ASSOCIATION OF HLA-DRB1*1501 TAGGING rs3135388 GENE POLYMORPHISM WITH MULTIPLE SCLEROSIS SUSCEPTIBILITY

Thesis submitted for the partial fulfilment for the requirement of the degree of DM Neurology

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DECLARATION

I, **Dr. Arun K** hereby declare that the thesis "**ASSOCIATION OF HLADRB1*1501 TAGGING rs3135388 GENE POLYMORPHISM WITH MULTIPLE SCLEROSIS SUSCEPTIBILITY"** was undertaken by me under the guidance and supervision of Dr C. Sarada, Professor, Department of Neurology at the Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram.

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CERTIFICATE

This is to certify that the thesis titled "**ASSOCIATION OF HLA-DRB1 *1501 TAGGING rs3135388 GENE POLYMORPHISM WITH MULTIPLE SCLEROSIS SUSCEPTIBILITY"**, is the bonafide work of Dr Arun K, Senior Resident, DM Neurology and has been done under my direct guidance and supervision at the Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram. He has shown keen interest in the research project and actively participated in all its phases.

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INTRODUCTION

Multiple sclerosis (MS) is an autoimmune disease of the central nervous system characterized by chronic inflammation, demyelination, gliosis, and neuronal loss. The course of the disease can be relapsing-remitting or progressive. Lesions of MS typically develop at different times and in different central nervous system locations. Majority of the patients are unemployed and disabled within 15 years of diagnosis of the disease and rates of depression, suicide, and divorce are substantially increased compared with the healthy population. About 50% of all patients need assistance for activities of daily living within 20 years of diagnosis, and approximately 50% of patients eventually develop substantial cognitive deficits. The disease often starts between 20 and 40 years of age, and affects women more frequently than men. According to the MS database, worldwide about 2·5 million people have MS, and figures from the MS International Federation states that in Europe alone the disease costs more than €15 billion / year in terms of direct health-care costs and lost productivity.

Although the precise etiology of MS remains unknown, in the recent years the data on the genetic variants affecting the development of the disease has grown substantially. Association with the human leukocyte antigen (HLA) genes has been confirmed in virtually all populations studied and the DRB1*1501 allele has been established as the main risk factor. In a collaborative GWAS it was proven that HLA-DRB1* 1501 has the strongest association with MS, with a consistent influence within the cohort ($P=1\times10^{-320}$, OR=3. 1)[3]. Stratification for this known risk factor is expected to aid in the identification of additional susceptibility factors and

to help discover their interactions. It is therefore reasoned that a simple and straightforward assay to establish DRB1*1501 genotype without the need for more elaborate classical HLA-DRB1 typing would be of immense value. Major efforts addressing the genetic variation across the major histocompatibility complex have confirmed the extremely polymorphic nature of the HLA-DRB1 gene. This exceptional degree of polymorphism contributes to the high failure rate of genotyping assays in this region. Zivkovic et al (2009) [18] found significantly higher frequency of rs3135388 A allele carriers in MS patients than in control group. Similar studies by Alcinia (2012)[30] et al and Benesova et al (2013)[6] also reported that rs3135388 gene polymorphism is a strong risk factor for MS susceptibility. Typing this SNP, they found a correlation coefficient (r^2) of 0. 97 between rs3135388*A and HLADRB1*1501. Aiming to reduce time and expenses in HLA MS risk allele genotyping, the present study has been designed for detection of HLA rs3135388 SNP and its association with MS susceptibility in South Indian population.

REVIEW OF LITERATURE

Introduction

Multiple sclerosis (MS) was initially described as a demyelinating disorder that predominantly affects the white matter tracts within the central nervous system. However the diagnosis, categorization, and treatment of multiple sclerosis (MS) and other demyelinating diseases have shifted over the past decade, and many of the fundamental principles of MS pathogenesis and clinical course are being rewritten. MS is no longer considered intrinsically a "white matter disease," as it is now recognized to be a disease affecting gray matter as pervasively as white. Indeed, gray matter involvement is likely a better predictor of clinical course and disability than the white matter lesions that have long been considered the imaging hallmark of this disease[31]. MS is no longer thought to be a purely T-cell driven disease, as both pathologic and clinical trial data implicate B-cell involvement in MS pathogenesis and as an apt therapeutic target. And MS is now understood to be a disease affecting cognition often and early in the course, rather than rarely and as a late consequence of disease. MS has been a treatable disease since the regulatory approval of disease-modifying therapies in the 1990s, but the explosion of therapeutics in the past 10 years is unprecedented and has brought with it both great opportunities and great challenges for optimal patient outcomes[7].

The variability in clinical presentation is well known in patients with MS. Lesions of MS typically develop at different times and in different central nervous system (CNS) locations (i. e., MS is said to be disseminated in time and space). Approximately 350,000 individuals in the United States and 2. 5 million individuals worldwide are affected with MS[31].

Clinical Manifestations of MS

The clinical course can be extremely variable, ranging from a benign condition to a rapidly evolving and incapacitating disease requiring profound lifestyle adjustments. MS is approximately threefold more common inwomen. The age of onset is typically between 20 and 40 years, but the disease can presentacross the lifespan. Approximately 10% of cases begin before age18 years, and a small percentage of cases begin before10 years of age[31].

