533

* Statistical significance is attained when $p \leq 0.05$

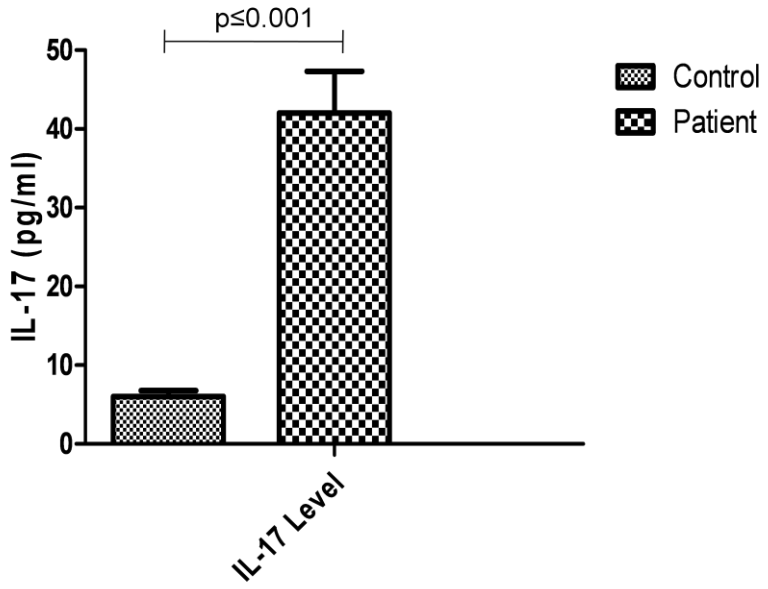


Figure 19: Level of IL-17 in serum of patients with GBS and healthy control.

5.5.4. Expression of adhesion molecule (ICAM-1) in GBS patients and healthy control: expression of ICAM-1 was evaluated at mRNA level and in serum of patients with GBS and healthy control.

5.5.4.1: Elevated mRNA level of ICAM-1 in GBS patient: In ICAM-1, mRNA expression increased in patients compared to controls (p=0.012, 2.04±0.261 vs 1.18±0.202) (Figure 20). Moreover, patient with heterozygous genotype G/A had significant more expression of mRNA (p=0.004, 2.19±0.33) compared to wild type homozygous genotype G/G (Table 23).

A representative gel image showing band patterns of mRNA expression in ICAM-1 along with other studied markers has been shown in Figure 21.

Table 23: mRNA level of ICAM-1 in GBS patients and healthy controls

Study groups	Level of ICAM-1 mean±SE	*p value*
Control	1.18±0.202	Reference
Patient	2.04±0.261	0.012*
ICAM-1 Genotype		
GG	1.97±0.10	Reference
GA	2.19±0.33	0.004
AA	-	-

* Statistical significance is attained when $p \leq 0.05$

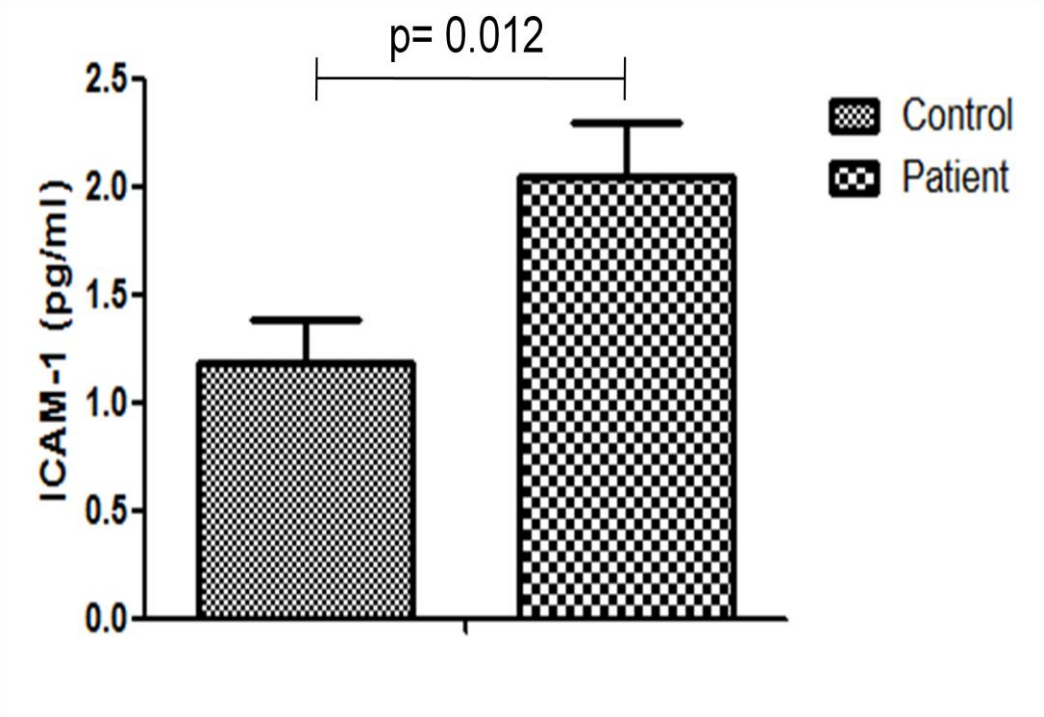


Figure 20: mRNA levels of ICAM-1 in patients with GBS and healthy controls.

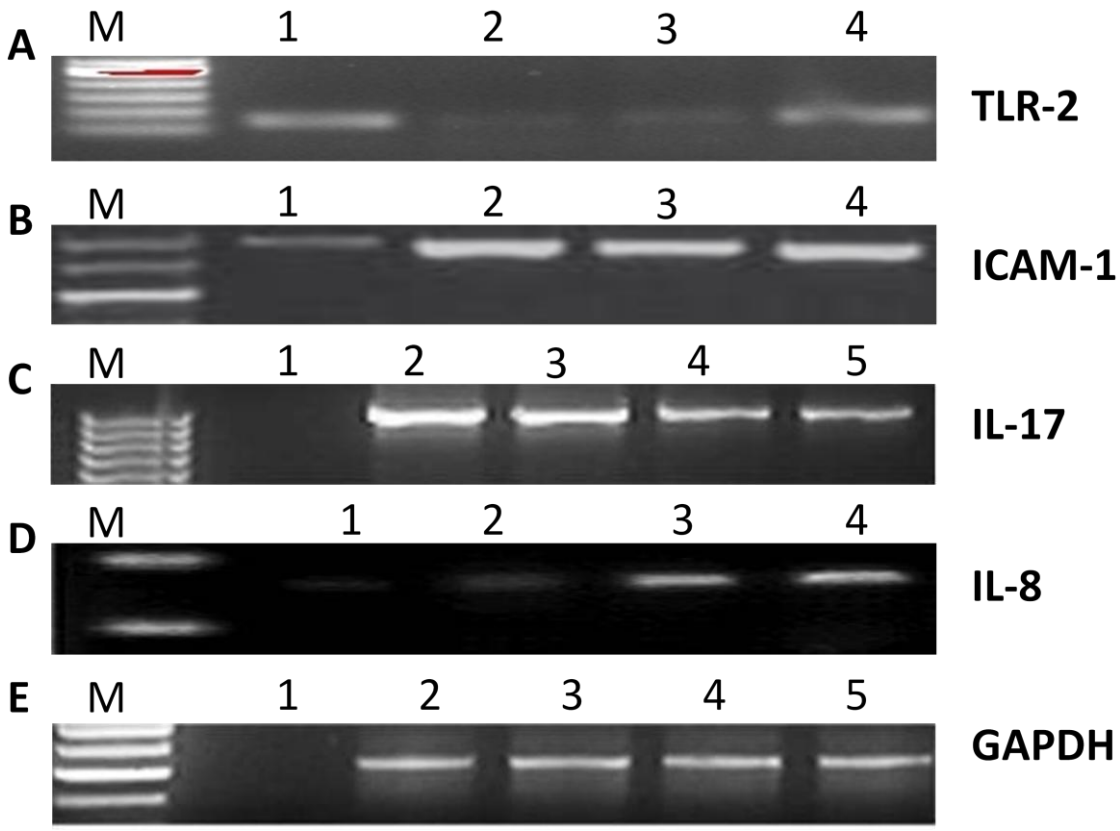


Figure 21: Band patterns of mRNA expression in TLR-2, ICAM-1, IL-17, IL-8 and GAPDH. Representation of band pattern of expressions of TLR-2, ICAM-1, IL-17, and GAPDH in control and GBS patients Densitometric scans from three experiments and GAPDH as internal marker.

A. mRNA expression of TLR-2: Lanes 1 and 4 show band pattern from patients, lanes 2 and 3 from controls.

B. mRNA expression of ICAM-1: lane 1 shows band pattern from control and lanes 2-4 from patients

C. mRNA expression of IL-17: lane 1 is blank, lane 2 and 3 show band pattern from patients, lanes 4 and 5 from healthy controls.

D. mRNA expression of IL-8: lanes 1 and 2 show band pattern from controls, lanes 3 and from patients.

E. mRNA expression of GAPDH: lane 1 is blank, lane 2 and 3 show band pattern from patients, lanes 4 and 5 from healthy controls.

5.5.4.2. Serum level of ICAM-1 in GBS patients and healthy controls

The levels of ICAM-1 in sera were elevated in GBS patients and the levels were significantly higher in GBS than controls ($P < 0.001$, 5.33±0.661 vs 2.25±0.288) (Figure 22). Patients with ICAM-1 wild type genotype (G/G) and heterozygous genotype (G/A) had almost similar levels of expressions (Table 24).

Table 24: ELISA expression of ICAM-1

Study groups	Level of ICAM-1 (pg/ml) mean±SE	*p value*
Control	2.25±0.29	Reference
Patients	5.33±0.66	0.001*
ICAM-1 Genotype		
GG	4.13±0.35	Reference
GA	4.92±0.42	0.152
AA	-	-

* Statistical significance is attained when $p \leq 0.05$

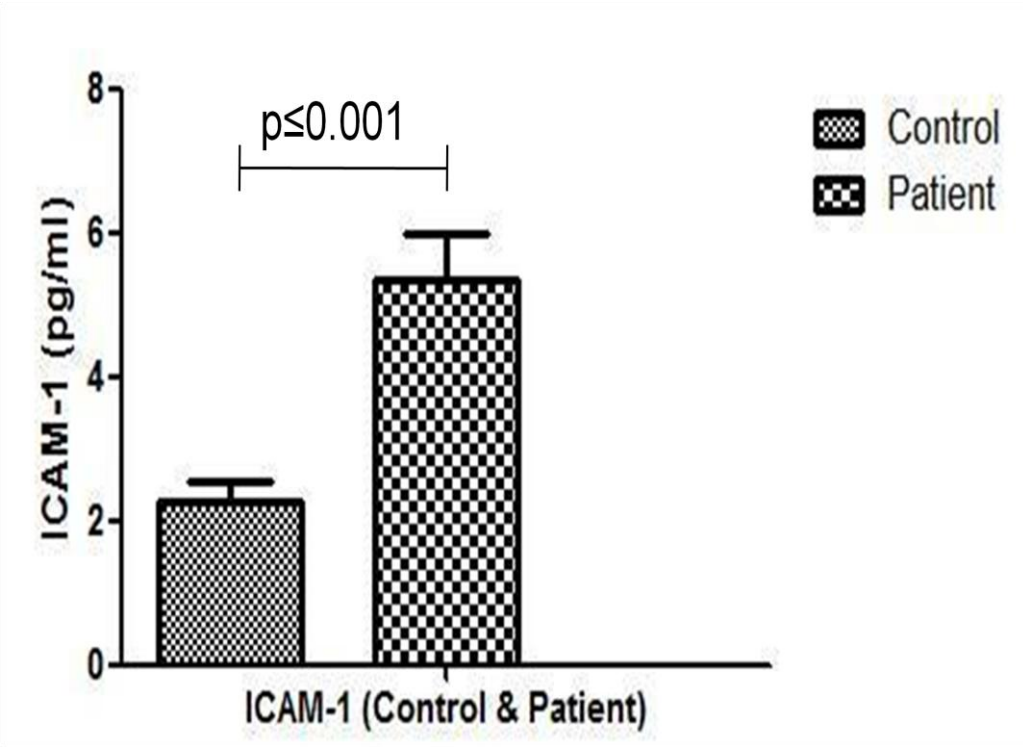


Figure 22: Enhanced levels of ICAM-1 in patients among GBS than healthy controls.

Chapter-VI

Discussion

Since the near eradication of poliomyelitis from most part of the world, GBS has emerged as the most common cause of acute flaccid paralysis with an annual incidence of 1-2 cases per 100,000 populations per year. The clinical spectrum of GBS comprises a heterogeneous group of pathological entities, each with different clinical features and pathogenesis (Hahn 1998). The two major sub-types of GBS, AIDP and axonal, also differ in their distribution in different parts of the world. GBS, being a post infectious autoimmune disorder, it is likely that type of preceding infection followed by subsequent immune response may have relation with onset of GBS and its subtype. Whether specific infections and immune responses mounted by the host could be considered as predictors of GBS, need to be investigated. Systemic study addressing these and many other issues are lacking in the world literature. Around half to two-thirds of the GBS cases, are preceded by numbers of bacterial and viral infections. The most commonly identified agents implicated for preceding infections of GBS are as follows: *C. jejuni, M. pneumoniae,* CMV, EBV, variacella-zoster virus and certain vaccines (rabies, swine influenza) (Hahn 1998). Serology is used for the detection of *C. jejuni* specific antibodies in patient's serum and IgG avidity ELISA was performed for the detection of CMV, EBV and *M. Pneumoniae* infection in patient serum using commercially available kits. Several drawbacks are associated with serology also: there is no consensus on the choice of antigens; most often a crude antigenic extract is used yielding low specificity, especially in endemic and hyperendemic countries (Tsang 2002); testing of paired sera and demonstration of significant increase and decrease in antibody titer may be required which is difficult and it also depends upon the time of sample collection. The disease is more often triggered by immune response against an infectious pathogen, which may lead to production of high titers of antibodies (Nachamkin, Allos et al. 1998). These antibodies target nerve antigen through a

molecular mimicry mechanism (Yuki 1994; van Doorn, Ruts et al. 2008). Cross reactive immune response is generated due to the molecular mimicry between *C. jejuni* cell wall antigens and ganglioside like epitopes in host nerve tissues (Yuki, Taki et al. 1993). It is estimated that only one in thousand individuals develops GBS after *C. jejuni* enteritis (Allos 1997). Besides the microbial factors, host susceptibility may also play an important role in the development of GBS. However, limited studies have identified potential host factors involved in the disease pathogenesis and impart susceptibility to an individual for the development of GBS. Therefore, this study was undertaken due to the lack of data regarding the potential host factors involved in the disease pathogenesis.

The present study analyzed serology for the detection of *C. jejuni* in patient's serum and IgG avidity ELISA was performed for the detection of CMV, EBV and *M. pneumoniae* infection in patient's serum. During the study, association of TLR-2, NLRs, Cytokine (IL-17), Chemokine (IL-8) and adhesion molecule (ICAM-1) polymorphisms were investigated. We further evaluated the expression of host factors including TLR-2, Cytokine (IL-17), chemokine and adhesion molecule in patients with GBS and healthy controls. In the present study, we also hypothesized that there might have some correlation between polymorphism and expression of host genes that might play important role in development of disease. The study was planned to understand the role of host factors in pathogenesis of GBS.

6.1 Study population and demographic details

We prospectively studied the presence of antecedent *C. jejuni*, CMV, EBV and *M. pneumoniae* infection in 105 GBS patients and 100 healthy controls. Several studies from the West have reported that more men than women are affected by GBS but the reason remain unexplained (Nachamkin et al. 1998). In present study also, male are

more affected than female. GBS affected wide range of age group (2 yrs- 69 yrs). A possible bimodal distribution of age in GBS cases has been suggested by some studies with peak in young adults and the elderly (Rees et al, 1998; Jiang et al. 1997; Hankey, 1987; de Pedro Cuesta et al. 1996). Some studies have also shown an increase in incidence with age, especially in the older age group (Jiang et al. 1997; Govani et al. 1996; McLean et al. 1994). However, we did not observe any such distribution of age in our GBS patients.

6.2. Presence of infectious illness preceding four weeks of GBS

Limited numbers of case control studies have been carried out demonstrating the presence of infectious illness in GBS. The clinical illness may be respiratory infection, diarrhea, fever etc. preceding 1-4 weeks of GBS onset. The infections are quite common in our general population and therefore their associations with GBS, if any, need to be studied. Literatures suggest that about half to two-thirds of the GBS patients have histories of antecedent infectious illness (Ropper, et. al. 1991). However, in our study 51 (48.6%) GBS patients reported some form of clinical illness in 1-3 weeks before GBS. The incidence of clinical illness in our patients appear to be low as compared to studies from the Netherlands (Jacobs, et.al. 1998), Japan (Koga, et.al. 2001) and Europe, North America and Australia (Hadden, et. al. 2001) where infectious illnesses were reported in 68%, 88% and 59% GBS patients respectively.

Respiratory (20%) infections were most commonly reported by our GBS patients followed by fever (13.3%) and diarrhea (9.5%). Several other studies have found respiratory infections as the most frequent prior event in GBS patients. In a Japanese study, 62% GBS patients had history of respiratory infections while 29% cases experienced gastrointestinal illness (Koga, et.al. 2001). Two case control studies for UK also demonstrated respiratory infection as the most common antecedent illness in

GBS patients (Melnick and Flewett, 1964; Winer et. al. 1998). This finding was confirmed by study from Netherlands (Jacobs, et.al. 1998) in which respiratory infection was reported by 44% and diarrheal illness 31% GBS patients. In a multicentric study by Hadden et. al. (2001) on 229 GBS patients from Europe, North America and Australia, 39% had prior respiratory illness followed by diarrhea which was present in 17% cases. However in contrast to all these studies including our present observation, diarrhea is the most predominant preceding illness in GBS patients from China.

6.3. Detection of *C. jejuni,* CMV, EBV and *M. pneumoniae* infection in 105 GBS patients and 100 healthy controls.

In general, 14-66% GBS patients have high prevalence of serum antibodies to *C. jejuni* and our finding falls within the range. In our study, 23.8% GBS patients and 3.8% HC had serological evidence of recent *C. jejuni* infection. The prevalence rate of 26% was reported from the West (Hadden, et. al.), 24-52% from Japan (Hao, et. al. 1998) and 47-72% from China (Ho, et. al. 1995).

Low prevalence rates of *M.pneumoniae* in GBS have been reported ranging from 5-6%. Interestingly, our GBS patient had higher incidence of *M.pneumoniae* infection. The infection was detected in 9.52% cases, which is at variation from studies carried out in western and East Asian countries. However, whether this unique feature is limited to our patients or more wide spread in other developing countries as well needs to be validated by other diagnostic methods.

In our study, EBV and CMV infection was detected by IgG avidity ELISA and found these infections in 12.4% and 14.28% of GBS patients respectively corroborating the studies from the West and East Asian countries. However, EBV infection have been

reported from 1-13% GBS cases and CMV infection rates in GBS patients have shown wide variation from study to study ranging from 5-22%. In contrast to the study of Jacob, et. al. (1998), the infection was not significantly greater in GBS patient as compared to controls. This feature in other developing countries really needs to be validated by serology and other detection/ diagnostic methods.

6.4. Association of TLR-2 (Arg753Gln & Arg677Trp) polymorphism in GBS patients and its expression

TLRs play central role in host defences and are involved in a number of autoimmune diseases including GBS. The magnitude of the association of polymorphisms with autoimmune disease varies depending on genetics, demographics and environmental factors. Many association studies reported that TLR-2 polymorphisms predisposed to autoimmune disease (Emonts, Hazes et al. 2011; Lee, Lee et al. 2012; Kaiser, Tang et al. 2014). However, some polymorphisms of TLR2 might not be associated with autoimmune disease like rheumatoid arthritis (RA) (Jaen, Petit-Teixeira et al. 2009). However, there is no data regarding TLR2 polymorphism with GBS till date.

The importance of TLR2 polymorphism in GBS is still largely unknown. In our study, the variant allele frequency of TLR2-753Gln and TLR2-677Trp was higher in GBS than controls (17.14% vs 6%, $p<0.0006$; OR, 3.24; 95% CI, 1.63-6.43 and 23.34% vs 0.5%, $p<0.0001$; OR, 60.56; 95% CI, 8.26-443.61). The reported rate of TLR2-Arg753Gln variant frequency was 1% in Spanish population (Sanchez, Orozco et al. 2004) whereas the TLR2-Arg677Trp was 30.3% in Tunisian population (Ben-Ali, Barbouche et al. 2004; Sanchez, Orozco et al. 2004). In separate studies, the Arg753Gln genotype was observed among 10.34% (Berdeli, Celik et al. 2005) and 12.3% (Berdeli, Emingil et al. 2007) of healthy Turkish subjects, while the Arg677Trp was not observed. Contrary to the study conducted by Kang et al (2001), other authors

failed to detect these TLR2 polymorphisms in the Korean population. In the Caucasian population, the TLR2-Arg753Gln SNP was detected in 9.4% of the German whites, while the Arg677Trp polymorphism was not observed at all (Schroder, Hermann et al. 2003).

The data regarding role of TLR2 gene polymorphism in inflammatory demyelinating diseases including GBS is limited. There is a single study that suggests association of TLR4 Asp299Gly and Thr399Ile polymorphisms with risk for development of GBS (Nyati, Prasad et al. 2010).

We conducted the present study in our population and a positive association of TLR2 polymorphism Arg753Gln ($p \leq 0.0001$; OR, 8.17; 95% CI, 3.26-20.48) and Arg677Trp ($p \leq 0.0001$; OR, 86.62; 95% CI, 11.63-644.77) was observed with GBS. Polymorphism in the TLR2 gene might cause inappropriate activation of the dendritic cells. These dendritic cells kick off cell maturation and increase the expression of major histocompatibility complex (MHC) and co-stimulatory molecule B7, and activate T cells which secrete increased amount of several chemokines and pro-inflammatory cytokines such as TNF-α responsible for demyelination. There are reports which show that increased level of TNF-α contributes to the pathogenesis of immune mediated demyelinating neuropathies and axonal degeneration by inducing damage to myelin (Redford, Hall et al. 1995; Zhu, Mix et al. 1998).

Besides the association of TLR2 with GBS, we also observed the enhanced expression of TLR2 in patient with GBS. The data regarding expression of TLR2 in GBS are very limited. So far, one study showed significantly elevated TLR2 expression in sciatic nerves during GBS (Wang, Liang et al. 2012). An increased expression of TLR2 had been shown in synovial tissues of patients with RA (Seibl, Birchler et al. 2003). In the present study, we found elevated TLR2 expression in PBMCs of patients with GBS

(fold change-1.112) when compared to healthy control (fold change 1). TLRs play a central role in the initiation of both innate and adaptive immune responses against microbial pathogens through myeloid differentiation (MyD88) dependent primary response gene or MyD88-independent transduction pathway (Akashi-Takamura and Miyake 2006). Each member of the TLR family has its own ligand for different pathogens, which helps in inducing a danger signal when pathogen invades the host and results in the activation of NF-κB and subsequent induction of signal transduction cascade. TLR2 can deliver co-stimulatory T cell signals for cell expansion and can induce proliferation of regulatory T cells (Mercier, Cottalorda et al. 2009); its signaling favors Th17 cell expansion. It was shown in the rat model of experimental autoimmune neuritis (EAN) that TLR2 was expressed in inflamed nervous tissue and NFκB was increased in activated T cells and macrophages (Zhang, Zhang et al. 2009). In EAN, potential endogenous TLR ligands generated following tissue damage or inflammation may also activate their TLRs and thereby play roles in the pathological progress of the disease. TLR2+, CD14+, and Hsp70+ cell accumulation was detected and positively correlated with neurologic disease severity in sciatic nerves of EAN rats, suggesting the involvement of innate immunity in the effector phase of disease (Zhang, Zhang et al. 2009). From our study, we hypothesize that the increased/enhanced level of TLR2 might deliver potent co-stimulatory signals to antigen activated T cells which secrete increased amount of several chemokine and pro-inflammatory cytokines such as TNF-α responsible for demyelination. However, no changes in expression were observed at genotype level.

6.5. Study of IL-8 expression and their gene polymorphism in GBS

Expression of IL-8 and its role in disease pathogenesis of GBS is still remains unknown. An earlier study had shown significantly higher IL-8 secretion from PBMCs

of MS patients compared to controls (Lund, Ashikian et al. 2004). In present study, we found the level of IL-8 significantly higher (112.55±13.96 vs 34.79±4.37; $p \leq 0.001$) in serum of GBS patients compared to healthy controls. Activation of TLRs enhances the transcription of several pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α via NF κB (Chow, Young et al. 1999; Kopp and Medzhitov 1999; Akira, Takeda et al. 2001). IL-1β in its turn stimulates expression of IL-8 (Zwerina, Redlich et al. 2005). It is also reported that IL-8 and CXCL1 production by human astrocytes at both the RNA and protein levels can be induced by IL-1β in MS patients (Omari, John et al. 2005). In another study, IL-1β and IL-17 induced the production of IL-8 in endothelial and parenchymal cell indicating an indirect role in polymorphonuclear neutrophil recruitment (Fossiez, Djossou et al. 1996). In another study, the level of IL-1β was found significantly higher in chicken model of GBS (Nyati, Prasad et al. 2012). Elevated expression of TLR2 in GBS as observed in our study indicates that TLR2 activation enhances the transcription of several pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α via NF κB, and IL-1β in turn stimulates the expression of IL-8 that may have indirect role in recruitment and activation of neutrophil at neuronal sites in GBS. However, no changes in expression were observed at genotype level.

Besides the IL-8 expression study in GBS, we also looked for the polymorphism of IL-8 gene in GBS but we did not observe the any association of IL-8-251A/T polymorphism (heterozygous genotype (A/T); 57.14% vs 44%, $p<0.0698$; OR, 1.69; 95% CI, 0.97-2.94 & homozygous mutant genotype (T/T); 25.71% vs 27%, $p<0.8748$; OR, 0.93; 95% CI, 0.50-1.74) with our GBS patients. Similar observation in another study reported that functional genetic variation in IL-8 did not play a major role in SLE susceptibility in the Spanish population (Sanchez, Sabio et al. 2006). In another study, the genetic polymorphisms of the CXCL8 gene were not associated with systemic

sclerosis. On contrary to these studies, association of IL-8-251A/T polymorphism with MS was reported in Iranian patients (Kamali-Sarvestani, Nikseresht et al. 2006).

In view of above observations on TLR-2, the present study indicates that TLR2 polymorphisms and their enhanced expressions were associated with GBS susceptibility in Indian patients. In addition, enhanced IL-8 production without its gene polymorphism was also observed in GBS patients. However, interaction between TLR2 activation and expression of IL-8 in absence of IL-8 gene polymorphisms, and their exact role in disease pathogenesis calls for further study. Similar studies in different ethnic populations are required to clarify the role of TLR2 and IL-8 in GBS patients.

6.6 Association of NLRs (NOD1& NOD2) Polymorphisms in GBS and GBS Subtypes:

NOD1 and NOD2 gene polymorphisms have been implicated in various autoimmune and inflammatory diseases (Economou, Trikalinos et al. 2004; Ghandil, Chelala et al. 2005; Hysi, Kabesch et al. 2005; Abraham and Cho 2006; Freire, Cardoso et al. 2014). However, their role in GBS patients largely remains unknown. Hence, we investigated NOD1 Glu266Lys and NOD2 (Arg702Trp and Gly908Arg) gene polymorphisms among patients with GBS. In our study, the variant allele frequency of NOD1 266Lys was higher among patients with GBS than controls (64.29% vs. 52.5%, p=0.016; OR 1.63; 95% CI, 1.10–2.42). Further, the frequency of NOD2 variant allele (702Trp and 908Arg) was higher among patients with GBS than controls (26.67% vs. 7.5%; p= 0.001; OR, 4.48; 95% CI, 2.44–8.24 and 30% vs. 21%; p=0.038 OR, 1.61; 95% CI, 1.03–2.53). The reported rate of NOD2 702Trp & 908Arg variant allele in Spanish population was 4% and 0%, and in Italian population 1.75% and 0.75 % respectively suggesting non co-segregation (Canto, Ricart et al. 2007), (Granzotto, Fabbro et al. 2007).

In our present study, a positive association of NOD1 Glu266Lys (homozygous mutant genotype p=0.013; OR, 2.90; 95% CI, 1.25-6.70) and NOD2 Arg702Trp (heterozygous genotype; p=0.001, OR=5.78, 95% CI=2.95-11.31) and Gly908Arg (heterozygous genotype p=0.010, OR=2.07; 95% CI=1.19-3.62) polymorphisms was observed among patients with GBS. It has been well documented that NOD2 mutations in Crohn's disease potentiate the activity of NF-κB and IL-1β processing, thereby enhancing the inflammation (Maeda, Hsu et al. 2005). NOD2 seems to be a negative regulator of TLR-2 mediated T helper cell type 1 responses and modulates ileal expression of antimicrobial peptides such as alpha-defensins and the expression of proinflammatory cytokines and chemokines in the intestinal mucosa (Watanabe, Kitani et al. 2004; Maeda, Hsu et al. 2005; Lakatos, Fischer et al. 2006). Our findings suggest that polymorphisms in NOD gene might lead to increased fold of oligomerization, and subsequent sensing of microbial ligands to activate the NF-κB. This may play an important role in inflammatory response and may be involved in the secretion of proinflammatory cytokines like IL-1β, IL-6 and TNF-α. The enhanced expression of TNF-α has been reported in GBS patients (Nyati, Prasad et al. 2010). One study showed that NOD2 was essential in the detection of bacterial muramyl dipeptide and capable of activating the adaptive immune system by acting as an adjuvant receptor for antibody production, either directly or by enhancing the production of α-defensins or other immunostimulatory molecules (Kobayashi, Chamaillard et al. 2005).

Our data support those studies that reported association of NOD2 polymorphism with other inflammatory diseases. NOD2 (Arg702Trp and Gly908Arg) polymorphisms were identified as risk factor for Crohn's disease (Brand, Staudinger et al. 2005) and further NOD2 variants being involved in pathogenesis of IBD (Schnitzler, Brand et al. 2006). In the present study, we found the significant association of variants of NOD2 gene

with GBS. On the contrary, NOD2 gene variants were not found to be involved in determining susceptibility to multiple sclerosis (Sawcer, Hellenthal et al. 2011). However, in one Indian study common NOD2 mutations were absent in patients with Crohn's disease (Pugazhendhi, Amte et al. 2008). In another study, individuals with homozygous or compound heterozygous for the common NOD2 mutations have about 20-fold increased risk for development of Crohn's disease whereas heterozygous subjects have only about two fold increased risk (Economou, Trikalinos et al. 2004). The association of NOD2 (Arg702Trp and Gly908Arg) polymorphisms with GBS in the present study suggests that variant of NOD2 gene increases the risk for disease development. NOD2 is expressed widely in macrophages, dendritic cells, paneth cells, keratinocytes, epithelial cells of the intestine and lung (Shigeoka, Kambo et al. 2010). Several studies indicate that cell mediated immune response to peripheral nerve may play an important role in the pathogenesis of GBS (Nyati and Prasad 2014). The inflammatory infiltrates in GBS are mainly composed of lymphocytes and macrophages, where this gene is constitutively expressed during acute phase of the disease (Wanschitz, Maier et al. 2003). Macrophages are the principal source of cytokines including TNF-α, a major pro-inflammatory cytokine that could be involved in early breakdown of the blood nerve barrier, up-regulation of endothelial adhesion molecules, leukocyte attraction to nerve tissue and myelin damage. Enhanced expression of TNF-α along with polymorphism has been reported in patients with GBS (Nyati and Prasad 2014). Therefore, it might be possible that mutations in NOD2 gene influence directly or indirectly TNF-α production that may lead to inflammation. Some other studies revealed that mutations in NOD2 gene were unable to activate the NF-κB signal transduction pathway upon binding of NOD2 ligands (Ogura, Inohara et al.

2001). NOD2 mutations were not found among patients with ulcerative colitis (Freire, Cardoso et al. 2014).

To the best of our knowledge, this is the first study suggesting that NOD1 Lys/Lys homozygote was significantly associated with AMAN (p=0.008) and AIDP (p=0.024) subtypes (Table 4). In addition, 702 Arg/Trp heterozygote was significantly associated with AMAN (p=0.001), AMSAN (p=0.029) and AIDP (p=0.001) subtypes while 908 Gly/Arg heterozygote showed association with AIDP (p=0.003) subtype only (Table 6). Association of TLR4 Gly299Gly homozygous genotype with AMAN subtype of GBS had been reported in an earlier study (Nyati, Prasad et al. 2009).

In the present study, NOD1 homozygous genotype (Lys/Lys) was significantly associated with GBS and it also increased the risk for AMAN and AIDP subtypes of GBS. In addition, there was significant association of NOD2 (702Arg/Trp and 908Gly/Arg) heterozygous genotypes with GBS, and 702 Arg/Trp heterozygous genotype increased the risk for all GBS subtypes; however, AIDP was associated with heterozygous genotype Gly908Arg.

6.7. Study of IL-17 gene polymorphism and their expression in GBS

In the present study, we investigated the association of IL-17 polymorphism (IL-17F Glu126Gly and His161Arg) with GBS and its relative expression. We also investigated the expression of ICAM-1 and its gene polymorphism (241Gly/Arg) in patients with GBS.

IL-17 is a proinflammatory cytokine produced by activated T cell, plays an essential role in immune host defenses and is involved in a number of autoimmune and inflammatory diseases including GBS. The magnitude of the association of polymorphisms with autoimmune disease varies depending on genetics, demographics

and environmental factors. Many association studies reported that IL-17 polymorphisms predisposed to autoimmune and inflammatory diseases (Kawaguchi, Takahashi et al. 2006; Arisawa, Tahara et al. 2008; Jang, Nam et al. 2008; Seiderer, Elben et al. 2008). However, some polymorphisms of IL-17F (Glu126Gly and His161Arg) might not be significantly associated with autoimmune disease like rheumatoid arthritis (Paradowska-Gorycka, Wojtecka-Lukasik et al. 2010). However, there is no data regarding IL-17 polymorphism with GBS till date.

The importance of IL-17 polymorphism in GBS is still largely unknown. In our study, the variant allele frequency of IL-17F (Glu126Gly) was higher in GBS than controls (71.43 % vs 53.5%, p=0.0002; OR, 2.17; 95% CI, 1.44-3.26) and allelic frequency of (IL-17F His161Arg) in GBS and control was 31.91% and 32.5% respectively with no difference between the groups. The allele frequency of His161Arg variants was lower for the Polish subjects (3.8%) than for the populations from Canada, United States of America, United Kingdom, China, Japan and Nigeria (Paradowska-Gorycka, Wojtecka-Lukasik et al. 2010). Moreover, in populations from Nigeria and Japan only wild-type allele for Glu126Gly was detected. In another study, the variant allelic frequency of (IL-17F His161Arg) was 4.8% in Munich population (Seiderer, Elben et al. 2008).

The data regarding role of IL-17F gene polymorphism in inflammatory demyelinating diseases including GBS is still not available. There is a single study that reported the enhanced level of IL-17 in cerebral spinal fluid of patients with GBS (Li, Yu et al. 2012).

We conducted the present study in our population and a positive association of IL-17 polymorphism (Glu126Gly) was observed with GBS. We observed significant association of IL-17F Glu126Gly polymorphism with GBS. Homozygous polymorphic (Gly/Gly; $p<0.0001$; OR=3.82, 95% CI= 2.09-6.99) was significantly associated with

susceptibility to GBS. Similarly in Korean population heterozygote genotype was associated with susceptibility to IBD (Jang, Nam et al. 2008). We did not found any association of IL-17F His161Arg gene polymorphism with GBS neither in homozygous mutant genotype (Arg/Arg; p<0.1018; OR=4.04, 95% CI= 0.83-19.52) nor in heterozygous genotype (His/Arg; p<0.0922; OR=0.60, 95% CI= 0.34-1.05). Our findings are in agreement with the previous study where His161Arg variant was associated neither with IBD susceptibility nor with Crohn's disease (CD) severity (Seiderer, Elben et al. 2008). However, in contrast another study showed correlation between IBD and IL-17F His161Arg gene polymorphism (Arisawa, Tahara et al. 2008).