Acute demyelinating optic neuritis is the presenting symptom in about 20% of MS patients. Weakness affects up to 80-90% of MS patients in the disease course. The brainstem is commonly affected in MS. The clinical syndromes produced by brainstem involvement in MS include: double vision, internuclear ophthalmoplegia, facial weakness or myokymia, vertigo or bulbar symptoms and facial sensory impairment. Involvement of cerebellar networks that connectwith the brainstem can lead to unilateral ataxia, dysmetria or dysdiadochokinesia. Acquired pendular nystagmus in MS is believed to be caused by a disruption of the cerebellopontine networks. The myelitis that occurs in MS is typically partial and usually presents sub acutely[7]. Tonic spasms are also seen in association with spinal cord and brainstem lesions. Numbness and paresthesias are common sensory symptoms experienced by patients with multiple sclerosis. Sensory complaints affect 80-90% of MS patients at some point in the course of disease and can be presenting syndrome in 30-40%. Pain and other unpleasant sensations were reported as troubling symptoms by 50-55% of MS patients with Lhermitte's symptom occurring in up to one-third. Subtle cognitive impairment can occur in 40–70% of MS patients. The most commonly affected cognitive domains are slowed information processing, executive

dysfunction, and impairment of long-term verbal and visual memory. Major depression occurs in about 30–45% of MS patients during the disease course. Fatigue is one of the most debilitating symptoms in MS and has been reported as a current symptom in 80-85% of patients. Neurogenic bladder and lower urinary tract impairment is an important cause of disability in MS. One of the most common manifestations of neurogenic bladder in MS is detrusor hyperreflexia which is present in about two-thirds of MS patients and about 20–25% exhibit symptoms of urinary frequency and incomplete emptying. The most difficult urinary condition to manage in MS is that of detrusor-sphincter dyssynergia. Bowel dysfunction in MS is less common and constipation is the most frequent manifestation. Sexual dysfunction is reported to affect up to one third of patients and up to 75-80% of men and 50–70% of women which include erectile dysfunction in men and loss of libido and/or fatigue in women. Heat characteristically aggravates MS symptoms. About two-thirds of MS patients complain of headaches and are attributable to migraine. Transient worsening of MS symptoms can occur in the context of infection and stressors[31].

1. Relapsing-remitting (RR) MS: Clearly defined relapses with full recovery or with sequelae and residual deficit on recovery. The periods between disease relapses are characterized by a lack of disease progression.

2. Secondary progressive (SP) MS: Initial relapsing-remitting disease course followed by progression with or without occasional relapses, minor remissions, and plateaus.

3. Primary progressive (PP) MS: Disease progression from onset, with occasional plateaus and temporary minor improvements allowed.

4. Progressive relapsing (PR) MS: Progressive disease from onset, with clear acute relapses with or without full recovery. The periods between relapses are characterized by continuing progression. [31]

Pathophysiology of MS

The symptoms and signs of MS are the manifestations of the pathological process seen in the CNS, namely demyelination and a moderate degree of axonal loss. Demyelination interrupts current flow by removing the insulator of internodal axon current flow. Long segments of demyelination can result in interruption of current flow. The low density of internodal Na+ channels, at least in the early stages of demyelination, inhibits impulse propagation. If conduction does occur, it is at a much reduced speed (5% to 10% of normal). The refractory period of demyelinated axons is prolonged, and repetitive volleys may be blocked when encountering an axon segment in arefractory period. [31] Persistent neurological deficits or negative symptoms of MS are caused by regions in which conduction block persists, such as in regions of large plaques, whereas transient worsening of function reflects a drop below the safety threshold for conduction because of physiological changes

involving the partially demyelinated axon (Uhthoff phenomenon, worsening with increased body temperature). Symptoms or signs may also arise from slowed conduction, producing temporal dispersion at time-critical synapses. Conduction block is absolute in transected axons. Mechanical stimulation of demyelinated axons can generate de novo action potentials in the axon and may explain the Lhermitte's sign, electric shock like sensations on flexing the neck. Spontaneous action potentials have been recorded from demyelinated axons and, if present in the CNS, could explain paroxysmal-positive symptoms of MS[31].

Pathology

The pathological hallmark of MS is the *cerebral or spinal plaque*, which consists of a discrete region of demyelination with relative preservation of axons, although spectroscopic and pathological studies suggest some axonal loss may be an integral part of the disease process. Plaques may be visible on the surface of the spinal cord on inspection. The cut surface of the brain reveals the plaques, which when active, appear whitish yellow or pink with somewhat indistinct borders. Older plaques appear translucent with a blue-gray discoloration and sharply demarcated margins. Plaques develop in a perivenular distribution and are seen most frequently in the periventricular white matter, brainstem, and spinal cord. One of the earliest features of acute MS lesions is a disruption of the blood–brain barrier (BBB). It can carry water, proteins, antibodies, and cytokines (and gadolinium) into the brain. The fate of oligodendroglia in MS lesions is disputed. Consensus is that oligodendroglia numbers are reduced proportionate to myelin loss in the plaque center, whereas at the plaque edge, oligodendroglia are preserved or even increased, suggesting an attempt at remyelination. Activated T cells and the microglia-macrophages can

contribute to tissue injury via non-antigen-restricted mechanisms. Each of these cell types releases an array of soluble factors that can contribute to tissue injury, including oligodendroglia. B cells and Ig are also found in MS lesions. To date, no specific antibody has been identified in MS, but antimyelin antibodies have been shown to enhance disease severity in the experimental allergic encephalomyelitis (EAE) model, suggesting that both cellular and humoral mechanisms may be needed for full expression of immune injury. Histological examination of active plaques reveals perivascular infiltration of lymphocytes (predominantly T cells) and macrophages, with occasional plasma cells. In the plaque, myelin is disrupted, resulting in myelin debris found in clumps or within lipid-laden macrophages. Macrophages appear to have an integral role in stripping myelin lamellae from axons. Reactive astrocytes are prominent in plaques. Immunohistochemical studies have found increased levels of cytokines in active plaques, indicative of on going immunore activity. Chronic inactive plaques are hypocellular and show astrocytic proliferation with denuded axons and an absence of oligodendroglia. Axonal loss of variable degree may be detected. Recent pathological studies have focused on the gray matter in MS and have found a lesion load within the cortex and deep gray structures. The nature of the intracortical plaques differs from those seen in white matter because there is less inflammation but considerable reactive microgliosis[31].

Risk factors for MS

Several viral and bacterial peptides share structural similarities with important proteins of myelin, and a few of them are able to activate specific T-cell clones derived from patients with MS. Myelin basic protein (MBP) has long been considered one of the primary candidates for an autoimmune attack. Several other

myelin proteins are also candidates for an autoimmune attack. Proteolipid protein accounts for 50% of CNS myelin protein and is an integral membrane protein of the myelin leaflets. Myelin-associated glycoprotein, myelin oligodendrocyte glycoprotein, and cyclic nucleotide phosphodiesterase are proteins that account for a few percent of myelin[7]. Although the possibility of autoimmunity as the causal mechanism for MS exists, the issue is not proven. The evidence for MS being a dysimmune condition is more compelling, with alterations in immune cell repertoire and activation state both in blood and CSF of MS patients compared to others. In recent decades pathogens such as human herpesvirus 6 (HHV6), Epstein–Barr virus (EBV), and *Chlamydia pneumoniae* have been the focus of interest as potential triggers for MS. Potential mechanisms invoking EBV in the development of MS include an inappropriate autoreactive immune response as the result of molecular mimicry or a more direct role of the EBV promoting persistent inflammation in the central nervous system. Although there is no definitive evidence for vitamin D deficiency as a causative factor in the pathogenesis of multiple sclerosis, low levels of vitamin D have now been associated with an increased risk for MS in many different studies. This led to the hypothesis that decreasing levels of vitamin D, related to lower levels of sun exposure, could explain this phenomenon. The association between smoking and an increased risk for MS has been established with evidence from multiple case control studies. There is also evidence, that smokers have a more severe course than nonsmokers. Smoking is thought to be a direct neurotoxin, but also may bring about immunomodulatory changes that promote inflammation[31].

Genetics of Multiple sclerosis

The Major Histocompatibility complex

Although associations between multiple sclerosis and variation in the genes encoding human leucocyte antigens (HLAs) contained within the major histocompatibility complex have been recognized for several decades, the extreme polymorphism and extensive linkage disequilibrium that characterize this region make the identification of these associations difficult. However, in the recent years the advent of high-throughput typing for single-nucleotide polymorphisms (SNPs), have enabled the study of several individuals, which in turn has allowed substantial progress to be made in this field. It is now established that the association with the haplotype exerting the greatest effect on risk is driven by the HLA-DRB1*15:01 allele, and that association with the other alleles of this haplotype is secondary only to their linkage disequilibrium with HLA-DRB1*15:01. Although these SNP-based studies have not yet provided convincing data to support the existence of complex interactions between these alleles and haplotypes, such interactions have been proposed in many studies. Such interactions almost does occur, but theoretical calculations points that very large sample sizes will be needed to reliably identify their nature and establish the alleles involved.

A large GWAS was conducted as part of the Wellcome Trust Case Control Consortium 2 (WTCCC2) project, cases recruited through the International Multiple Sclerosis Genetics Consortium (IMSGC) and they found that DRB1*15:01 has the strongest association with multiple sclerosis amongst all classical and SNP alleles ($p < 1 \times 10^{-32^{0}}$). Conditioning on DRB1*15:01, they confirmed the presence of aprotective Class I allele and identified the signal as being driven by HLA-A*02:01

($p = 9.\ 1\times10^{-23}$) and conditioning on both DRB1*15:01 and A*02:01 disclosed additional risk associated with the strongly linked alleles DRB1*03:01 and DQB1*02:01 ($p = 3.\ 6\times10^{-10}$)[3].