Our hypothesis suggests that polymorphisms in the IL-17 gene may cause redundant production of some proinflammatory cytokines, such as IL-1β and TNF-α which can mediate inflammatory pathology in many autoimmune diseases, including GBS. In addition, in autoimmune diseases, TNF-α is responsible for the inflammatory and protective aspects, and IL-1β is responsible for the destructive processes (Paradowska-Gorycka, Grzybowska-Kowalczyk et al. 2010). There are reports which show that increased level of TNF-α contributes to the pathogenesis of immune mediated demyelinating neuropathies and axonal degeneration by inducing damage to myelin (Redford, Hall et al. 1995; Zhu, Mix et al. 1998).

Besides the association of IL-17 with GBS, we also observed the enhanced expression of IL-17 in patient with GBS. The data regarding expression of IL-17 in GBS is very limited. So far, one study showed significantly elevated IL-17 expression in cerebral spinal fluid of patients during GBS (Li, Yu et al. 2012). An increased expression of IL-17 had been shown in Crohn's disease and chronic inflammatory demyelinating polyradiculoneuropathy (Seiderer, Elben et al. 2008; Chi, Xu et al. 2010). In the present

study, we found elevated IL-17 level ($p \leq 0.001$) in serum of patients with GBS (42.00±5.33) when compared to healthy controls (6.0±0.768). In addition to this, elevated mRNA level was also found in GBS patients (3.66±0.792) as compared to healthy controls (2.05±0.299). IL-17 is produced by Th17 cells that stimulates the production of proinflammatory cytokines such as IL-1β and IL-6 from monocytes and therefore further amplifies the inflammatory cascade (Chabaud, Aarvak et al. 2001). IL-17 producing Th17 cells are associated with immunopathology in autoimmune diseases. Recently, the role of Th17 cells has been shown and correlated with the pathogenesis of GBS. IL17, a signature cytokine produce by Th17 cells, may have synergistic effects with proinflammatory cytokines such as TNF-α, IFNγ, and IL1β. IFNγ can prevent IL23 triggered expansion of Th17 cells (Harrington, Hatton et al. 2005); therefore, IFNγ sometimes plays a protective role in GBS/EAN which might be due to its ability to suppress Th17 cells development. Moreover, IFNγ increases the over-expression of which in turn leads to a robust reduction of IL-17 generation (Mathur, Chang et al. 2006) .

Further, IL-17 was detected in sciatic nerves of EAN, and the accumulation of IL-17 was correlated with the severity of neurological signs (Zhang, Zhang et al. 2009), which suggests a pathological contribution of IL17 to the development of EAN. The frequency of Th17 cells in CSF and the level of IL-17 in plasma were significantly higher in active chronic inflammatory demyelinating polyradiculoneuropathy (CIDP) (Chi, Xu et al. 2010). Further the levels of IL17 and IL22 in CSF were correlated with GBS severity (Li, Yu et al. 2012). TIM-3 pathway influenced IL17 release and Th17 and Th1 differentiation along with their cytokine expressions during the pathogenesis of GBS (Liang, Wang et al. 2012). Pelidou et al., 2000 reported enhanced acute phase of EAN in Lewis rats by intranasal administration of recombinant mouse IL17, along

with increased infiltration of inflammatory cells into the sciatic nerves and severe demyelination (Pelidou, Zou et al. 2000). Collectively, these findings indicate thatTh17 cells and their effector cytokines might be involved in the pathogenesis of GBS and EAN. Although the mechanism of action of IL17 in GBS and EAN remains unclear, it mainly acts as a proinflammatory cytokine that upregulates the expression of inflammatory genes including proinflammatory chemokine, hematopoietic cytokines, acute phase response genes, and antimicrobial substances(Shen and Gaffen 2008) in neutrophils, macrophages, and endothelial cells (Zepp, Wu et al. 2011). However, no changes in expression were observed at genotype level.

6.8. Studies of ICAM-1 gene polymorphism and their expression in GBS

In present study, we investigated the ICAM-1 polymorphism in patient with GBS and observed the significant association of ICAM-1 (241Gly/Arg) heterozygous genotype ($p<0.0001$; OR=4.05, 95% CI= 2.26-7.26) in patient with GBS when compared to the healthy controls. ICAM-1, adhesion molecules belong to the cytokine inducible immunoglobulin-like (Ig-like) superfamily and ICAM-1 dependent engagement and signalling in lymphocyte, blood brain barrier endothelial cell, microglia, and astrocytes, which can activate effector mechanism that promotes the progression of neuro-inflammmation(Lee and Benveniste 1999). Polymorphisms of ICAM-1 G241R are common genetic variation in populations and associated with several autoimmune diseases, such as multiple sclerosis, Crohn's disease (Killestein, Schrijver et al. 2000) whereas other studies failed to find a significant contribution of the 721A allelic variant in inflammatory diseases (Wenzel, Ernst et al. 1996; Taylor, Tang et al. 2002).

The data regarding role of ICAM-1 gene polymorphism in inflammatory demyelinating diseases including GBS is still not available. No study is available in literature on the role of ICAM-1 in development of GBS.

In our GBS group, a significant association with the 721A allele frequency of ICAM-1 polymorphism (p=0.0001; OR=2.42, 95% CI= 1.53-3.84), was found. Similar result was observed in the study of Macchioni et al., reported association with the 721A (R241) allele in Italian RA patients (Macchioni, Boiardi et al. 2000) whereas another study of Korean RA patients this polymorphism was not identified (Lee, Kim et al. 2004). These differences between populations can be explained by the genetic background that influences the inter-population variability of the population. Thus it is evident from our finding that ICAM-1 polymorphism might have significant contribution in GBS.

Besides the ICAM-1 polymorphism study in GBS, we also looked for the expression of ICAM-1 in GBS and we observed that the level of ICAM-1 was significantly enhanced ($p \leq 0.001$) in sera of the GBS patients (5.33±0.661) when compared to healthy control (2.25±0.288). The enhanced mRNA level (p=0.012) was also observed in GBS patients (2.04±0.261) compared to healthy control (1.18±0.202). Similar result was reported in another association study in healthy subjects where a significant effect of ICAM1 (721G>A/241Gly>Arg) polymorphisms with serum sICAM-1 levels but the association was weak (Ponthieux, Lambert et al. 2003).

Increased expression of ICAM-1 has been shown on endothelial cell, microglia, and astrocytes in active MS and EAE lesion (Mycko, Kwinkowski et al. 1998; Carrithers, Visintin et al. 2000). Free cICAM-1 contains almost all extracellular domains of membrane ICAM-1, as well as the ability to bind specifically to the adhesion receptor LFA-1 and to promote endothelial signaling to lymphocytes by engaging LFA-1 or to facilitate trans-endothelial migration of leukocytes into inflammatory sites. Conversely, in view of its ability to specifically bind to LFA-1, cICAM-1 may play an inhibitory role in cell to cell interaction by competing with membrane-associated ICAM-1 for

LFA-1. cICAM-1 may provide insight into the pathophysiology of inflammatory cell involvement in MS, particularly the mechanisms of lymphocyte homing and cellular trafficking across the blood-brain barrier (Constantin, Piccio et al. 1999; Nejentsev, Laaksonen et al. 2003).The elevated levels of ICAM-1 and TNF-α in serum of patient with GBS clearly demonstrate that ICAM-1 plays a central role in the development of demyelinating disease. TNF-α as well as IL-1β is able to up-regulate ICAM-1 on schwann cell (Lisak and Bealmear 1997). Elevated level of TNF-α and IFN-γ during acute phase of disease have been reported in patient with GBS (Prasad, Nyati et al. 2010). . In the present study, our results suggest that up-regulation of adhesion molecules on Schwann cells during the inflammation of disease inducing damage to myelin, may have a role in the pathogenesis of inflammation in the peripheral nerve.

Another explanation for the supports of our study is that IL-17 facilitates T cell activation and infiltration into tissues by upregulating the expression of ICAM-1 and amplifies the immune response by inducing the production of IL-6, prostaglandin E2, granulocyte-macrophage colony stimulating factor, and granulocyte colony stimulating factor (Cai, Gommoll et al. 1998; Albanesi, Cavani et al. 1999; Schwarzenberger, Huang et al. 2000). However, in another in-vitro study, intervertebral disc cells exposed to IL-17, IFN-γ or TNF-α showed a remarkable increase in inflammatory mediator release and ICAM-1 expression. Addition of IFN-γ or TNF-α to IL-17 demonstrated a synergistic increase in inflammatory mediator release. Macrophages and synoviocytes have increased inflammatory phenotypes in response to IL-17 alone or IL-17 combined with IFN-γ or TNF-α (Fossiez, Djossou et al. 1996; Jovanovic, Di Battista et al. 1998) and marked increase in ICAM-1 expression.

Increased expression of adhesion molecules on brain endothelial cells can lead to altered BBB permeability facilitating the entry of some immune cells in the brain

(Zameer and Hoffman 2001). Elevated level of TNF-α and IFN-γ during acute phase of disease have been reported in patient with GBS (Prasad, Nyati et al. 2010). The findings of this work reveal a significant role for IL-17 in upregulating inflammatory mediator release and ICAM-1 and suggest that IFN-γ and TNF-α act synergistically to elevate ICAM-1 expression in the presence of IL-17. However, no changes in expression were observed at genotype level.

In present study in the view of findings, we presume several potential mechanisms of the origin in plasma of these cytokines: (i) the elevation of IL-17 and ICAM-1 in plasma might be related to the local inflammation and systemic release of cytokines in peripheral nerves that causes demyelination and axon degeneration and (ii) reactive Th17 cells in peripheral blood may cross the BBB/BNB and secret relative cytokines that synergistically can induce ICAM1 expression. This indicates that the elevation of IL-17 and ICAM-1 in serum and development of disease have some correlation that may coordinate in the pathogenesis of the disease. Besides this, IL-17 and ICAM-1 could be a genetic marker to GBS susceptibility which, however, should be confirmed in future studies with a large sample size

Chapter-VII

Summary and Conclusions

7.1. Summary

Guillain Barré syndrome (GBS) is an immune-mediated inflammatory disease mainly affecting the myelin and axons of peripheral nerves with heterogeneous pathological features. GBS is often triggered by an aberrant immune response towards an infectious pathogen and is preceded by an infectious illness usually 1-3 weeks before the onset of neurological symptoms. Epidemiological studies linked it with *Campylobacter jejuni*, Cytomegalovirus, Epstein Barr virus and *Mycoplasma pneumoniae*. The mechanisms involved in immunopathogenesis of GBS are still unclear. The hypothesis put forward for the immunopathogenesis of GBS points to molecular mimicry between lipopolysaccharide (LPS) and ganglioside like epitopes in host nerve cells, which leads to cross reactivity of immune response after the infection. Besides microbial factors, host susceptibility may also play an important role in the etiology of GBS, because not all infected individuals develop this disorder. It is estimated that only 1:1000 people develop GBS after *C. jejuni* enteritis, thus highlighting the role of host genetic factors. However, studies demonstrating for identifying the potential host factors that may impart susceptibility to GBS are least understood. In present study, we investigated the polymorphism (TLR-2, IL-8, NLRs, IL-17 and ICAM-1) and expression (TLR-2, IL-8, IL-17 and ICAM-1) of host factor molecules to define and understand the role in GBS.

Keeping the above observations in mind, the present study was planned. The major features of the present study are summarized as follows:

1. One hundred five patients with GBS (male/female, 84/21; mean age ± SD, 30.20±10.98 years) were enrolled for the study.
2. Age and sex matched 100 individuals (male/female, 78/22; mean age ± SD, 28.12 ± 16.97 years) without any history of apparent infectious illnesses within the last four weeks, were included as healthy control group.

3. History of preceding infection was found in 51 (48.6%) patients. Either alone or in combination, 21(20%) patients had respiratory infection or 10 (9.5%) patients had diarrhoea prior to the onset of GBS.
4. 25 (23.8%) of the 105 GBS cases had infection with *C.jejuni* and 10 (9.52%) of GBS patients had *M.pneumoniae* infection and 2(1.9%) in HC.
5. EBV-VCA infection was detected in 13 (12.4%) of GBS patients however, 15 (14.28%) of GBS patients had CMV infection.
6. Genotypic distribution of TLR2 Arg753Gln and Arg677Trp polymorphism demonstrated increased risk of GBS patient for heterozygous genotypes Arg/Gln (34.3% vs 6%) and Arg/Trp (46.67% vs 1%), when compared with healthy control.
7. Frequency of wild homozygous genotypes was observed at 65.7% vs 94% for TLR2Arg753Gln and 53.33% vs 99% for Arg677Trp polymorphisms in a patient and control wise manner.
8. Patients with GBS showed significantly higher frequency in comparison to controls for allele 753Gln (17.14% vs 6%) and allele 677Trp (23.34% vs 0.5%).
9. The genotypic frequencies of both homozygous (Lys/Lys) and heterozygous (Glu/Lys) variants of NOD1 were higher in GBS patients than healthy controls.
10. Homozygous variant showed increased risk to GBS (40% vs 29%) and heterozygous variant also showed a trend towards risk to GBS than healthy controls (48.57% vs 47%).
11. The frequency of variant allele (NOD1 266Lys) was also higher among GBS patients (64.29%) than healthy controls (52.5%) and showed 1.63 fold risk association with GBS.
12. NOD1 variant allele (266Lys) showed increased susceptibility to AMAN and AIDP subtypes.

13. Increased risks for the development of GBS were revealed with heterozygote genotypes of NOD2, Arg702Trp and Gly908Arg.

14. NOD2 alleles i.e., 702Trp & 908Arg also showed significant risk association with GBS.

15. Significant protective association for AMAN, AMSAN and AIDP subtypes were found with NOD2 Arg702Trp heterozygote genotype. On the contrary, predisposing association was observed with NOD2 702Trp allele for AMAN, AMSAN and AIDP subtypes.

16. Heterozygote (Gly/Arg) genotype of NOD2 Gly908Arg revealed protective association and variant allele (Arg) of Gly908Arg polymorphism showed risk associations with AIDP subtype.

17. The occurrence of Arg702-Gly908 haplotype comprising of wild genotype (Arg/Gly) among control and patients was 79% and 68.5% respectively.

18. Significant risk with Trp702-Arg908 haplotype and protective association with Arg702-Arg908 haplotype for GBS were observed.

19. The LD analysis showed five significant associations in this study and it is evident from the analysis that significant risk was associated with GBS in the presence of mutant alleles.

20. Pair-wise LD showed risk for GBS in the presence of Glu (Glu266Lys)-Lys(Glu266Lys), Glu (Glu266Lys)-Trp (Arg702Trp), Glu (Glu266Lys)-Arg (Gly908Arg), Trp (Arg702Trp)-Arg (Gly908Arg) and Trp (Arg702Trp)-Arg (Gly908Arg) alleles among the studied individuals.

21. Only homozygous variant (Gly/Gly) of IL-17F (Glu126Gly) showed increased susceptibility to GBS (53.34% vs. 23%, $p \leq 0.0001$). Further, G allele also showed increased risk to GBS (71.43% vs. 53.5%, $p=0.0002$).

22. IL-17F His161Arg gene polymorphism did not show any association with GBS susceptibility.
23. The logistic regression analysis revealed no significant association of IL-8-251A/T polymorphism (polymorphic and heterozygous) with GBS (p=0.8748 and p=0.0698).
24. The logistic regression analysis revealed that heterozygous genotype (G/A) of ICAM-1 Gly241Argpolymorphism had association only with GBS (p<0.0001) while not any homozygous polymorphic (AA) genotype was detected either in patients or in controls and frequency of homozygous wild type was (30.47% vs 64%) between patients and controls.
25. In expression studies, mRNA expression of TLR 2 was increased significantly in GBS patients than controls (1.727±0.412 vs 0.319±0.113).
26. Enhanced expression of TLR-2 was observed in GBS patients (fold change-1.112) compared to healthy controls (fold change-1) by immunoblotting.
27. mRNA expression of IL-8 was increased significantly in GBS patients than controls (3.47±0.14 vs 1.27±0.07).
28. Level of IL-8 was elevated in sera of GBS Patients than healthy controls (112.55±13.96 vs 34.79±4.373; p≤ 0.001).
29. The mRNA level of IL-17 was found increased trend (p=0.066, 3.66±0.792 vs 2.05±0.299) in patient than control but statically not significant.
30. The level of IL-17 in sera was elevated in GBS patients when compared with healthy control (42.00±5.33Vs 6.0±0.77; p≤ 0.001).
31. In ICAM-1, mRNA expression (p=0.012, 2.04±0.261 vs 1.18±0.202) increased in patient compared to control.
32. The elevated level was observed for ICAM-1(5.33±0.661 vs 2.25±0.288) in serum, when compared with HC.

7.2. Conclusion

1. Elevated expression of TLR2 in GBS as observed in our study indicates that TLR2 activation enhances the transcription of several pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α via NF κB, and IL-1β in turn stimulates the expression of IL-8 that may have indirect role in recruitment and activation of neutrophil at neuronal sites in GBS.

2. The increased/enhanced level of TLR2 might deliver potent co-stimulatory signals to antigen activated T cells which secrete increased amount of several chemokine and pro-inflammatory cytokines such as TNF-α responsible for demyelination.

3. TLR2 polymorphisms (Arg677Trp & Arg753Gln) could be a genetic marker to GBS susceptibility.

4. NOD1 homozygous genotype (Lys/Lys) was significantly associated with GBS and it also increased the risk for AMAN and AIDP subtypes of GBS.

5. There was significant association of NOD2 (702Arg/Trp and 908Gly/Arg) heterozygous genotypes with GBS, and 702 Arg/Trp heterozygous genotype increased the risk for all GBS subtypes; however, AIDP was associated with heterozygous genotype Gly908Arg.