Studies in Lithuanian population, showed that HLA DRB1*1501 allele were frequently associated in patients with progressive MS, with the beginning of the disease, female gender and a very bad prognosis[2]. Smestad et al. (2007)[34], Hensiek et al (2002)[12], Van der Walt et al (2011)[2], , Wu et al. (2010)[36] have previously proposed that this allele may be strongly associated with the disease, its first symptoms in younger individuals and more aggressive manifestations. However, Romero-Pinel et al (2011)[33] haven't found any significant association between age of MS development and the different HLA alleles in the Spanish population. On the contrary, studies conducted by Masterman et al, 2000[51]; Smestad et al. , 2007[34] ; Hensiek et al. , 2002[12] found a significant association between a lower age at onset and the HLA-DR15 haplotype.

Balnyte et al. (2013)[2] published that, in more than 80% of Lithuanian patients with MS, oligo clonal Bands (OCBs) were detected in their cerebrospinal fluid (CSF). Other studies in Australia (Wu et al. , 2011)[36], Turkey (Idiman et al. , 2009)[38], and Spain (Romero-Pinel et al. , 2011)[33]also reported similar conclusions. OCBs were more frequently found in those patients with HLA DRB1*15 allele. The study of Romero-Pinel et al. (2011)[33] also showed the association between HLA DRB1*15 and oligoclonal bands in MS, in the Spanish population - as the reports of Kikuchi et al. (2003)[39], Wu et al. (2010)[36], Imrel et al. (2006)[42].

In Sardinia, where MS have a high incidence, a genomic association study of the Inter- national Multiple Sclerosis Genetic Consortium, in 2011, confirmed that

DRB1*1501 is the most significant genetic factor for developing MS. Subsequently small number of studies in non-European populations, reported lower prevalence of DRB1*1501 allele in MS patients. On contarary, studies conducted by Masterman et al. , 2000[51]; Barcellos et al. , 2006[4]; Hensiek et al. , 2002[12]; Weinshenker et al. [37], 1998and Runmarker et al. ,[52] 1994 haven't found any association between DRB1*15 allele and the severity of the disease. Meanwhile, and Wu et al. (2010)[36], in dis-agreement have reported that patients with positive HLA DRB1*15 were related to a worst prognosis, not a better one. In Irish patients, HLA-DR15 positivity were associated with earlier beginning of MS and to female gender. Irizar et al. (2012)[54], studying the Japanese population, reported that HLA DRB1*15 allele confers greater risk of MS only for women.

Kollaee et al. (2012)[32] compared the alleles of HLA-DRB1*15 in the Iranian population, and reported that this allele is associated with relapsing–remitting multiple sclerosis (RRMS), compared to the control group. This findings were consistent with some other reports in Iranian relapsing–remitting and primary progressive MS patients (Kalanie et al. , 2000[58]), but it is inconsistent with the study conducted by Amir-Zargar et al. (1998[59]).

Kollae et al. (2012)[32], also pointed that the HLA DRB1*1501 allele is not the only predisposing factor to MS. In MS patients, the odds ratio (OR = 7. 792) for the DRB1*1501-DQB1*0602 haplotype have shown to be more significant in the patients that present the DRB1*1501 allele alone (OR = 3. 203). Studies conducted by Cocco et al (2012)[43] in the Sardinian population have confirmed that a higher MS susceptibility is associated with the*13:03-*03:01 (OR = 2. 9), *04:05- *03:01 (OR = 2. 4) and *03:01-*02:01 (OR = 2. 1) haplotypes. The *16:01-*05:02 haplotype,

presenting in its recessive form in Sardinian population besides the *15:02-*06:01 haplotype, are negatively associated to MS susceptibility. Jones et al. (2006) suggested that the associations between the DRB1 and DQB1 alleles may influence the severity of the disease through unknown mechanisms. Dyment et al. (2005)[10], Ramagopalan et al. (2007)[25], Barcellos et al. (2003)[4]and Barcellos et al. (2006)[5] reported that some alleles influence the effect of *1501 in MS susceptibility. The presence of two copies of *1501 represents a higher risk of MS. Link et al. (2012), proposed that groups of Class I HLA alleles may interact with Class II HLA-DRB1*15, neutralizing its negative effect as a predisposing factor of MS. The conclusions of Irizar et al. (2012)[14] have indicated that the DRB1, DRB5 and DQA1 genes are expressed significantly in the samples positives for DRB1*1501 in their genotypes. The expression of DRB5 gene have been shown to be specific of the DRB1*1501 allele, once the super expression of that gene have demonstrated to be significantly higher in the individuals with positive allele.

Zivkovic et al. (2009)[18] and Benesova et al. (2012)[6], De Bakker et al[19]. have reported an association between DRB1*15 and the rs3135388 polymorphism. The rs3135388 is useful marker for the DRB1*1501 allele, helping in the detection of this polymorphism in clinical tests. SNP rs3135388 is located 197 base pairs downstream of the terminal exon of the HLA-DRA gene. The very high degree of correlation observed between SNP rs3135388 and HLADRB1 is remarkable. They are separated by a distance of nearly 120 kb. This fragment is located within a 158-kb highly conserved segment that is most extensively preserved on the HLA-DR15 haplotype.

Gorris et al (2008)[60] studied the DR1501 allele and genotype frequencies as deduced from the tagging SNP and the results were concordant with those reported previously for full HLA-DRB1 typing in different samples. The observed distribution was in line with a gradient seen throughout Europe. Association of the DRB1*1501 allele with MS was confirmed ($P = 5 \times 10^{-21}$), and the estimated odds ratios of 3. 03 for heterozygotes and 5. 10 for homozygotes overlap with previous estimates in other populations.