6. The elevation of IL-17 and ICAM-1 in plasma might be related to the local inflammation and systemic release of cytokines spinal roots and peripheral nerves that causes demyelination and axon degeneration.

7. Reactive Th17 in peripheral blood may cross the BBB/BNB and secret relative cytokines that synergistically can induce ICAM1 expression. This indicates that

the elevation of IL-17 and ICAM-1 in serum and development of disease have some correlation that may coordinate in the pathogenesis of the disease.

8. IL-17 and ICAM-1 polymorphisms could be a genetic marker to GBS susceptibility.

9. *C. jejuni* is one of a potential infectious agents implicated in triggering GBS through antigen mimicry. Infection generates immune response against the agent and induces neuronal inflammation through production of cytokines.

10. The present study of Host factor associated GBS may present a good observation for understanding the pathogenesis and immune response during development of the disease.

7.3. Future Directions

1. Studies on animal models are required to provide direct insights into putative immunopathogenesis of the disease associated with host factors.

2. Multi-centre studies are needed to determine the magnitude of GBS and its link to host factors in addition to mentioned in study.

3. Role of others adhesion molecules, chemokines and matrix metalloproteinases (MMPs) need to be studied to correlate with neurophysiologic changes, disease severity and outcome.

4. Study to elucidate the intricate interaction of inflammatory molecules such as MMPs and its inhibitors, and different other cytokines, especially IL-23. Although this cytokine play important role in various autoimmune diseases, no such studies are available in relation to GBS. Such studies are likely to improve the strategies of management for neuro-inflammatory disease like GBS.

5. Study with more extensive sampling of the peripheral and central nervous system, including spinal roots and motor nerve terminals to understand the exact pathological mechanism of the disease.

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Annexures

Annexure I

Patient Proforma Sheet

Name of patient .....CR N.....

Age/Sex ..... Address.....

Occupation .....

Dr.I/C .....

Ward/OPD .....

Present illness.....

Onset

- Girdle pains
- Proximal weakness
- Gen lower limb weakness
- Acroparesthesia
- Numbness
- Generalised weakness

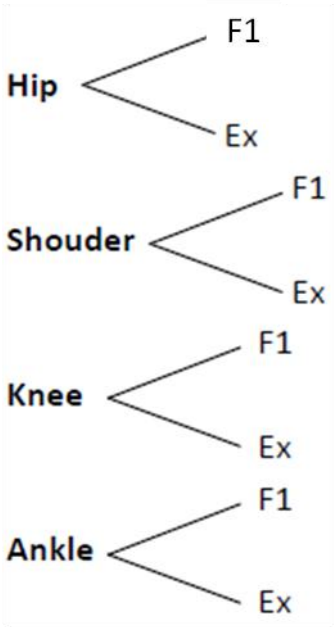
Progression: For how many days-

Recovery: After how many days-

MRC grade for Muscle Power:

At maximum disability (Days.....)

At maximum recovery (Days .....)



Family history of similar illness (if so details).....

Personal habit: .....

History of diarrhea/dysentery.....

History of respiratory illness .....

History of any infective episode .....

Treatment (Antibiotic if any).....

Clinical diagnosis.....

NCV/EMG.....

Final diagnosis (AMAN/AMSAN/AIDP):.....

Investigation:

Blood

Antibodies against Campylobacter (IgG,IgM).....

Isolation of DNA.....

Isolation of RNA.....

Isolation of lymphocytes.....

ELISA.....

RT-PCR.....

Signature of Investigator

Date:

Annexure II

SOLUTIONS FOR DNA EXTRACTION

Solution I

Glucose 50 mM

Tris Cl (pH 8.0) 25 mM

EDTA (pH 8.0) 10 mM

Autoclaved and stored at 4ºC

Solution II

NaOH 0.2 N
SDS 1%

Phenol

Tris saturated phenol (pH 7.5), stored in dark at 4ºC

Chloroform: Isoamyl alcohol (24:1)

Chloroform 24 ml
Isoamyl alcohol 1 ml

Ethanol (70%)

95% Ethanol (Merck) 75 ml
Distilled water 25 ml

Tris EDTA (TE buffer)

1M Tris buffer (pH 8.0) 5 ml
0.5M EDTA 1 ml

Distilled water 494 ml

SOLUTIONS FOR AGAROSE GEL ELECTROPHORESIS

10X Tris Borate EDTA (TBE)

Tris Base 108 gm
Boric acid 55 gm
0.5M EDTA (pH 8.0) 40 ml
Volume made upto 1000 ml with H2O. Working concentration was 0.5X

Ethidium bromide (EtBr)

EtBr 10 mg
Distilled water 1 ml
Solution stored in dark at 4ºC. Working concentration was 0.5 µg/ml

Loading buffer

Bromophenol blue 0.25%
Xylene cyanol 0.25%
Glycerol 30%

SOLUTIONS FOR EXTRACTION OF OUTER MEMBRANE PROTEINS

1M Phosphate-buffered Saline (PBS pH 7.4)

Na2HPO4 1.44 gm

KH2PO4 0.24 gm

NaCl 8.0 gm

KCL 0.2 gm

Dissolved in 800 ml distilled water, pH adjusted and volume made upto 1000 ml. Autoclaved at 121ºC for 15 min and stored at 4ºC.

10 mM PMSF

1.75mg in 1ml Isopropanol, aliquoted and stored at 20ºC

1.67% Sodium lauroyl sarcosine

N-lauroyl-sarcosine 1.67 gm

Tris-HCl (pH 7.6) 11.1 mM

N-lauroyl-sarcosine dissolved in 100 ml 11.1mM Tris-HCl

Publications

1. **Kharwar NK**, Prasad KN, Paliwal VK, Modi DR. Association of NOD-1and NOD-2 polymorphism with Guillain-Barr´e syndrome in Northern Indian population. J. Neurol Sci. 2016; 363:57-62.
2. **Kharwar NK**, Prasad KN, Singh K, Paliwal VK, Modi DR. Polymorphism of IL-17 and ICAM-1 and their expression in Guillain-Barr´e syndrome. Int. J. of Neuro Sci. 2016; PMID 27595159.
3. **Kharwar NK**, Prasad KN, Rai M, Paliwal VK, Modi DR. Association of TLR-2and IL-8 polymorphism and their expression in Guillain-Barr´e syndrome. Int. J. of Pharma. Sci and Res.2016;7(9): 3695-3702.
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Association of NOD1 and NOD2 polymorphisms with Guillain-Barré syndrome in Northern Indian population

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ABSTRACT

Background: Nucleotide oligomerization domain (NOD) proteins are cytosolic pattern recognition receptors that respond to bacterial substrate and induce NF-κB activation in host. Association of NOD polymorphisms have been studied in many autoimmune disorders, however its role in Guillain-Barré syndrome (GBS) remains unknown. We have investigated NOD1 Glu266Lys and NOD2 (Arg702Trp and Gly908Arg) gene polymorphisms among patients with GBS.

Materials and method: Polymorphisms in NOD-1 (Glu266Lys) and NOD-2 (Arg702Trp and Gly908Arg) genes were studied using polymerase chain reaction-restriction fragment length polymorphism in 105 patients with GBS and 100 healthy controls.

Results: Homozygous genotype (Lys/Lys) of NOD1 was significantly associated with GBS ($p = 0.013$); and its subtypes viz. acute motor axonal neuropathy (AMAN) and acute inflammatory demyelinating polyneuropathy (AIDP) ($p = 0.008$ and $p = 0.024$ respectively) than controls. In NOD2 (Arg702Trp and Gly908Arg) polymorphisms, only heterozygous genotype (Arg/Trp and Gly/Arg) showed significant association with GBS ($p = 0.001$ and $p = 0.01$ respectively); subtypes AMAN, acute motor-sensory axonal neuropathy (AMSAN) and AIDP showed association with heterozygote Arg702Trp ($p = 0.001$; $p = 0.029$ and $p = 0.001$ respectively) whereas only AIDP was associated with heterozygote genotype Gly908Arg ($p = 0.003$).

Conclusion: NOD1 (Glu266Lys) and NOD2 (Arg702Trp and Gly908Arg) polymorphisms were associated with an increased susceptibility to GBS. These polymorphisms could be genetic marker to GBS susceptibility.

1. Introduction

Guillain-Barré syndrome (GBS) is the most common acute inflammatory neuropathy of peripheral nervous system (PNS). Clinically and electrophysiologically GBS is divided into three subtypes: acute inflammatory demyelinating polyneuropathy (AIDP), acute motor axonal neuropathy (AMAN), and acute motor and sensory axonal neuropathy (AMSAN) [1]. *Campylobacter jejuni* (*C. jejuni*) is considered to be the most common agent leading to GBS. The disease is more often triggered by immune response against an infectious pathogen, which may lead to production of high titres of antibodies [2]. These antibodies target nerve antigen through a molecular mimicry mechanism [3,4]. Cross reactive immune response is generated due to the molecular mimicry between *C. jejuni* cell wall antigens and ganglioside like epitope in host nerve tissues [5]. It is estimated that only one in thousand individual develops GBS after *C. jejuni* enteritis [6]. Besides the microbial factors, host susceptibility may also play an important role in the development of GBS. However, limited studies have identified potential host factors involved in the disease pathogenesis and impart susceptibility to an individual for the development of GBS.

Nucleotide-binding oligomerization domain like receptors (NLRs) comprising of a large family of pathogen recognition molecules (PRMs) that are characterized by the presence of a conserved nucleotide-binding oligomerization domain (NOD) [7]. Among NLRs, NOD1 and NOD2 are composed of a *C*-terminal series of leucine-rich repeats (LRRs) and a central nucleotide-binding site (NBS) domain [8]. At the *N*-terminus, NOD1 has one caspase-activating and recruitment domain (CARD), while NOD2 has two of these domains [9,10]. Gene encoding NOD1 (CARD 4) is located on chromosome 7p14–p15 [11]. NOD1 gene mutations are found to be associated with asthma and inflammatory bowel disease outcome [12]. Structural modifications in NOD1 and NOD2 genes may activate nuclear factor-κB (NF-κB), mitogen-activated protein kinases (MAPKs), and interferon (IFN) regulatory factors (IRFs) to stimulate innate immunity [13,14]. The association of *NOD1* gene with innate immune system defects like

Abbreviation: AIDP, acute inflammatory demyelinating polyneuropathy; AMAN, acute motor axonal neuropathy; AMSAN, acute motor and sensory axonal neuropathy; GBS, Guillain-Barré syndrome; LD, linkage disequilibrium; NOD, nucleotide-binding oligomerization domain.

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atopic dermatitis [15], inflammatory bowel disease [13], sarcoidosis [16] point towards its clinical relevance. Several studies have also reported that the variations in NOD2 gene are associated with Crohn's disease (CD) and inflammatory bowel disease (IBD) [12,17–23]. Taking the importance of NOD1 and NOD2 in genetic susceptibility of various diseases, we hypothesized that host factors might determine the intensity of immune response towards microbial ligands, which might play a pivotal role in GBS development. In the present study we investigated the genetic polymorphisms in NOD1 (Glu266Lys) and NOD2 (Arg702Trp and Gly908Arg) genes in patients with GBS.

2. Methods

2.1. Study population

A total of 105 patients of GBS and 100 age/sex matched healthy controls were recruited for the present study. All the patients were selected according to the diagnostic criteria for the diagnosis of GBS adapted from Asbury and Cornblath [24]. The patients were classified into AIDP, AMSAN and AMAN based on the electrophysiological criteria adapted from Ho and colleagues [25]. The study protocol was approved by institutional ethics committee and the consent was obtained from each study participants prior to their inclusion in the study.

2.2. DNA extraction and genotyping

Blood sample was taken from each study subject and genomic DNA was extracted from EDTA anti-coagulated peripheral blood by salting-out method [24]. Further, the DNA was quantified and stored at −20 °C until use. Genotyping of both NOD1 and NOD2 genes was performed by polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (RFLP). The primer sequences used in the study are given in Table 1. All the PCR amplification reactions were performed in 20 μl volume containing 12 μl 2× PCR master mixes (Fermentas, Vilnius, Lithuania), 0.1 mM of each primer and 100 ng of extracted DNA. The thermal cycling conditions were as follows: an initial denaturation at 94 °C for 10 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at (64 °C and 56.2 °C for 1 min for NOD1 and NOD2 respectively) extension at 72 °C for 1 min and a final extension at 72 °C for 10 min. Template free water was used as negative control. Details of the primers and enzymes used in the study for each NOD1 and NOD2 single nucleotide polymorphisms (SNPs) are given in Table 1. The amplified products were tested on 2% agarose gel pre-stained with ethidium bromide (EtBr) with a migrating distance of approximately 3 cm. Samples were then used for restriction fragment length polymorphism (RFLP) study. NOD1 Glu266Lys, NOD2 Arg702Trp and NOD2 Gly908Arg polymorphisms were studied by PCR-RFLP method using restriction enzymes *Eco*881, *Msp*I and *Hha*1 (10 U) (New England Biolabs) respectively; were incubated at 37 °C overnight. Further, the digested NOD1 Glu266Lys, NOD2 Arg702Trp and NOD2 Gly908Arg products were checked on 15% polyacrylamide gel electrophoresis (PAGE), stained with EtBr (Fig. 1).

2.3. Statistical analysis

The level of significance of the genotypes and allele frequencies were analyzed by using Fischer's exact test or χ^2 test as appropriate. Power of the study was calculated using Quanto software version 1.0 (http://hydra.usc.edu/gxe) to achieve more than 80% of the statistical power at significance level (α) $p < 0.05$. Independent student *t*-test was performed to compare continuous data. Logistic regression was applied to compare genetic and clinical data; the results were expressed as odds ratios (OR) with 95% confidence intervals (95% CI). A *p*-value of <0.05 was considered significant. SPSS (statistical packages of social sciences, SPSS for Windows, version 15.0, Chicago, IC, USA) was used for the statistical analysis. Haplotypes and pair-wise linkage disequilibrium were generated using Arlequin software (v 3.5) (University of Dusseldorf, Germany) [26]. A negative Tajima's D signifies an excess of low frequency polymorphisms relative to expectation, indicating population size expansion or purifying selection. A positive Tajima's D signifies low levels of either low and high frequency polymorphisms, indicating a decrease in population size or balancing selection [24].

3. Results

3.1. Characteristics of GBS patients and control subjects

A total of 105 patients with GBS and 100 healthy controls were included in this study. Patients with GBS and controls were comparable in age (30.20 ± 10.97 vs. 28.12 ± 16.97 years). Based on electrophysiology, 37 (35.23%) patients were classified as AMAN, 30 (28.57%) as AMSAN and 38 (36.19%) as AIDP (Table 2). NOD1 (Glu266Lys) and NOD2 (Arg702Trp and Gly908Arg) gene polymorphisms were in Hardy-Weinberg Equilibrium in controls.

3.2. NOD1 (Glu266Lys) polymorphism

The genotypic frequencies of both homozygous (Lys/Lys) and heterozygous (Glu/Lys) variants of NOD1 were higher in GBS patients than healthy controls (Table 3). Further, the homozygous variant showed increased risk to GBS ($p = 0.013$, OR = 2.89; 95% CI = 1.25–6.70) and heterozygous variant also showed a trend towards risk to GBS than healthy controls ($p = 0.057$, OR = 2.17 95% CI = 0.98–4.82). The frequency of variant allele (266Lys) was also higher among GBS patients (64.29%) than healthy controls (52.50%) and showed 1.63 fold risk association with GBS ($p = 0.016$, OR = 1.63, 95% CI = 1.10–2.42).

3.3. Association of NOD1 (Glu266Lys) polymorphism with GBS subtypes

We further analyzed the data to determine association of NOD1 (Glu266Lys) polymorphisms with GBS subtypes. Both heterozygous and homozygous variants did not show risk association with any of the GBS subtypes (AMAN, AMSAN and AIDP) when compared with healthy controls (Table 4). However, NOD1 variant allele (266Lys) showed increased susceptibility to AMAN ($p = 0.001$, OR = 2.61, 95% CI = 1.45–4.73) and AIDP ($p = 0.018$, OR = 1.6, 95% CI = 1.22–3.42) subtypes.

Table 1
List of primer sequences for NOD1 and NOD2 gene polymorphisms.

S. No	Polymorphism	Primer sequence (5′ → 3′)	PCR product size	Enzyme	After digestion
1.	NOD1 Glu266Lys (rs2075820)	5′-AAGTGACAGGCTGTGCTGC-3′ 5′-CTTCCCACTGAGCAGGTTG-3′	232 bp	*Eco*881	Wild (G) – 232 bp Mutant (A) – 170 bp
2.	NOD2 Arg702Trp (rs2066844)	5′-GGCGCCCCTGGAATTC-3′ 5′-CCTCACCCGGTGCAGC-3′	185 bp	*Msp*I	Wild (*R*702) – 20 + 35 + 54 + 76 bp Mutant – 20 + 35 + 130 bp
3.	NOD2 Gly908Arg (rs2066845)	5′-CCCAGCTCCTCCCTCTTTC-3′ 5′-AAGTCTGTAATGTAAACGCCAC-3′	163 bp	*Hha*I	Wild – 163 bp Mutant – 27 + 136 bp Heterozygous – 27 + 136 + 163 bp

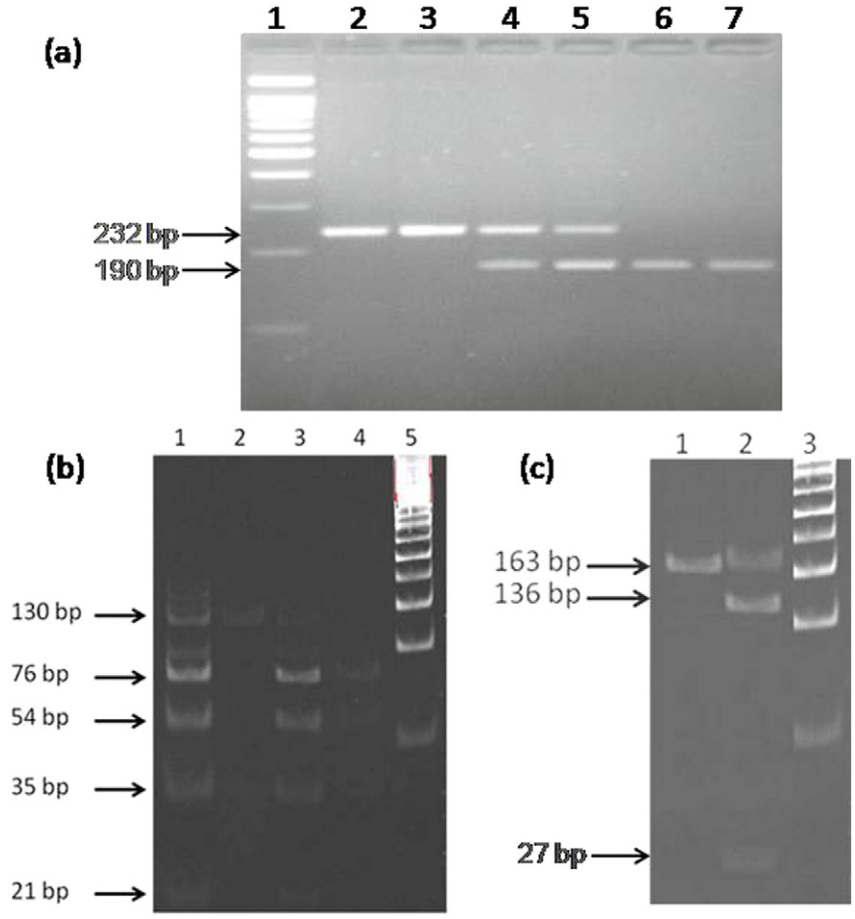

Fig. 1. Gel images of (a) NOD1 Glu266Lys genotypes; *Eco*881 digestion of PCR product: wild-type shows band of 232 bp, homozygous shows 190 bp, whereas a heterozygous shows 232&190 bp, (b) NOD2 Arg702Trp genotypes; *Msp*I digestion of PCR product (185 bp): wild-type shows bands of 76, 54, 35 & 21 bp, homozygous shows 130, 35 & 21 bp, whereas a heterozygous shows 130, 76, 54, 35 &21 bp and (c) NOD2 Gly908Arg genotypes; *Hha*I digestion of PCR product (163 bp): wild-type shows bands of 163 bp, and heterozygous shows 163, 136 & 27 bp.

3.4. NOD2 (Arg702Trp and Gly908Arg) polymorphisms and GBS

Increased risks for the development of GBS were revealed with heterozygote genotypes of NOD2, Arg702Trp ($p = 0.001$, OR = 5.78, 95% CI = 2.95–11.31) and Gly908Arg ($p = 0.010$, OR = 2.07; 95% CI = 1.19–3.62) (Table 5). NOD2 alleles i.e., 702Trp ($p = 0.001$, OR = 4.48, 95% CI = 2.44–8.24) & 908Arg ($p = 0.038$, OR = 1.61, 95% CI = 1.02–2.53) also showed significant risk association with GBS.

3.5. Association of NOD2 polymorphisms (Arg702Trp and Gly908Arg) with GBS subtypes

Significant protective association for AMAN ($p = 0.001$, OR = 0.12, 95% CI = 0.05–0.28), AMSAN ($p = 0.029$, OR = 0.35, 95% CI = 0.14–0.90) and AIDP ($p = 0.001$, OR = 0.14, 95% CI = 0.06–0.33) subtypes were found with NOD2 Arg702Trp heterozygote genotype. On the contrary, predisposing association was observed with NOD2 702Trp allele for AMAN ($p = 0.001$, OR = 6.29, 95% CI = 3.08–12.84), AMSAN ($p = 0.039$, OR = 2.47, 95% CI = 1.05–5.82) and AIDP ($p = 0.001$, OR = 4.71, 95% CI = 2.27–9.75) subtypes. Heterozygote (Gly/Arg) genotype revealed protective association ($p = 0.003$, OR = 0.30, 95% CI = 0.13–0.66) and variant allele (Arg) of Gly908Arg polymorphism showed risk associations with AIDP subtype ($p = 0.014$, OR = 2.07, 95% CI = 1.16–3.70) (Table 6).

3.6. Distribution of NOD2 haplotype frequency among patients with GBS and control subjects

Haplotype analysis was carried out in order to evaluate whether the NOD1 and NOD2 gene polymorphisms have any additive effect on GBS

Table 2
Demographic details of study subjects.

	Patients (105)	Controls (100)
Characteristics		
Age in years (mean ± SD)	30.20 ± 10.97	28.12 ± 16.97
Gender (male)	84 (80%)	66 (66%)
GBS subtypes		
AMAN	37 (35.23%)	
AMSAN	30 (28.57%)	
AIDP	38 (36.19%)	

AMAN, acute motor axonal neuropathy; AMSAN, acute motor-sensory axonal neuropathy; AIDP, acute inflammatory demyelinating polyneuropathy.

Table 3
Influence of NOD1 polymorphism on GBS susceptibility.

Gene polymorphism	Patients (%)	Controls (%)	*p* value	OR# (95% CI)
rs2075820 genotype				
Glu/Glu	12 (11.43%)	24 (24%)	–	Reference
Glu/Lys	51 (48.57%)	47 (47%)	0.057	2.17 (0.98–4.82)
Lys/Lys	42 (40%)	29 (29%)	0.013	2.90 (1.25–6.70)
Allele				
Glu	75 (35.71%)	95 (47.5%)	–	Reference
Lys	135 (64.29%)	105 (52.5%)	0.016	1.63 (1.10–2.42)

Table 4
NOD1 (rs2075820) genotype distribution in GBS subtypes and controls.

NOD1 genotype	GBS subtypes				[a and d]		[b and d]		[c and d]	
	AMAN [a] (%)	AMSAN [b] (%)	AIDP [c] (%)	CONTROL [d] (%)	*p* value	OR 95% CI	*p* value	OR 95% CI	*p* value	OR (95% CI)
Glu266Lys										
Glu/Glu	2 (5.4)	7 (23.33)	3 (7.89)	24 (24)	–	References	–	References	–	References
Glu/Lys	15 (40.55)	18 (60)	18 (47.37)	47 (47)	0.091	0.26 (0.06–1.23)	0.594	0.76 (0.28–2.07)	0.096	0.33 (0.09–1.22)
Lys/Lys	20 (54.05)	5 (16.67)	17 (44.74)	29 (29)	0.008	0.12 (0.03–0.57)	0.417	1.69 (0.48–6.02)	0.024	0.21 (0.06–0.82)
Allele										
Glu	19 (25.68)	32 (53.33)	24 (31.58)	95 (47.5)	–	References	–	References	–	References
Lys	55 (74.32)	28 (46.67)	52 (68.42)	105 (52.5)	0.001	2.62 (1.45–4.73)	0.428	0.79 (0.44–1.41)	0.018	1.60 (1.22–3.42)

Table 5
Influence of NOD2 polymorphisms on GBS susceptibility.

Gene polymorphism	Patients (%)	Controls (%)	*p* value	OR# (95% CI)
NOD2 Arg702Trp (rs2066844) genotype				
Arg/Arg	51 (48.57%)	85 (85%)	–	Reference
Arg/Trp	52 (49.52%)	15 (15%)	0.001	5.78 (2.95–11.31)
Trp/Trp	2 (1.9%)	0 (0%)	0.999	NC
Allele				
Arg	154 (73.33%)	185 (92.5%)	–	Reference
Trp	56 (26.67%)	15 (7.5%)	0.001	4.48 (2.44–8.24)
NOD2 Gly908Arg (rs2066845) genotype				
Gly/Gly	42 (40%)	58 (58%)	–	Reference
Gly/Arg	63 (60%)	42 (42%)	0.010	2.07 (1.19–3.62)
Arg/Arg	0 (0%)	0 (0%)	–	–
Allele				
Gly	147 (70%)	158 (79%)	–	Reference
Arg	63 (30%)	42 (21%)	0.038	1.61 (1.03–2.53)

(Table 7). The occurrence of Arg702-Gly908 haplotype comprising of wild genotype (Arg/Gly) among control and patients was 79% and 68.5% respectively. Total four haplotypes were found among patients while three were found among controls. Significant risk with Trp702-Arg908 ($p \leq 0.001$, OR = 3.87, 95% CI = 2.09–7.18) haplotype and protective association with Arg702-Arg908 ($p = 0.022$, OR = 0.40, 95% CI = 0.19–0.86) haplotype for GBS were observed.

3.7. Linkage disequilibrium analysis

The standardized linkage disequilibrium (LD) coefficient (D′), the conventional measure of LD (r^2) and the *p* value were measured among the GBS cases (Table 8). The LD analysis showed five significant associations in this study. It is evident from the analysis that significant risk was associated with GBS in the presence of mutant alleles. Pair-wise LD showed risk for GBS in the presence of Glu (Glu266Lys)-Lys (Glu266Lys) (D′ = 0.99, *p*-value = <2e−16), Glu (Glu266Lys)-Trp (Arg702Trp) (D′ = 0.99, *p*-value = 2.89e−07), Glu (Glu266Lys)-Arg (Gly908Arg) (D′ = 0.99, *p*-value = 1.92e−07), Trp (Arg702Trp)-Arg (Gly908Arg) (D′ = 0.99, *p*-value = <2e−16) and Trp (Arg702Trp)-Arg (Gly908Arg) (D′ = 0.99, *p*-value = <2e−16) alleles among the studied individuals.

4. Discussion

NOD1 and NOD2 gene polymorphisms have been implicated in various autoimmune and inflammatory diseases [12,17,19–21]. However, their role in GBS patients largely remains unknown. Hence, we investigated NOD1 Glu266Lys and NOD2 (Arg702Trp and Gly908Arg) gene polymorphisms among patients with GBS. In our study, the variant allele frequency of NOD1 266Lys was higher among patients with GBS than controls (64.28% vs. 52.5%, $p = 0.016$; OR 1.629; 95% CI, 1.096–2.419). Further, the frequency of NOD2 variant allele (702Trp and 908Arg) was higher among patients with GBS than controls (26.67% vs. 7.5%; $p = 0.001$; OR, 4.485; 95% CI, 2.440–8.243 and 30% vs. 21%; $p = 0.038$ OR, 1.612; 95% CI, 1.028–2.530). The reported rate of NOD2 702Trp & 908Arg variant allele in Spanish population was 4% and 0%, and in Italian population 1.75% and 0.75% respectively suggesting non co-segregation [18,27]. The genetic frequencies of these two alleles in the present study were different, suggesting the non co-segregation.

In the present study, a positive association of NOD1 (Glu266Lys) and NOD2 (Arg702Trp and Gly908Arg) polymorphisms was observed among patients with GBS. It has been well documented that NOD2 mutations in Crohn's disease potentiate the activity of NF-κB and IL-1β processing, thereby enhancing the inflammation [23].

Our data support those studies that reported association of NOD2 polymorphism with other inflammatory diseases. NOD2 (Arg702Trp and Gly908Arg) polymorphisms were identified as risk factor for

Table 6
NOD2 Genotype distribution in GBS subtypes and controls.

NOD2 genotypes	GBS subtypes				[a and d]		[b and d]		[c and d]	
	AMAN [a] (%)	AMSAN [b] (%)	AIDP [c] (%)	CONTROL [d] (%)	*p* value	OR 95% CI	*p* value	OR 95% CI	*p* value	OR 95% CI
NOD2 Arg702Trp (rs2066844)										
Arg/Arg	14 (37.84)	20 (66.67)	17 (44.74)	85 (85)	–	References	–	References	–	References
Arg/Trp	21 (56.76)	10 (33.33)	21 (55.26)	15 (15)	0.001	0.12 (0.05–0.28)	0.029	0.35 (0.14–0.90)	0.001	0.14 (0.06–0.33)
Trp/Trp	2 (5.4)	0	0	0	0.999	NC	–	–	–	–
Allele										
Arg	49 (66.21)	50 (83.33)	55 (72.37)	185 (92.5)	–	References	–	References	–	References
Trp	25 (33.79)	10 (16.67)	21 (27.63)	15 (7.5)	0.001	6.29 (3.08–12.84)	0.039	2.47 (1.05–5.82)	0.001	4.71 (2.27–9.75)
Gly908Arg (rs2066845)										
Gly/Gly	15 (40.54)	16 (53.33)	11 (28.95)	58 (58)	–	References	–	References	–	References
Gly/Arg	22 (59.46)	14 (46.67)	27 (71.05)	42 (42)	0.071	0.49 (0.23–1.06)	0.651	0.83 (0.37–1.88)	0.003	0.30 (0.13–0.66)
Arg/Arg	0	0	0	0	–	–	–	–	–	–
Allele										
Gly	52 (70.27)	46 (76.67)	49 (64.47)	158 (79)	–	References	–	References	–	References
Arg	22 (29.73)	14 (23.33)	27 (35.53)	42 (21)	0.131	1.59 (0.87–2.97)	0.700	1.15 (0.58–2.28)	0.014	2.07 (1.16–3.70)

Table 7
NOD2 haplotype frequency distribution between patients and control subjects.

Haplotype	Patient ($N = 105$)	Control ($N = 100$)	p-Value	OR	95% CI
Arg702-Gly908	144 (68.5%)	158 (79.0%)	Reference		
Trp702-Gly908	3 (1.4%)	–	–	–	–
Trp702-Arg908	53 (25.2%)	15 (7.5%)	<0.001	3.87	2.09–7.18
Arg702-Arg908	10 (4.7%)	27 (13.5%)	0.0221	0.40	0.19–0.86

Crohn's disease [28] and further NOD2 variants being involved in pathogenesis of IBD [26]. In present study, we found the significant association of variants of NOD2 gene with GBS. On the contrary, NOD2 gene variants were not found to be involved in determining susceptibility to multiple sclerosis [29]. However, in one Indian study common NOD2 mutations were absent in patients with Crohn's disease [30]. In another study, individuals with homozygous or compound heterozygous for the common NOD2 mutations have about 20-fold increased risk for development of Crohn's disease whereas heterozygous subjects have only about two fold increased risk [19]. The association of NOD2 (Arg702Trp and Gly908Arg) polymorphisms with GBS in the present study suggests that variant of NOD2 gene increases the risk for disease development. NOD2 is expressed widely in macrophages, dendritic cells, paneth cells, keratinocytes, epithelial cells of the intestine and lung [31]. Several studies indicate that cell mediated immune response to peripheral nerve may play an important role in the pathogenesis of GBS [32]. The inflammatory infiltrates in GBS are mainly composed of lymphocytes and macrophages, where this gene is constitutively expressed during acute phase of the disease [33]. Macrophages are the principal source of cytokines including TNF-α, a major pro-inflammatory cytokine that could be involved in early breakdown of the blood nerve barrier, up-regulation of endothelial adhesion molecules, leukocyte attraction to nerve tissue and myelin damage. Enhanced expression of TNF-α along with polymorphism has been reported in patients with GBS [32]. Previous studies in non-gestational tissues have shown that TNF and IL1β up-regulate *NOD1* and/or *NOD2* mRNA expression in various cell lineages [34–36]. Therefore, it might be possible that mutations in NOD2 gene influence directly or indirectly to TNF-α production that may lead to inflammation. Some other studies revealed that mutations in NOD2 gene were unable to activate the NF-κB signal transduction pathway upon binding of NOD2 ligands [37]. NOD2 mutations were not found among patients with ulcerative colitis [38].

To the best of our knowledge, this is the first study suggesting that NOD1 Lys/Lys homozygote was significantly associated with AMAN ($p = 0.008$) and AIDP ($p = 0.024$) subtypes (Table 4). In addition, 702 Arg/Trp heterozygote was significantly associated with AMAN ($p = 0.001$), AMSAN ($p = 0.029$) and AIDP ($p = 0.001$) subtypes while 908 Gly/Arg heterozygote showed association with AIDP ($p = 0.003$) subtype only (Table 6). Association of TLR4 Gly299Gly homozygous genotype with AMAN subtype of GBS had been reported in an earlier study [39].

In conclusion, the present study showed that NOD1 homozygous genotype (Lys/Lys) was significantly associated with GBS and it also increased the risk for AMAN and AIDP subtypes of GBS. In addition, there was significant risk association of NOD2 (702Arg/Trp and 908Gly/Arg) heterozygous genotypes with GBS. Further heightened risk for 702 Arg/Trp heterozygous genotype was observed for all GBS subtypes; while, AIDP was associated with heterozygous genotype Gly908Arg.

Conflict of interest

The authors declare that they have no conflict of interest.

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Table 8
Linkage disequilibrium association among NOD1 Glu266Lys (rs2075820), NOD2 Arg702Trp (rs2066844), NOD2 Gly908Arg (rs2066845) polymorphisms in GBS patients.

		Glu (Glu266Lys)	Lys (Glu266Lys)	Arg (Arg702Trp)	Trp (Arg702Trp)	Gly (Gly908Arg)	Arg (Gly908Arg)
Glu (Glu266Lys)	D’	*	0.9995	0.4852	0.9997	0.5657	0.9997
	r^2	*	0.8072	0.3096	0.8112	0.4228	0.785
	p-Value	*	<2e−16	1.97e−05	2.89e−07	3.903–09	1.92e−07
Lys (Glu266Lys)	D′	*	*	0.9991	0.5508	0.9992	0.7353
	r^2	*	*	−0.3175	0.3609	−0.3719	−0.4663
	p-Value	*	*	5.74e−07	8.82e−05	2.33e−09	1.65e−07
Arg (Arg702Trp)	D′	*	*	*	0.9995	0.8911	0.9995
	r^2	*	*	*	0.5175	0.7609	0.5008
	p-Value	*	*	*	1.89e−08	<2e−16	3.17e−08
Trp (Arg702Trp)	D′	*	*	*	*	0.9226	0.9998
	r^2	*	*	*	*	0.5594	0.9676
	p-Value	*	*	*	*	6.44e−10	<2e−16
Gly (Gly908Arg)	D′	*	*	*	*	*	0.9994
	r^2	*	*	*	*	*	0.5865
	p-Value	*	*	*	*	*	<2e−16
Arg (Gly908Arg)	D′	*	*	*	*	*	*
	r^2	*	*	*	*	*	*
	p-Value	*	*	*	*	*	*

D′: Values generated using Lewontin's principle; r^2: Values generated using Mattiuz's principle; $p < 0.05$ = statistical significance.