Zivkovic et al. (2009)[18] found significantly higher frequency of rs3135388 A allele carriers in MS patients than in control group (OR 2. 14). The carriers of one A allele had OR 2. 09 and the AA homozygotes had OR 4. 37 for MS susceptibility, adjusted for gender. They have not found any difference in rs3135388 G/A genotype distribution with respect to gender. There was no significant difference in allele carriership frequency between relapsing remitting and secondary progressive patients. Also, they have not found any significant influence of genotype on age at onset.

Alciniaetal [30](2012) reported that homozygotic carrier for rs3135388 risk AA allele has shown 15. 7-, 5. 2-, and 8. 3-fold higher expression with respect to the GG carrier, and 1. 6-, 1. 5- and 1. 8-fold higher expression with respect to the AG carrier for the DQB1, DRB5 and DRB1 genes, respectively. Benesova et al (2012)[6] reported that rs3135388 gene polymorphism is a strong risk factor for MS susceptibility. A significant increase of the A allele in MS patients was proved and A allele carriers were more frequent in patients with MS (OR=3. 69); the OR for GA heterozygotes and AA homozygotes was 4. 27. Their study confirmed previous results indicating that rs3135388 A allele carriers are more frequent in patients with

MS. They demonstrated that the A allele carriers were more frequent in female patients with MS. Their study did not reveal any significant association of genotypes with disability and disease severity in the whole study group and when separated by gender. After stratification of the patient group with MS according to disease type, only marginally significant associations of genotypes with disability in female RRMS were found. However studies conducted by Sombekke et al. , 2009[49]; provided some evidence that HLA DRBl*1501 might also be associated with a more severe course of the disease.

Modifying effects of HLA-DRB1 allele interactions on age at onset of multiple sclerosis

The study conducted by Wu et al (2010)[36] in a cohort of 461 multiple sclerosis patients from the Perth Demyelinating Diseases Database showed that carriage of the HLA-DRB1*1501 risk allele was not significantly associated with age at onset but HLA-DRB1*0801 was associated with a later onset of the disease. The HLA-DRB1*0401 allele was associated with a reduced age at onset when combined with DRB1*1501 but may delay age at onset when combined with DRB1*0801. These findings indicate that epistatic interactions at the HLA-DRB1 locus have significant modifying effects on age at onset of multiple sclerosis and demonstrate the value of high-resolution genotyping in detecting such associations. The biological mechanisms which underlie these interactive effects of HLA-DRB1 alleles remain speculative. In view of the known role of the MHC Class II alleles in antigen presentation and the fact that the two alleles at the DRB1 locus are co-dominant and are both expressed, it might be hypothesized that the combination of

certain alleles, such as DRB1*1501/*0401, has a more permissive effect on the induction of an immune response in the face of an appropriate environmental triggering agent, while other combinations such as DRB1*1501/ *0801 have a less permissive effect. These postulated effects could be mediated through differential binding and presentation of antigenic epitopes by the two DRB1 proteins. In other words, as has been postulated in type 1 diabetes, one allele may be involved in presentation of the disease-specific antigenic epitope while the other may act through its linkage disequilibrium with polymorphisms in other MHC genes which may act by modifying the immune response through other mechanisms such as cytokine networks. Similar results were obtained by Balnyte et al (2013)[2] from Lithuanian population, Lima et al (2015)[26] from Scopus database. Hensiek et al (2002)[12], Romero-Pinel et al (2001)[33], noted that positive DR15 were associated with earlier beginning of MS and to female gender. Irizar et al. (2012)[14], studying the Japanese population, suggested that DRB1*15 allele confers greater risk of MS only for women.

Associations of multiple sclerosis susceptibility genes with brain magnetic resonance imaging

There are several studies that have explored whether susceptibility genes can explain differences in disease severity in multiple sclerosis. Large variation can be found between patients with MS with regard to the number and volume of T2 lesions in the brain. An association between HLA-DRB1*1501, and T2 lesion volume was found by Okuda and colleagues (2009)[44]. However, this association was not confirmed in other studies. Likewise, although some studies by Zivadinov et al[45]

have reported associations between susceptibility genes with T1 black holes (BH), in other studies conducted by Kalincik et[46] al no significant relations could be found. In patients with CIS, associations between HLA status and number and volume of gadolinium enhancing lesions were found by Horakova et al[46]. However, in a longitudinal study by the same group with 179 patients with CIS and 16 susceptibility SNPs, including HLA-DRB1, no relations with T2 lesion load (T2LL) or BV (brain volume) were found. In majority of the studies using BV as an outcome measure, no associations with known susceptibility genes have been found[46].

In addition to the large range of number and volume of lesions in patients with MS, anatomic location of lesions within the brain varies widely among patients with multiple sclerosis. This differences in involvement of the cerebrum, brainstem, and cerebellum may partly be explained by genetic differences. This possibility was investigated by Sombekke et al (2011)[49]by examining the effect of 69 candidate SNPs on a lesion probability map of 208 patients with MS, showing increased probability for lesions in certain brain areas for 5 SNPs and decreased probability for lesions in 6 SNPs. The most statistically robust finding was the increased probability of having a lesion in the cerebral white matter against the frontal and occipital horn of the left lateral ventricle, for the heterozygous genotype of rs2227139, located within the MHC class II region. In another study comparing T1BH and T2 lesion probability maps between 50 patients with MS with negative and positive HLA-DR2 status (determined by the presence of the HLA-DRB1*1501 allele, present in 30% of patients),by Sepulcre et al[50] no significant differences in lesion distribution were found, and neither did gray or white matter atrophy differ. A

GWAS conducted by Gourraud et al (2013) found several SNPs associated with one of the lesion distribution patterns found in a group of 284 patients with MS. The genes involved have immunity-related but also neural functions[3].

Association between spinal MRI lesions and rs3135388 polymorphism

In a study conducted by Madelaine et al (2009), they noted that five single-nucleotide polymorphisms within the major histocompatibility complex region were associated with the number of focal abnormalities in the spinal cord. The most significant was rs3135388 (surrogate marker for the HLA-DRB1*1501 allele). Carriers of HLA-DRB1*1501 had a median of 4 spinal cord lesions compared with 2 lesions for non-carriers (P<. 001). No significant association was noted between the single nucleotide polymorphisms and T2-weighted lesion load in the brain. Some other studies gave inconsistent results. More research is warranted in this area, especially because spinal cord pathology in MS are strongly associated with clinical disability.

Association between HLA DR B1*15 01 and CSF oligo clonal bands

Balnyte et al (2011)[2] noted that 55% were positive for OCBs and 56% for HLA DRB1*1501. OCB positive patients with multiple sclerosis had higher EDSS scores than their OCB-negative counterparts at onset of the disease (P=0. 02) and during the last visit (P=0. 009). The mean relapse rate was higher in the OCB-positive group compared with OCB-negative group (P=0. 001). OCB-positive patients had higher IgG index compared with OCB-negative patients (P=0. 0001).

However no relationship was found between HLA DRB1*1501 antigen status and the clinical features or EDSS score, and presence or absence of OCB.

In a meta-analysis conducted by Maurizio et al[53] among Italian, Scandinavian and Belgian population, HLA-*DRB1*15* is associated with OCB positivity (p=0. 03). None of the 52 non-HLA MS susceptibility loci was associated with OCB, except one SNP (rs2546890) near *IL12B* gene. The weighted Genetic Risk Score mean was significantly (p=0. 0008) higher in OCB positive (7. 668) than in OCB negative (7. 412) patients. After meta-analysis on the three datasets for the best associated signals resulted from the Italian GWAS, the strongest signal was a SNP (rs9320598) on chromosome 6q ($p=9.\ 4\times10^{-7}$) outside the HLA region (65 Mb) which points to the fact that genetic factors predispose to the development of OCB positivity[3].

Diagnostic tests in MS

MRI has revolutionized the diagnosis and management of MS. The characteristic abnormalities are found in >95% of patients, although > 90% of the lesions seen in MRI are asymptomatic. Lesions are mostly oriented perpendicular to the ventricular surface, corresponding to the pathologic pattern of perivenous demyelination. Lesions are multifocal within the brain, brainstem, and spinal cord. Lesions larger than 6 mm located in the corpus callosum, periventricular white matter, brainstem, cerebellum, or spinal cord are particularly useful for diagnostic purposes. The total volume of T2-weighted signal abnormality – i. e., "burden of disease" shows a significant correlation with clinical disability, as do measures of brain atrophy. Black holes are considered as markers of irreversible demyelination and axonal loss, although even this measure depends on the timing of the image

acquisition. CSF abnormalities found in MS include a mononuclear cell pleocytosis and an increased intrathecal synthesized IgG levels. A mild CSF pleocytosis (>5 cells/μL) is present in up to approximately 25% of cases. The total CSF protein is usually normal. The measurement of oligo clonal bands (OCBs) in the CSF assesses intrathecal production of IgG. OCBs may be absent at the onset of MS. In individual patients, the number of bands may increase with time. An abnormal visual EP would permit a diagnosis of clinically definite MS. Abnormalities on one or more electrophysiological modalities occur in 80–90% of MS patients which are not specific to MS[31].

Multiple sclerosis in India

In India, multiple sclerosis was recognized only in the 1960s when physicians who received training in Neurology in the West, returned to India. Baldev Singh, Bharucha and Ramamurthy[28] were the pioneers who first described the manifestations of multiple sclerosis in the Indian context. Statistics based on the hospital data in the 1970s suggested an approximate prevalence rate of 0. 17 to 1. 33/100,000 in various parts of India. With advanced awareness about the disease, a significant increase in the number of trained neurologists and relatively easy availability of magnetic resonance imaging (MRI) the current estimate stands at about 7 to 10/100,000. This figure may still be higher as major sections of the Indian population still have only limited access to adequate medical facilities particularly in the rural areas. No large epidemiological studies have been reported from India so far. In a small Parsi population of approximately 70,000, Bharucha et al[28]. , reported a higher prevalence of approximately 21/100,000. Another study from the same Parsi community by Wadia etal[21] reported a similar higher incidence of

approximately 26/100,000. In a recent epidemiological survey, from urban Mangalore the prevalence of 8/100,000 was noted.