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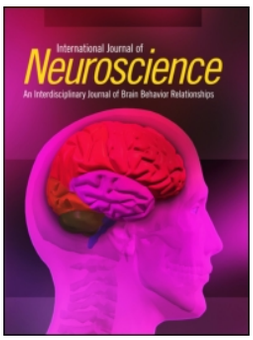

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Polymorphisms of IL-17 and ICAM-1 and their expression in Guillain-Barré syndrome

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Abstract:

Purpose: Guillain Barré syndrome (GBS) is an acute inflammatory, autoimmune disorder of peripheral nervous system (PNS). Interleukin-17 (IL-17) and intercellular adhesion molecule-1 (ICAM-1) polymorphisms with higher expression levels have already been studied in many inflammatory and autoimmune diseases. However, the possible role of IL-17 and ICAM-1 polymorphisms in GBS remains unknown. Therefore, the current study investigated IL-17 (His161Arg, Glu126Gly) and ICAM-1 (Gly241Arg) polymorphisms.

Materials and method: In present study total 80 GBS patients and 75 normal healthy controls were included. IL-17 (His161Arg, Glu126Gly) and ICAM-1 (Gly241Arg) polymorphisms were performed using polymerase chain reaction-restriction fragment length polymorphism analysis. Further, the expression of ICAM-1 and IL-17 were determined by reverse-transcriptase PCR and enzyme-linked immunosorbent assay.

Results: IL-17 (Glu126Gly) mutant and ICAM-1 (Gly241Arg) heterozygous genotypes were strongly associated with increased risk of GBS (p<0.016; OR=3.706, 95% CI=1.28-10.67; p<.001; OR=4.148, 95% CI=2.119-8.119 respectively). IL-17 and ICAM-1 genes showed significantly higher expression in GBS when compared with healthy controls.

Conclusion: IL-17 and ICAM-1 polymorphisms showed significant association with GBS and their enhanced expressions have possible role in GBS development. IL-17 and ICAM-1 polymorphisms could be genetic markers to GBS susceptibility.

Keywords: Guillain-Barr´e syndrome, gene polymorphism, cytokine IL-17 and adhesion molecule, ICAM-1

Introduction:

Guillain Barré syndrome (GBS) is an immune-mediated inflammatory disease mainly affecting the myelin and axons of peripheral nerves with heterogeneous pathological features, often triggered by an aberrant immune response towards an infectious pathogen [1]. Based on clinical features, etiology, and pathologic studies, GBS may be sub-classified into several forms namely acute inflammatory demyelinating polyradiculoneuropathy (AIDP); axonal forms of GBS, which include acute motor-sensory axonal neuropathy (AMSAN) and acute motor axonal neuropathy (AMAN) [1]. Epidemiological studies linked it with *Campylobacter jejuni,* cytomegalovirus, Epstein Barr virus and *Mycoplasma pneumoniae,* [2]. The mechanisms involved in immunopathogenesis of GBS are still unclear. The hypothesis puts forward for the immunopathogenesis of GBS points to molecular mimicry between lipopolysaccharide (LPS) and ganglioside like epitopes in host nerve cells, which leads to cross reactivity after the infection. Besides microbial factors, host susceptibility may also play an important role in the etiology of GBS, because not all infected individuals develop this disorder. It is estimated that only 1:1000 people develop GBS after *C. jejuni* enteritis, thus highlighting the role of host genetic factors [3]. However, limited studies have been conducted for identifying the potential host factors that may impart susceptibility to GBS.

IL-17 is the signature cytokine of the "Th17" T helper cell population and is produced by several cell types including activated T cell subsets (CD4+ and CD8+), natural killer cells, macrophage and neutrophils [4]. IL-17F was discovered as member of IL-17 family (IL-17A-IL-17F) mapped on the same chromosome position at 6p12 and share highest degree of homology with IL-17 [5] and Human Th17 cells could involve in disruption of blood brain barrier, stimulating the inflammation, macrophage activation, chemotaxis of neutrophils and myelin damage [6, 7]. IL-17 induces cytokines and chemokine expression and plays an essential role in immune host defences and also implicated in various autoimmune and inflammatory diseases [8, 9, 10, 11]. IL-17 induces T cell activation and infiltration into tissues by up-regulating the expression of ICAM-1 and amplifies the immune response by inducing the production of IL-6 and prostaglandin E2 [12]. ICAM-1 is a surface glycoprotein, expressed on vascular endothelium, macrophages, and human neuronal cells, activated lymphocytes. It mediates leukocyte circulation and extravasations from the blood into the areas of inflammation and macrophage differentiation [13, 14].

IL-17F (Glu126Gly) polymorphism was significantly associated with an increased disease activity in Korean patients with Behcet's disease (BD) [10]. In another study, polymorphism in IL-17 (His161Arg) was not associated with Crohn's disease (CD). In addition to this, Serum and synovial fluid levels of IL- 17 were in correlation with disease activity in patients with rheumatoid arthritis (RA) [11, 15, 16, 17]. However, another study, reported augmented mRNA expression for interleukin-17 in blood and CSF mononuclear cells among multiple sclerosis cases [18]. Several studies also revealed predisposition for autoimmune diseases due to IL-17 polymorphisms [10, 11, 16, 18].

Several studies have suggested higher IL-17 concentrations in the acute-stage GBS than stable-stage GBS [19, 20, 21]. Similarly significance of inhibiting IL17-A like receptors has been suggested as therapeutic intervention in **experimental autoimmune neuritis (EAN)** to prevent GBS [22]

Association of ICAM-1 polymorphism (Gly241Arg) was reported in RA of Mexican population [23]. However, many studies revealed the enhanced expression of ICAM-1 in autoimmune diseases like multiple sclerosis and experimental autoimmune encephalomyelitis (EAE) etc [24, 25, 26].

Looking at the importance of IL-17 and ICAM-1 in genetic susceptibility to various diseases, we hypothesized that host factors might determine the intensity of immune response towards microbial ligands, which might play a pivotal role in progression and development of GBS. The precise role of polymorphism & expression of IL-17 and ICAM-1 are yet not deciphered in context to GBS. In present study, we therefore investigated the genetic association of IL-17 (Glu126Gly and His161Arg) and ICAM-1 polymorphisms (Gly241Arg), and their expressions with GBS.

Material and Methods

Study population

Patients admitted to Neurology ward, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow were enrolled for the study. The Institutional ethics committee granted approval for this study and consent was obtained from all the study subjects. A total of 80 patients (male 64) with GBS, mean age ± SD, 30.20±10.98 years, and 75 gender and age matched normal healthy controls (male 44), mean age 28.12 ± 16.97 years were included. GBS patients were selected on the basis of criteria as described earlier [27]. Normal healthy

controls were individuals without any history of apparent infectious illness within the preceding four weeks. Patients with GBS had not received any immunosuppressive or immunomodulatory treatment in the last two months prior to sample collection. Further GBS was classified into its subtypes: AIDP, 29 (36.25%); AMAN, 28 (35%); and AMSAN, 23 (28.75%) based on clinical and electrophysiological criteria described by Hadden et al. [28]. All the patients with GBS were asked for the presence of symptoms and signs of any apparent infectious illness within the last 4 weeks. History of preceding infection was available in 38 (47.5%) patients. GBS patients (48.6%) reported clinical history of infectious illness; these illnesses were as follows: respiratory 16 (20%), diarrhoea with and without abdominal pain 7 (8.75%), both respiratory and diarrhoea 5 (6.25%), fever 14 (17.5%) and chicken pox 1 (1.25%).

Sample Collection

Blood samples were collected through peripheral vein puncture during the first 2 weeks after the onset of GBS. Blood in ethylene diamine tetra acetate (EDTA) was stored at -20°C for DNA extraction. Sera from clotted blood were separated and stored at -80°C till further use for enzyme-linked immunosorbent assay (ELISA).

Genomic DNA isolation

Genomic DNA was extracted from whole blood using salting out method [29]. DNA samples were stored at -20ºC till further use for genotyping.

IL-17 genotyping (Glu126Gly & His161Arg)

Single nucleotide polymorphisms (SNPs) were detected by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis. DNA samples of 100 ng/µl concentration were used for SNP detection; primer sequences for IL-17 (Glu126Gly & His161Arg) amplifications are shown in Table 1. PCR amplifications were performed in a 25 µL volume containing 10 X assay buffer, 200 mM each of dATP, dCTP, dGTP and dTTP along with, 0.1 mM of sense and antisense primers and 1.0 U of Taq DNA polymerase (Bangalore Genei, Bengaluru, India). PCR protocols were as follows: initial denaturation for 10 minutes at 95ºC followed by 35 PCR cycles of denaturation for 30 seconds at 94ºC, annealing for 30 seconds at 64º C, extension for 30 seconds at 72ºC with final extension of 5 minutes at 72ºC. Amplification products (20 μl) were digested with 1U of AvaII (Fermentas, Burlington, Canada) restriction enzyme for the Glu126Gly polymorphism, and with NlaIII

(Fermentas, Burlington, Canada) restriction enzyme for the His161Arg polymorphism at 37ºC overnight, electrophoresed on 3.5% agarose (Sigma–Aldrich, St Louis, MO, USA) gel, visualized under UV illumination and stained with ethidium bromide.

ICAM-1 (721 G>A) genotyping

ICAM-1 genotype was determined by PCR-RFLP as mentioned above differing only at the annealing temperature of 58.5ºC. ICAM-1 (721 G>A) specific primers are shown in Table 1. For RFLP analysis, the PCR products (15μL) were digested with 1 U of BsrGI restriction enzyme (Fermentas Life Science, USA) at 37ºC overnight, electrophoresed on 3.5% agarose (Sigma–Aldrich, St Louis, MO, USA) gel, visualized under UV illumination and stained with ethidium bromide.

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

RNA was extracted from the frozen blood using the Qiagen RNeasy kit (Qiagen Inc, Valencia, California) according to the manufacturer's instructions. Complementary DNA was synthesized by the RevertAid H minus first strand complementary DNA synthesis kit (Fermentas Life Sciences, Glen Burnie, Maryland) taking 100 ng of RNA for each sample. After reverse transcription, PCR was performed using specific primer of IL-17 and ICAM-1 (Table1); and GAPDH was used as the housekeeping gene. To normalize messenger RNA (mRNA) levels, densities of IL-17, ICAM-1 and GAPDH bands from the same samples were measured, and data were calculated as the ratios of band intensity values relative to the band intensity value of GAPDH.

Serum ELISA (Enzyme-Linked Immunosorbent Assay) for IL-17 and ICAM-1

IL-17 and ICAM-1 levels were measured in the serum using commercial ELISA kit (Invitrogen, Carisbad, USA) following manufacturer's instruction. All samples were measured in triplicates. For IL-17 & ICAM-1, serum samples were diluted to a ratio of 1:100 using assay buffer. The detection limits of the kits were 250 pg/ml. The optical density of the wells was determined using a microplate reader set at 450 nm.

Statistical analysis

The SPSS 16.0 statistical package (Chicago, IL, USA) was used for data management and analysis. "Quanto software version 1.0 (http://hydra.usc.edu/gxe) was used to determine the statistical power. The present study achieved statistical power higher than 80% with a

significance level (α) < 0.05"). Logistic regression analysis was applied to estimate association with GBS susceptibility after adjusting for age and gender and considered significant if the p-values were ≤ 0.05. Hardy-Weinberg equilibrium was checked in controls by goodness of fit χ^2 test. For comparisons between the groups of study populations χ^2 test was used. ELISA data was expressed as mean ± SD of triplicate experiments performed independently for each sample. One-way ANOVA: Post Hock (Bonferroni) test was performed to determine the expression level of IL-17 & ICAM-1 and to compare continuous data (age).

Results:

Genetic polymorphisms

Genotypes and allele frequencies in GBS patients and normal healthy controls are shown in tables 2 and 3. Both IL-17 and ICAM-1 polymorphisms were in agreement with Hardy-Weinberg equilibrium in controls.

Frequency distributions of the IL-17F Glu126Gly and His161Arg genotypes in GBS patients and controls.

Genotypic distributions of IL-17F Glu126Gly polymorphism demonstrated increased risk of GBS for homozygous variant genotypes Gly/Gly (52.5% vs 22.67%; p < 0.016; OR=3.70; 95% CI=1.28-10.67). The frequency of wild homozygous genotypes was observed at 10% vs 16% for IL-17F Glu126Gly polymorphism in patients and controls. Patients with GBS showed significantly higher frequency in comparison to controls for allele 126Gly (71.25% vs 53.33%, p < 0.001; OR=2.16; 95% CI=1.35-3.46) (Table 2). However, the logistic regression analysis revealed no significant association of IL-17 His161Arg polymorphism (polymorphic and heterozygous) with GBS (p=0.142 and p=0.306).

Frequency distribution of the ICAM-1 241Gly/Arg genotype in GBS patients and controls

To analyze the association of ICAM-1 polymorphisms with GBS, the genotype frequency was compared between normal healthy controls and GBS patients. Logistic regression analysis revealed susceptible association for heterozygous genotype (G/A) of ICAM-1 241Gly/Arg polymorphism with GBS (p < 0.001; OR=4.14, 95% CI=2.11-8.11). We found no incidence of the mutant homozygous Arg/Arg genotype either among patients or controls.

Patients with GBS showed significantly higher frequency in comparison to controls for allele 241 Arg (35% vs 18%, p<0.001; OR=2.16; 95% CI=1.35-3.46) (Table3).

Elevated mRNA level of IL-17 and ICAM-1 in GBS patient and normal healthy controls

The mRNA level of IL-17 was found to be increased (p=0.066, 3.66±0.792 vs 2.05±0.299) in GBS cases than controls but no statically significance was concurred. However, in ICAM-1, mRNA level (p=0.012, 2.04±0.261 vs 1.18±0.202) was found to be increased among patients with GBS compared to controls (Figure 1).

Serum levels of IL-17 and ICAM-1 in GBS Patients and normal healthy controls

Elevated level of IL-17 was observed among GBS patients when compared to healthy controls (p≤ 0.001, 42.00±5.33 vs 6.0±0.768). Similarly heightened level of ICAM-1 was also noted in sera of GBS cases (p≤0.001, 5.33±0.661 vs 2.25±0.288) as shown in Figure 2.

Discussion:

In the present study, we have investigated the association of IL-17 polymorphism (IL-17F Glu126Gly and His161Arg) with GBS and evaluated their relative expressions. We also investigated the expression of ICAM-1 and its gene polymorphism (241Gly/Arg) among GBS patients.

IL-17 is a pro-inflammatory cytokine produced by activated T cell. It plays an essential role in immune host defence and is involved in a number of autoimmune and inflammatory diseases including GBS. The magnitude of the association of polymorphisms with autoimmune disease varies depending on genetics, demographics, and environmental factors. Many association studies reported that IL-17 polymorphisms predisposed to autoimmune and inflammatory diseases [8, 9, 10, 11]. However, some polymorphisms of IL-17F (Glu126Gly and His161Arg) might not be significantly associated with autoimmune disease like that of RA [30]. However, there is no data reported till date in context to IL-17F polymorphism with GBS.

The importance of IL-17F polymorphism in GBS is still largely unknown. In our study, the variant allele frequency of IL-17F (Glu126Gly) was observed to be higher in GBS than controls (71.25 % vs 53.33%, p=0.001; OR=2.16; 95% CI=1.35-3.46). However, the allelic frequency of IL-17F His161Arg was almost similar in GBS patients and controls (31.8% vs. 32.6% respectively). The allele frequency of His161Arg variant was reportedly lower for the Polish subjects (3.8%) in comparison to patients from Canada, United States of America,

United Kingdom, China, Japan, and Nigeria [30]. Moreover, in populations from Nigeria and Japan only the wild type allele for Glu126Gly had been reported. In another study, the variant allelic frequency of (IL-17F His161Arg) was 4.8% in Munich population [11]. Data pertaining to the role of IL-17F gene polymorphism in inflammatory demyelinating diseases including GBS is still not available. There is a single study that reports the enhanced level of IL-17 in cerebral spinal fluid of patients with GBS [31]. The present study findings revealed nearly four-fold risk association for homozygous (Gly/Gly) genotype ($p<0.016$) of IL-17F Glu126Gly polymorphism with GBS. Meanwhile we did not find any significant association for the heterozygous His161Arg genotype of IL-17F SNP with GBS. In the similar light, Seiderer et al., have found no risk association for His161Arg variant with IBD and CD [11]. On the contrary no association for the heterozygote His161Arg genotype has been observed in Korean BD [10] and Japanese ulcerative colitis cases [9].

It may be suggested that polymorphisms in IL-17F gene may cause enhanced production of IL-1β [30] and TNF-α [32, 33] like pro-inflammatory cytokines, which can mediate inflammatory pathology in GBS like other autoimmune diseases. We have found elevated level for IL-17 ($p=0.001$) in serum along with increased mRNA expression in GBS patients.

IL-17, a signature cytokine produced by Th17 cells, may have synergistic effects with TNF-α, IFN=γ, and IL1β like pro-inflammatory cytokines. IL-17 was detected in sciatic nerves of **EAN** rat models, and the accumulation of IL-17 was correlated with the severity of neurological signs [34], suggesting a pathological contribution of IL-17 to the development of EAN. The frequency of Th17 cells in cerebrospinal fluid (CSF) and the level of IL-17 in plasma were significantly higher in active chronic inflammatory demyelinating polyradiculoneuropathy (CIDP) [6]. Further the levels of IL17 and IL22 in CSF were found to be in correlation with GBS severity [31]. Collectively, these findings indicate that Th17 cells and their effector cytokines might be involved in the pathogenesis of GBS and EAN. IL17 up-regulates the expression of inflammatory genes including pro-inflammatory chemokines, hematopoietic cytokines, acute phase response genes, and antimicrobial substances [35] in neutrophils, macrophages, and endothelial cells [36].