There are limited reports of HLA linkage among Indian population. Kankonkar et al[15], in a small study from Mumbai demonstrated the association between DRB1*1501 allele and multiple sclerosis susceptibility and also suggested association for two novel DRB1*15 alleles, DRB1*1506 and DRB1*1508. In a more recent study, Pandit et al[22], concluded that the risk, attributable to the HLA-DRB1*1501 seen in Europeans are also seen in Indian population.

The first Indian article using international criteria of Schumacher was published by Mathew et al. , from Vellore, subsequently by Singhal and Wadia[21] describing the clinical features in multiple sclerosis. Subsequently, several papers from other parts of India were published, describing the clinical features and demographic data. Singhal et al[16]. , Jain and Maheshwari reviewed the published cases from India till 1985 summed up and commented on the higher frequency of optic nerve involvement and low yield of oligo clonal bands in Indian multiple sclerosis patients. The demographic features in the Indian population were similar to those seen in the West. The average age of onset was 25 to 35 years with females about two times more affected than men.

To study the differences in the clinical presentations of multiple sclerosis in the Asian patients as compared to that of West, Professor Kuroiwa[28] organized meetings in Japan with delegates from Asian countries. These discussions were concluded in two publications titled: Multiple Sclerosis in Asia and Multiple Sclerosis East and West. The essential differences noted were:

1) Frequent initial clinical presentation with optic nerve or spinal cord involvement

2) Often bilateral optic nerve affection

3) Severe myelopathy with sensory level

4) Less frequent clinical presentation to suggest cerebral or cerebellar involvement

5) More frequent painful tonic spasms.

The term optico-spinal multiple sclerosis (OS-MS) was used to highlight the clinical features in Asian multiple sclerosis. The term OS-MS continues to be used even today, though many of these patients are now diagnosed as cases of Neuromyelitis optica (NMO)[28].

The disease course of multiple sclerosis in Indian subcontinent is largely similar to that seen in the West though there has been no well-defined study reported from India on this subject. In Indian patients with 'Radiologically Isolated Syndrome', 'Clinically Isolated syndrome', a large majority having 'Remitting Relapsing (RR) course' has been reported. In some RR MS patients, phase of 'Secondary Progressive MS' sets in, during the course of disease. Though the precise statistics are not known there are reports of 'Primary Progressive MS' (PPMS) in Indian population[28].

Gradients in Distribution of DRB1* Alleles in Castes and Tribes of South India

Populations from Kerala:

Studies conducted by Balakrishnan et al [57] revealed, among Nairs, the most common allele was DRB1*15 (28. 57%) followed by DRB1*13 (16. 07%),

DRB1*10 (13. 39%), DRB1*04 (10. 71%), DRB1*14 (10. 71%), DRB1*07 (7. 14%), DRB1*03 (5. 35%) and DRB1*01 (3. 57%). In the same study alleles DRB1* 11 and DRB1*12 (both sub-types of DR5) were observed in lower frequencies with DRB1*08, DRB1*09 and DRB1*16 were completely absent. They also reported that among Namboothiris the commonest allele was DRB1*10 (18. 57%) followed by alleles DRB1*15 (17. 14%), DRB1*01 (12. 85%), DRB1*13 (11. 42%), DRB1*11 (10%), DRB1* 07 (8. 57%), DRB1*03 (7. 14%). Moderate frequencies were detected for DRB1*04 and DRB1*14 (each 5. 71%). DRB1*08 and DRB1* 09 were observed in lower frequencies with DRB1*16 and DRB1*12 were completely absent.

Populations of Tamil Nadu:

Epidemiological studies conducted by Balakrishnan et al[57] reported that among Iyers, the most frequent alleles were DRB1*10 (19. 32%), DRB1*07 and DRB1*15 (18. 18% each). In Kallars, the most frequently observed alleles were DRB1* 07 (23. 58%), DRB1*15 (18. 86%), DRB1*04 (12. 26%), DRB1*14 (10. 37%), DRB1*08 (6. 60%) and DRB1*12 (5. 66%). Among Vanniyars the most common allele was DRB1*03 (36. 27%) followed by DRB1*15 and DRB1*10 (each 21. 56%). In Sourashtrans, the predominant alleles reported were DRB1*15 (30. 76%), DRB1*07 (19. 23%), DRB1*10 (11. 53%) and DRB1*03 (6. 41%). In Pallars, the most common allele observed was DRB1*15 (43%) followed by alleles DRB1*07 (11%), DRB1*04 (10%), DRB1*10 (7%), DRB1*11 (7%), DRB1* 16 (6%). Among Narikuravars, the most frequent allele was DRB1*04 (41. 46%) followed by alleles DRB1*03 (14. 63%), DRB1*07 (12. 19%), DRB1*11 (9. 75%), DRB1*01 (8. 53%) and DRB1*13 (7. 31%).