Increased expression of ICAM-1 has been shown on endothelial cell, microglia, and astrocytes in active MS and EAE lesion [37, 38]. Free cICAM-1 contains almost all extracellular domains of membrane ICAM-1, as well as the ability to bind specifically to the adhesion receptor LFA-1 and to promote endothelial signalling to lymphocytes by engaging LFA-1 or to facilitate transendothelial migration of leukocytes into inflammatory sites.

Conversely, in view of its ability to specifically bind to LFA-1, cICAM-1 may play an inhibitory role in cell to cell interaction by competing with membrane-associated ICAM-1 for LFA-1. cICAM-1 may provide insight into the pathophysiology of inflammatory cell involvement in MS, particularly the mechanisms of lymphocyte homing and cellular trafficking across the blood-brain barrier [39, 40]. The elevated levels of ICAM-1 and TNF-α were measured in serum of patients with acute inflammatory demyelinating polyneuropathy (AIDP) and CIDP. Further the level was elevated in the CSF of GBS patients compared to patients with headache [41]. Immunohistochemical analysis of frozen sural nerves biopsies from GBS patients (AIDP variant) showed localization of ICAM-1 both on endothelial cells and macrophages [42]. In animal studies, increases in ICAM-1 mRNA and protein were detected in cauda equina harvested from Lewis rats with EAN [43, 44]; antibody-mediated neutralization of ICAM-1 attenuated the course of EAN [45]. Kelly A et al (2013) proposed that increased CCL2 and ICAM-1 expressions in response to TNFα might facilitate recruitment and trafficking of autoreactive leucocytes across the **blood nerve barrier (BNB)** in autoimmune disorders, including GBS [46]. Increased levels of soluble E-selectin, ICAM-1 and VCAM-1 had been described in the sera of GBS patients, with higher levels temporally associated with disease activity [47].

The above studies clearly demonstrate that ICAM-1 plays a central role in the development of demyelinating disease [48]. TNF-α and IL-1β are able to up-regulate ICAM-1 on schwann cell [49]. Elevated levels of TNF-α and IFN-γ during acute phase of disease have been reported in patients with GBS [50]. In present study, our results suggest that up-regulation of adhesion molecules induce damage to myelin and thus may have a role in the pathogenesis of inflammation in the peripheral nerve.

We have observed significant risk association of ICAM-1 (241Gly/Arg) heterozygous genotype ($p<0.001$) with GBS. It is evident that the ICAM-1 adhesion molecules can activate effector mechanism to promote progression of neuro-inflammmation [51]. Polymorphisms of ICAM-1 G241R are common genetic variation in different populations and associated with multiple sclerosis and CD [52] whereas other studies failed to find a significant contribution of the 721A allelic variant in inflammatory diseases [53, 54]. Significantly susceptible association was concurred for 721A allele of ICAM-1 polymorphism ($p=0.001$), with GBS. Our data is supported by previous findings by Macchioni et al. in Italian RA patients [55]. However, it remained unidentified among Korean RA subjects [56]. Such contradictory findings among populations may be due to variability in genetic background. Significantly induced level of ICAM-1 ($p\leq0.001$) in sera of the GBS patients along with enhanced mRNA level ($p=0.012$) was also observed in our north Indian GBS patients. This finding is in concurrence with a previous study [57].

The present study findings may be justified by the fact that IL-17 facilitates T cell activation and infiltration into tissues by up-regulating the expression ICAM-1 and amplifying the immune response by inducing production of IL-6, prostaglandin E2, granulocyte-macrophage colony stimulating factor (GM-CSF), and granulocyte colony stimulating factor [12, 58, 59]. However, in another in-vitro study, inter vertebral disc cells exposed to IL-17, IFN-γ, or TNF-α showed a remarkable increase in inflammatory mediator release and ICAM-1 expression. Synergistic increase in inflammatory mediator release from macrophages and synoviocytes in response to IL-17 alone or IL-17 combined with IFN-γ or TNF-α had been demonstrated [60, 61]. Increased expression of adhesion molecules on brain endothelial cells can lead to altered blood brain barrier permeability facilitating the entry of some immune cells in the brain [62]. Elevated level of TNF-α and IFN-γ during acute phase of GBS had been reported in patient with GBS [50].

In conclusion, IL-17 and ICAM-1 polymorphisms could be considered as potential genetic markers to GBS susceptibility upon further validation in ethnically different populations. IL-17 and ICAM-1 gene polymorphisms and their significantly higher expressions in GBS patients suggest their role in disease pathogenesis through demyelination and axonal degeneration. It might be possible that reactive Th17 cells cross the **BNB** and secret cytokines that synergistically induce ICAM1 expression and local inflammation. However, further studies are required to know the exact interaction between IL-17 and ICAM-1, and their involvement in demyelination and axonal degeneration in GBS patients.

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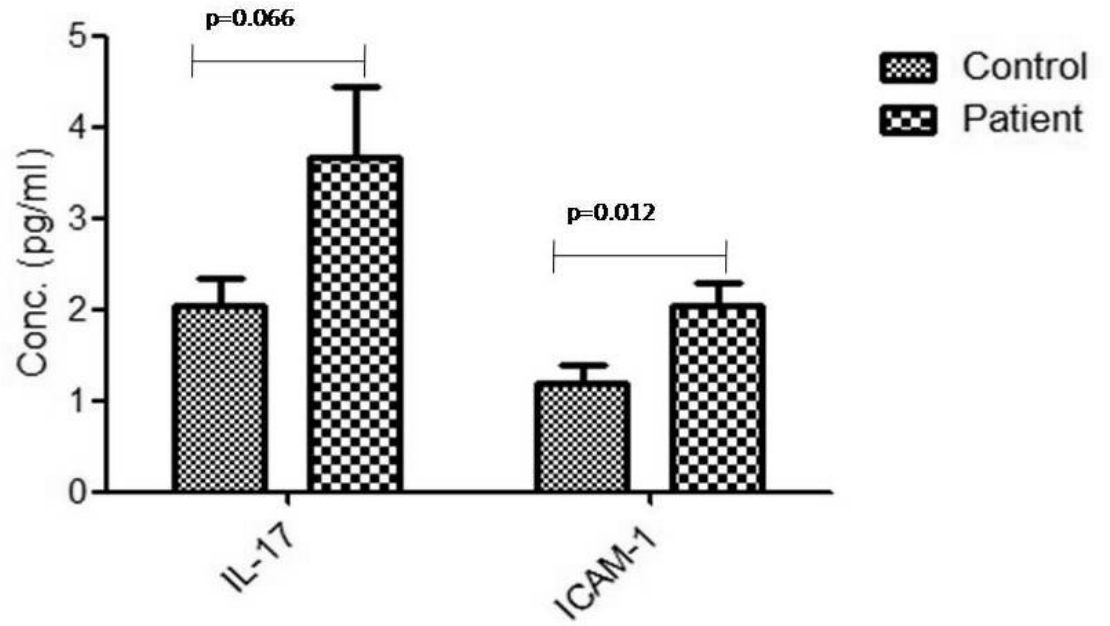

Fig 1: mRNA level of IL-17 and ICAM-1 in GBS patients and healthy controls.

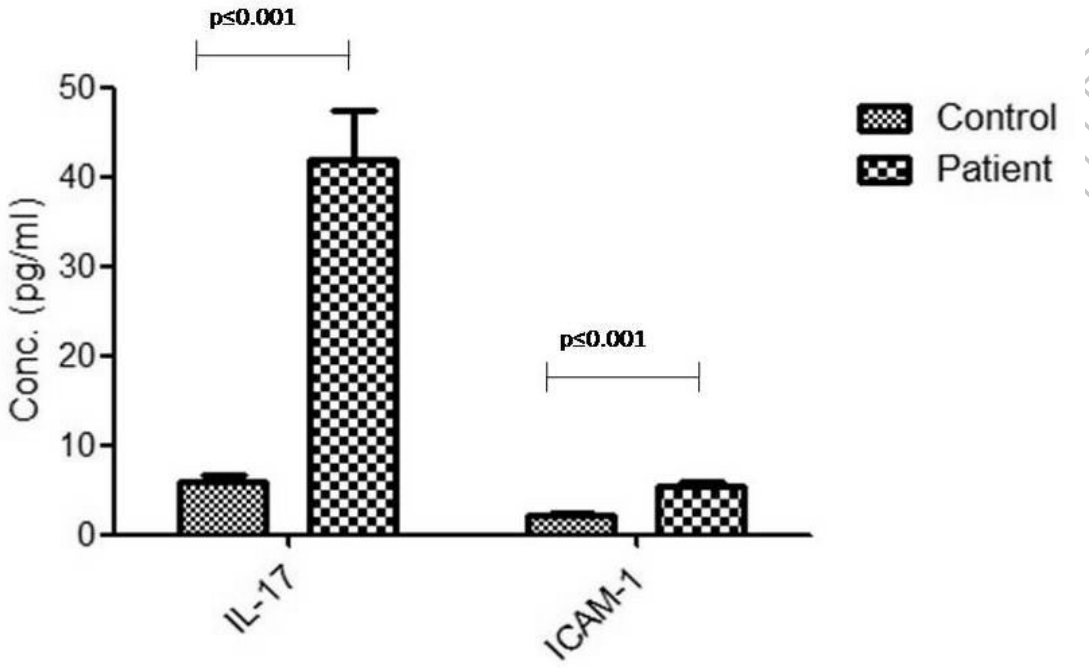

Fig 2: Serum level of IL-17 and ICAM-1 in GBS patients and healthy controls

Table1. List of primer sequences for IL-17 and ICAM-1 gene polymorphisms and RT-PCR study

Gene/Gene polymorphisms	**Methods**	**Primer sequence**	
		Forward primer	Reverse primer
IL-17 Glu126Gly	PCR-RFLP	GTGTAGGAACTTGGGCTGCATCAT	AGCTGGGAATGCAAACAAC
IL-17 His161Arg	PCR-RFLP	GTGTAGGAACTTGGGCTGCATCAT	AGCTGGGAATGCAAACAAC
ICAM1Gly241Arg	PCR-RFLP	CCGTGGTCTGTTCCCTGTAC	GAAGGAGTCGTTGCCATAG
IL-17	RT-PCR	ATGACTCCTGGGAAGACCTCATTG	TTAGGCCACATGGTGGACAATCGG
ICAM-1	RT-PCR	AATGTCATCCTGCCCCGGGGG	AGGGCAGTTTGAATAGCAA
GAPDH	RT-PCR	GTGGGCGCCCAGGCACCA	CTCCTTAATGTCACGCACGATTT

Table2. Influence of IL-17 polymorphisms on GBS susceptibility

Gene polymorphism	Patients (%)	Controls (%)	*p* value	OR[#] (95% CI)
IL-17 (Glu126Gly) Genotype				
Glu/Glu	8(10%)	12(16%)	--	Reference
Glu/Gly	30(37.5%)	46(61.33%)	0.966	0.978(0.357-2.68)
Gly/Gly	42(52.5%)	17(22.67%)	0.016	3.706(1.28-10.67)
Allele				
Glu	46(28.75)	70(46.67)	--	Reference
Gly	114(71.25)	80(53.33)	0.001	2.168(1.356-3.467)
IL-17 (His161Arg) Genotype				
His/His	35(43.75%)	28(37.34%)	--	Reference
His/Arg	39(48.75%)	45(60%)	0.274	0.693 (0.360-1.337)
Arg/Arg	6(7.5%)	2(2.6%)	0.306	2.40(0.449-12.822)
Allele				
His	109(68.2%)	101(67.34%)	--	Reference
Arg	51(31.8%)	49(32.66%)	0.882	0.964(0.599-1.553)

Table3. Influence of ICAM-1 polymorphism on GBS susceptibility.

Gene polymorphism	Patients (%)	Controls (%)	*p* value	OR[#] (95% CI)
ICAM-1 (Gly241Arg) Genotype				
Gly/Gly	24(30%)	48(64%)		Reference
Gly/Arg	56(70%)	27(36%)	≤0.001	4.148(2.119-8.119)
Arg/Arg	0	0	--	--
Allele				
Gly	104(65%)	123(82%)		Reference
Arg	56(35%)	27(18%)	0.001	2.443 (1.446-4.160)

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ASSOCIATION OF TLR2 AND IL-8 POLYMORPHISMS AND THEIR EXPRESSION IN GUILLAIN-BARRÉ SYNDROME

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ABSTRACT: Guillain-Barré syndrome (GBS) is an acute inflammatory, autoimmune disorder of peripheral nervous system. Association of TLR2 and IL-8 polymorphism with higher expression level has already been studied in many inflammatory and autoimmune diseases. However, the possible role of TLR2 and IL-8 polymorphism in GBS remains unknown. Therefore, the current study investigated the TLR-2 (Arg677Trp & Arg753Gln) and IL-8 (-251A/T) polymorphisms in 105 GBS patients and 100 healthy controls using polymerase chain reaction-restriction fragment length polymorphism analysis. Further, the expression of TLR-2 and IL-8 gene was determined by western blotting and enzyme-linked immunosorbent assay respectively. TLR2 (Arg677Trp & Arg753Gln) heterozygous genotypes were strongly associated with increased risk of GBS. TLR2 and IL-8 genes showed significantly higher expression in GBS when compared with healthy controls. In conclusion, TLR2 polymorphisms showed significant association with GBS, and their enhanced expressions and increased level of IL-8 have possible role in GBS development. TLR2 polymorphisms could be a genetic marker to GBS susceptibility.

INTRODUCTION: Guillain Barré syndrome (GBS) is an immune-mediated inflammatory disease mainly affecting the myelin and axons of peripheral nerves with heterogeneous pathological features, often triggered by an aberrant immune response towards an infectious pathogen [1]. Epidemiological studies linked it with *Campylobacter jejuni*, Cytomegalovirus, Epstein Barr virus and *Mycoplasma pneumonia* [2].

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The mechanisms involved in immunopathogenesis of GBS are still unclear. The hypothesis put forward for the immunopathogenesis of GBS points to molecular mimicry between lipopolysaccharide (LPS) and ganglioside like epitopes in host nerve cells, which leads to cross reactivity of immune response after the infection. Besides microbial factors, host susceptibility may also play an important role in the etiology of GBS, because not all infected individuals develop this disorder.

It is estimated that only 1:1000 people develop GBS after *C. jejuni* enteritis, thus highlighting the role of host genetic factors [3]. However, limited studies have been conducted for identifying the potential host factors that may impart susceptibility to GBS.

Toll-like receptors (TLRs) family plays a fundamental role in innate immunity and signal the activation of adaptive immunity [4]. One of the human Toll homologues, TLR2, has been shown to be involved in LPS signalling [5, 6, 7]. In addition to this, TLR2 is activated primarily by peptidoglycan, spirochetal glycolipids, lipoproteins and lipoarabinomannan [8, 9, 10]. Activation of TLRs enhances the transcription of several pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α via NF-κB [11, 12, 13]. IL-8 chemokine was characterized for its ability in recruitment and activation of neutrophils at inflammatory sites [14, 15]. IL-8 also promotes inflammatory processes by attracting some subsets of T lymphocytes to the site of inflammation, inducing cytokine production as well as releasing tissue damaging mediators by neutrophils [16].

TLR2 polymorphism at position 753 (Arg753Gln), an exchange of arginine by glutamine was correlated with the incidence of sepsis caused by gram-positive bacteria in human [17]. Another polymorphism in TLR2 at position 677 (Arg677Trp) was associated with susceptibility to lepromatous leprosy [18]. Several studies reported that TLR2 polymorphisms predisposed to autoimmune diseases [19, 20, 21]. So far, only one study had shown association of TLR4 polymorphism with GBS [22]. Association of IL-8-251A/T polymorphism was studied in autoimmune inflammatory diseases like multiple sclerosis (MS), systemic lupus erythromatosus (SLE) and rheumatoid arthritis (RA) [23, 24, 25].

Taking the importance of TLR2 and IL-8 in genetic susceptibility of various diseases, we hypothesized that host factors might determine the intensity of immune response towards microbial ligands, which might play a pivotal role in GBS development. The precise role of polymorphism & expression of TLR2 and IL-8 are yet not understood in GBS. In present study, we therefore investigated the association of TLR2 (Arg677Trp and Arg753Gln) and IL-8 polymorphisms (-251A/T), and their expressions with GBS.

METHODS:

Study population: Patients admitted to Neurology ward, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow were enrolled for the study. The Institutional ethics committee granted approval for this study and consent was obtained from all the study subjects. A total of 105 patients (male 80) with GBS, mean age ± SD, 30.20 ± 10.98 years, and sex/age matched 100 healthy controls (male 76), mean age 28.12 ± 16.97 years were included in study. GBS patients were selected on the basis of criteria as described earlier [26] and healthy controls were individuals without any history of apparent infectious illness within the preceding 4 weeks. Patients with GBS had not received any immunosuppressive or immunomodulatory treatment in the last 2 months prior to sample collection.

Sample Collection:

Blood samples were collected through peripheral vein puncture during the first 2 weeks after the onset of GBS. Blood in ethylene diamine tetra acetate (EDTA) was stored at -20°C for DNA extraction. Sera from clotted blood were separated and stored at -80°C till further use for enzyme-linked immunosorbent assay (ELISA). Peripheral blood mononuclear cells (PBMCs) were separated from heparinised blood and stored at -20°C for protein extraction.

Genomic DNA isolation:

Genomic DNA was extracted from whole blood using salting out method. DNA samples were stored at -20ºC till further use for genotyping.

TLR-2 genotyping (Arg677Trp& Arg753Gln):

Single nucleotide polymorphisms (SNPs) were detected by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis. DNA samples of 100 ng/µl concentration were used for SNP detection; primer sequences for TLR-2 (Arg677Trp& Arg753Gln) amplifications are shown in table1. PCR amplifications were performed in a 25 µL volume containing 10 X assay buffer, 200 mM each of dATP, dCTP, dGTP, dTTP, 0.1 mM of each primer, 1.0 U of Taq DNA polymerase (Bangalore Genei, Bengaluru, India). PCR protocols were as follows: initial denaturation for 10 min at 95^0C followed by 35 PCR cycles of denaturation for 30 s at 94^0C, annealing for 30 s at 58^0C, extension for 30 s at 72 ^{0}C with final extension of 5 min at 72^0C. Amplification products

(20 µl) were digested with 1U of AciI (Fermentas, Burlington, Canada) at 37^0C overnight, electrophoresed on 3.5% agarose (Sigma–Aldrich, St Louis, MO, USA) gel, visualized under UV illumination and stained with ethidium bromide.

IL-8-251 A/T genotyping:

IL-8-251A/T genotype was determined by PCR–RFLP as mentioned above. IL-8 specific primers are shown in **Table 1**. For RFLP analysis, the PCR products (15 μL) were digested with 1 U of MunI restriction enzyme (Fermentas Life Science, USA) at 37^0C overnight, electrophoresed on 3.5% agarose (Sigma–Aldrich, St Louis, MO, USA) gel, visualized under UV illumination and stained with ethidium bromide.

TABLE 1: LIST OF PRIMER SEQUENCES FOR TOLL-LIKE RECEPTOR 2 (TLR2) AND INTERLEUKIN 8 (IL-8) GENE POLYMORPHISMS

Gene polymorphism	Method	Primer sequence	
		Forward primer	Reverse primer
TLR2 (Arg677Trp)	PCR-RFLP	GGGACTTCATTCCTGGCAAGT	GGCCACTCCAGGTAGGTCTT
TLR2 Arg753Gln	PCR-RFLP	GCCTACTGGGTGGAGAACCT	GGCCACTCCAGGTAGGTCTT
IL-8(-251T > A)	PCR-RFLP	CATGATAGCATCTGTAATTAACTG	CTCATCTTTTCATTATGTCAG AG

Western Blotting:

Total protein extracts from PBMCs were prepared by whole cell lysate preparation procedure [27] and supernatants were stored at-20°C. Protein concentration of the cell lysate was determined by Bradford assay method [28]. The constituent proteins of the PBMCs were separated by SDS-PAGE on a 10% separating gel and then transferred to nitrocellulose membranes (Sartorius, Gottingen, Germany). In order to verify the equivalent loadings of proteins in the wells, the gel and the nitrocellulose membrane were stained with Coomassie brillant blue and Ponceau S respectively (Sigma). Membranes were blocked by incubation in tris-buffered saline (150 mM NaCl, 25 mM Tris pH 7.5), containing 0.05% Tween 20 (Sigma) and 5% non-fat dry milk and incubated overnight at 4^0C. Following morning, the membrane was washed in TBS-T (TBS containing 0.05% Tween-20) for 5 min with three changes.

The membrane was then blotted by transferring into a primary anti-rabbit antibody solution (1:1000) and kept at 40^0C for 6 hrs. The blotted membrane was washed thrice as described before and then transferred into the secondary antibody solution and probed with anti-goat horseradish peroxidase (HRP) conjugated IgG antibody and kept at room temperature for 1 hr. The membrane was washed thrice with TBS-T. HRP development reagent (ECL detection kit, GE Healthcare, Buckinghamshire, UK) was supplied as two solutions, one containing Lumiglo and the other containing peroxide. These two solutions were mixed in the ratio of 1:40 and the membrane was immersed in the mixture for 1 min, wrapped with saran wrap exposed to X-ray film and developed. Density of the proteins on the autoradiogram was quantified by Bio-Rad model GS-700 imaging densitometer using molecular analyst software, version 1.5 (Bio-Rad, CA, USA). The autoradiograms were scanned in the transmittance mode at a resolution setting of 150 dpi, using a gray filter. The Intensity of bands were compared on the basis of adjusted volume (mean optical density area in square millimetres).The densitometric scans from three experiments normalized with native β-actin are shown (**Fig.1**).

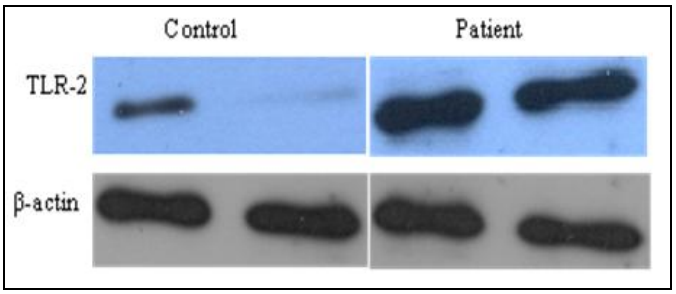

FIG. 1: DENSITOMETRIC SCANS OF REPRESENTATIVE WESTERN BLOTS SHOWING EXPRESSION OF TLR-2 IN CONTROL AND GBS PATIENT FROM THREE EXPERIMENTS, NORMALIZED TO NATIVE β -ACTIN LEVELS

ELISA:

Human IL-8 was measured in the serum using commercial ELISA kit (Invitrogen, Carisbad, USA) following manufacturer's instruction. All samples were measured in triplicates. For IL-8 serum

samples were diluted to a ratio of 1:100 using assay buffer. The detection limit of the kit for IL-8 is 0·31 ng/ml. The optical density of the wells was determined using a microplate reader set at 450 nm.

Statistical analysis:

The SPSS 16.0 statistical package (Chicago, IL, USA) was used for data management and analysis. Power of the study was calculated using Quanto software version 1.0 (http://hydra.usc.edu/gxe) to achieve 80% of the statistical power for Odds Ratio (OR) ≥ 2.0 at significance level (α) <0.05. Logistic regression analysis was applied to estimate association with GBS susceptibility after adjusting for age and gender and considered significant if the *p* values were <0.05. Hardy-Weinberg equilibrium was checked in controls by goodness of fit χ^2 test. For comparisons between the groups of study populations $\chi 2$ test was used. ELISA data was expressed as mean ± SD of triplicate experiments performed independently for each sample. One-way anova: Post Hock (Bonferroni) test was performed to determine the expression level of IL-8 and to compare continuous data (age). The statistical significance of the data of Western blot was determined using Student's *t* test with Graph Pad Prism software (San Diego, CA, USA) and triplicate experiments performed independently for each sample.

RESULTS:

Genetic polymorphism:

Genotype and allele frequencies in GBS and healthy controls are shown in **Tables 2** and **3.** Both polymorphisms were in agreement with Hardy-Weinberg equilibrium in controls.

Frequency distribution of the TLR2 Arg753Gln and Arg677Trp Genotype variants in patients with GBS and control groups:

Genotypic distributions of TLR2 Arg753Gln and Arg677Trp polymorphisms demonstrated increased risk of GBS for heterozygous genotypes Arg/Gln (p<0.0001; OR, 8.17; 95% CI, 3.26-20.48) and Arg/Trp (p<0.0001; OR, 86.62; 95% CI, 11.63-644.77). The frequency of wild homozygous genotypes was observed at 65.7% vs 94% for TLR2 Arg753Gln and 53.33% vs 99% for Arg677Trp polymorphisms in patients and controls. Patients with GBS showed significantly higher frequency in comparison to controls for allele 753Gln (17.14% vs 6%, p<0.0006; OR, 3.24; 95% CI, 1.63-6.43) and allele 677Trp (23.34% vs 0.5%, p<0.0001; OR, 60.56; 95% CI, 8.26-443.61) (**Table 2**).

TABLE 2: TOLL-LIKE RECEPTOR 2 (TLR-2) POLYMORPHISMS IN GBS PATIENTS AND HEALTHY CONTROLS.

Gene polymorphism	Patients (%)	Controls (%)	*p* value	OR# (95% CI)
TLR2Arg753Gln Genotype				
Arg/ Arg	69(65.7%)	94(94%)	≤0.0001	0.12 (0.04-0.30)
Arg /Gln	36(34.3%)	6(6%)	≤0.0001	8.17(3.26-20.48)
Gln/Gln	0(0%)	0(0%)	--	--
Allele				
Arg	174(82.86%)	188(94%)	0.0006	0.30(0.15-0.61)
Gln	36(17.14%)	12(6%)	0.0006	3.24 (1.63—6.43)
TLR2Arg677Trp Genotype				
Arg / Arg	56(53.33%)	99(99%)	≤0.0001	0.01 (0.001-0.08)
Arg /Trp	49(46.67%)	1(1%)	≤0.0001	86.62(11.63-644.77)
Trp / Trp	0(0%)	0(0%)	--	--
Allele				
Arg	161(76.66%)	199(99.5%)	<0.0001	0.01(0.002-0.12)
Trp	49(23.34%)	1(0.5%)	<0.0001	60.56(8.26-443.61)

Frequency distribution of the IL-8-251 A/T variant in patients with GBS and healthy controls: To analyze the association of IL-8 polymorphisms with GBS, the genotype frequency was compared between controls and patients with GBS. The logistic regression analysis revealed no significant association of IL-8-251A/T polymorphism (polymorphic and heterozygous) with GBS (p=0.8748 and p=0.0698) (data is shown in **Table 3**).

TABLE 3: IL-8-251A/T POLYMORPHISMS AND GBS SUSCEPTIBILITY

Gene polymorphism	Patients (%)	Controls (%)	*p* value	OR# (95% CI)
IL-8(-251A/T) Genotype				
A/A	18 (17.14%)	29 (29%)	0.0476	0.50 (0.26-0.98)
A / T	60(57.14%)	44(44%)	0.0698	1.69 (0.97-2.94)
T /T	27(25.71%)	27(27%)	0.8748	0.93 (0.50-1.74)
Allele				
A	96(45.71%)	102(51%)	0.322	0.80 (0.54-1.19)
T	114(54.28%)	98(49%)	0.322	1.23 (0.83-1.82)

Expressions of IL-8 and TLR2:

Enhanced expression of TLR-2 was observed in GBS patients (fold change-1.112) compared to healthy controls (fold change-1) shown in **Fig. 2**. Level of IL-8 was elevated in sera of GBS patients than healthy controls (112.55±13.96 vs 34.79±4.373; $p \leq 0.001$) shown in **Fig.3.**

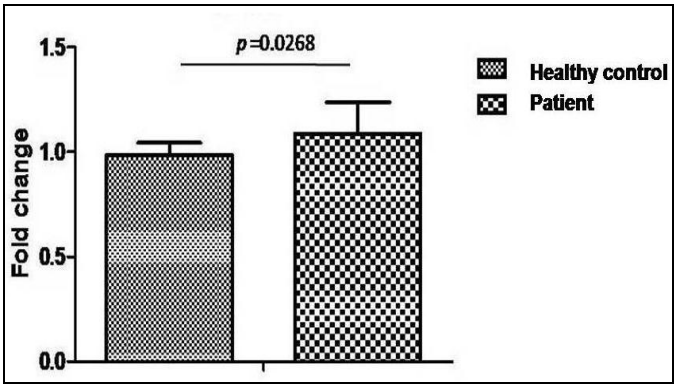

FIG. 2: DATA SHOWS THE ENHANCED EXPRESSION OF TLR-2 IN GBS PATIENTS (FOLD CHANGE-1.112) COMPARED TO CONTROLS (FOLD CHANGE 1).

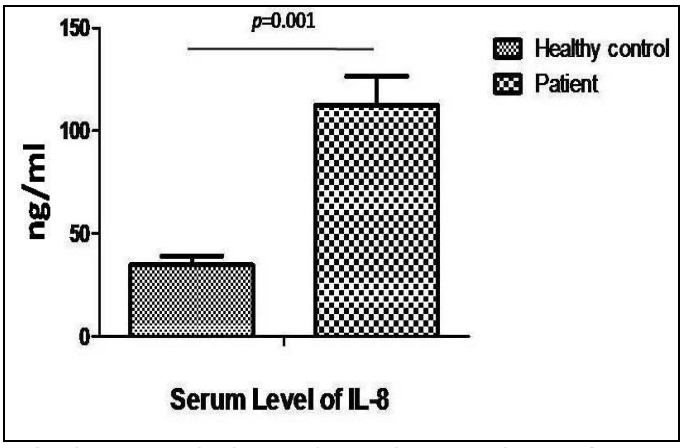

FIG. 3: LEVELS OF IL-8 IN SERUM OF HEALTHY CONTROLS AND GBS PATIENTS

DISCUSSION: In the present study, we investigated the association of TLR2 polymorphism (Arg753Gln and Arg677Trp) with GBS and its relative expression. We also investigated the expression of IL-8 and its gene polymorphism in patients with GBS. TLRs play central role in host defences and are involved in a number of autoimmune diseases including GBS. The magnitude of the association of polymorphisms with autoimmune disease varies depending on genetics, demographics and environmental factors. Many association studies reported that TLR-2 polymorphisms predisposed to autoimmune disease [19, 20, 21]. However, some polymorphisms of TLR2 might not be associated with autoimmune disease like rheumatoid arthritis (RA) [29]. However, there is no data regarding TLR2 polymorphism with GBS till date.

The importance of TLR2 polymorphism in GBS is still largely unknown. In our study, the variant allele frequency of TLR2-753Gln and TLR2-677Trp was higher in GBS than controls (17.14% vs 6%, p<0.0006; OR, 3.24; 95% CI, 1.63-6.43 & 23.34% vs 0.5%, p<0.0001; OR, 60.56; 95% CI, 8.26-443.61). The reported rate of TLR2-Arg753Gln variant frequency was 1% in Spanish population [30] whereas the TLR2-Arg677Trp was 30.3% in Croatian population [30, 31]. In separate studies, the Arg753Gln genotype was observed among 10.34% [32] and 12.3% [33] of healthy Turkish subjects, while the Arg677Trp was not observed. Contrary to the study conducted by Kang et al (2001), other authors failed to detect these TLR2 polymorphisms in the Korean population. In the Caucasian population, the TLR2-Arg753Gln SNP was detected in 9.4% of the German whites, while the Arg677Trp polymorphism was not observed at all [34].

The data regarding role of TLR2 gene polymorphism in inflammatory demyelinating diseases including GBS is limited. There is a single

study that suggests association of TLR4 Asp299Gly and Thr399Ile polymorphisms with risk for development of GBS [22].

We conducted the present study in our population and a positive association of TLR2 polymorphism (Arg753Gln and Arg677Trp) was observed with GBS. Polymorphism in the TLR2 gene causes inappropriate activation of the dendritic cells. These dendritic cells kick off cell maturation and increase the expression of major histocompatibility complex (MHC) and co-stimulatory molecule B7, and activate T cells which secrete increased amount of several chemokines and pro-inflammatory cytokines such as TNF-α responsible for demyelination. There are reports which show that increased level of TNF-α contributes to the pathogenesis of immune mediated demyelinating neuropathies and axonal degeneration by inducing damage to myelin [35].

Besides the association of TLR2 with GBS, we also observed the enhanced expression of TLR2 in patient with GBS. The data regarding expression of TLR2 in GBS is very limited. So far, one study showed significantly elevated TLR2 expression in sciatic nerves during GBS [36]. An increased expression of TLR2 had been shown in synovial tissues of patients with RA [37].

In the present study, we found elevated TLR2 expression in PBMCs of patients with GBS (fold change-1.112) when compared to healthy control (fold change 1). TLRs play a central role in the initiation of both innate and adaptive immune responses against microbial pathogens through myeloid differentiation (MyD88) dependent primary response gene or MyD88-independent transduction pathway [38]. Each member of the TLR family has its own ligand from different pathogens, which helps in inducing a danger signal when pathogen invades the host and results in the activation of NF-κB and subsequent induction of signal transduction cascade. TLR2 can deliver co-stimulatory T cell signals for cell expansion and can induce proliferation of regulatory T cells [39]; its signalling favours Th17 cell expansion.

It was shown in the rat model of experimental autoimmune neuritis (EAN) that TLR2 was expressed in inflamed nervous tissue and NFκB was increased in activated T cells and macrophages [40]. In EAN, potential endogenous TLR ligands generated following tissue damage or inflammation may also activate their TLRs and thereby play roles in the pathological progress of the disease.TLR2+, CD14+, and Hsp70+cell accumulation was detected and positively correlated with neurologic disease severity in sciatic nerves of EAN rats, suggesting the involvement of innate immunity in the effect or phase of disease [40]. From our study, we hypothesize that the increased/enhanced level of TLR2 might deliver potent co-stimulatory signals to antigen activated T cells which secrete increased amount of several chemokine and pro-inflammatory cytokines such as TNF-α responsible for demyelination.

Expression of IL-8 and its role in disease pathogenesis in GBS still remain unknown. An earlier study had shown significantly higher IL-8 secretion from PBMCs of MS patients compared to controls [41]. In present study, we found the level of IL-8 significantly higher (112.55±13.96 vs 34.79±4.37; p=0.001) in GBS patients compared to healthy controls. Activation of TLRs enhances the transcription of several pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α via NF-κB [11, 12, 13].

It is also reported that IL-8 and CXCL1 production by human astrocytes at both the RNA and protein levels can be induced by IL-1β in MS patients [42]. In another study, IL-1β and IL-17 induced the production of IL-8 in endothelial and parenchymal cell indicating an indirect role in polymorphonuclear neutrophil recruitment [43]. In another study, the level of IL-1β was found significantly higher in chicken model of GBS [44]. Elevated expression of TLR2 in GBS as observed in our study indicates that TLR2 activation enhances the transcription of several pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α via NFκB and IL-1β in turn stimulates the expression of IL-8 that may have indirect role in recruitment and activation of neutrophil at neuronal sites in GBS.

Besides the IL-8 expression study in GBS, we also looked for the polymorphism of IL-8 gene in GBS but we did not observe the any association of IL-8-251A/T polymorphism with our GBS patients.

Similar observation in another study reported that functional genetic variation in IL-8 did not play a major role in SLE susceptibility in the Spanish population [24]. In another study, the genetic polymorphisms of the CXCL8 gene were not associated with systemic sclerosis. On contrary to these studies, association of IL-8-251 A/T polymorphism with MS was reported in Iranian patients [23].

In summary, our study indicates that TLR2 polymorphisms and their enhanced expressions were associated with GBS susceptibility in Indian patients. In addition, enhanced IL-8 production without its gene polymorphism was also observed in GBS patients. However, interaction between TLR2 activation and expression of IL-8 in absence of IL-8 gene polymorphisms, and their exact role in disease pathogenesis calls for further study. Similar studies in different ethnic populations are required to clarify the role of TLR2 and IL-8 in GBS patients.

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