Research Needs in the Indian context

There is no large scale data of the usage of disease modifying agents from India. We cannot as yet accurately predict the course of events in a given patient and the search for biomarkers still continues. The entity of benign multiple sclerosis is still remaining a matter of debate. We do not have data to guide patients who remain free of illness for two to three years, who enquire if they can discontinue the costly drugs. Although we are diagnosing more patients with multiple sclerosis today, much effort need to be done in the Indian context. We need greater awareness programs, more infrastructure especially for rehabilitation, specialized multiple sclerosis clinics, multiple sclerosis registry, government support, insurance coverage and availability of effective and affordable disease modifying agents. Multiple sclerosis Society has been in existence for over 25 years in India to provide support for the patients and their caregivers.

Recent discovery of a high-resolution HLAand SNP map have thrown hopes in the field of multiple sclerosis genetics. The analysis provided informative tag SNPs, capturing much of the common variation in the MHC region. This concept enabled detection SNPs, making it "surrogate" markers for haplotype associated with certain disease, particularly MS. The SNP rs3135388 was proposed as a tagging SNP for DRB1*1501/DQB1*0602 alleles. The presence of this SNP has predicts a relative risk of 4 for MS and coefficient (r 2) of determination between identified HLA predictor and the HLA risk allele of 0. 97, which was confirmed through MS GWAS study. Aiming to reduce time and expenses in HLAMS risk allele genotyping, a dozen of study were done worldwide. No data is available from the south Indian population regarding such an association and we designeda study

for high throughput detection of HLA rs3135388 SNP genotypes and investigated its association with MS in patients from South India.

AIM OF THE STUDY

To determine the association of HLA-DRB1*1501 tagging rs3135388 gene polymorphism with multiple sclerosis susceptibility.

MATERIALS AND METHODS

Subjects of the study were those diagnosed with relapsing and remitting Multiple sclerosis (RRMS) according to the Mc Donald's criteria with the age more than >13 years. Minor, pregnant woman, neonates, person incompetent to give informed consent, prisoners were not included in the study. Controls were healthy volunteers > 13 years of age. Relatives of patients were not included among controls. Controls were selected by small advertisements/ fliers which were attached on various places in the hospital. Separate consent forms were prepared for cases and controls and informed consent was obtained from both.

The objectives of the study, study methods and the likely benefits were discussed with the patient in detail by the principal investigator. Strict confidentiality was assured and adequate time was given to the patient to decide on participation in the study. The patients were made to understand that they do not have to participate if they are unwilling and this will not influence their further treatment in the hospital in any way. No incentives was offered to participate in the study. The study was conducted during the routine review of the patients and they were not asked to visit the hospital for the sole purpose of the study. The expense for the investigations for the patients was met by the investigator.

INCLUSION CRITERIA:

1. All patient with relapsing and remitting multiple sclerosis according to the Mc Donald's criteria (Poser et al 2011) with age >13 years. Patient or legally authorized representative willing to sign consent form.

2. Controls were healthy volunteers > 13 years of age. Relatives of patients were not included among controls.

EXCLUSION CRITERIA:

1. Patients with Progressive forms of multiple sclerosis were excluded.
2. Patients younger than 13 years, pregnant ladies and those not able to give an informed consent.
3. Subject diagnosed with another etiology causing white matter disease

RECRUITMENT

The subjects were recruited from the OP and IP of Department of Neurology, Sree Chitra Tirunal Institute for Medical sciences and Technology, Trivandrum by the principal investigator.

DATA COLLECTION PROCEDURES

30 consecutive patients with relapsing and remitting multiple sclerosis more than 13 years, who are on regular follow up in the neuromuscular disorders clinic of SCTIMST participated in the study, looking for genetic polymorphisms in them. 60 normal controls who were friends of the patients or other normal healthy volunteers who are willing to participate in the study and can provide informed consent were included to assess the frequency of the polymorphisms in the healthy population.

Consecutive relapsing and remitting multiple sclerosis (RRMS) patients attending neuromuscular disorders clinic and IP satisfying the inclusion and exclusion criteria, were screened by the principal investigator for eligibility for participation in the study. Voluntary informed consent were obtained from each

subject prior to enrolling to the study. Each subject were given both verbal and written information describing the nature of the study, need for participating in the study and potential benefits of the study. The informed consent process was done in a place where the subject has ample time to consider the risks and benefits associated with his/her participation in the study. He/she was informed that the participation in the study is voluntary and that he/she may refuse to participate or withdraw from the trial, at any time. Subjects were not allowed to participate in the study until the subject has signed an approved informed consent written in a language that is understandable to the subject.

The IEC approved informed consent form was signed and personally dated by the subject and the person who conducts the informed consent discussion. The informed consent procedure was done according to the guidelines provided in the Declaration of Helsinki and the ICH E6 Guideline for Good Clinical Practice. The original informed consent was retained in the Investigator's file. The blood sample collection was done after taking informed written consent from the patient and ensuring confidentiality.

BLOOD SAMPLE COLLECTION AND ISOLATION OF GENOMIC DNA

The SNP analysis was done in 25 patients only due to technical reasons. Peripheral blood (10ml) was collected from all the individuals in EDTA vials and stored at 4°C and for later DNA isolation. Clinical data from the patients were collected in specific data sheets. DNA was isolated from lymphocytes obtained from anticoagulated blood. A modified standard organic extraction method was used for DNA extraction (Sambrook and Russell, 2006). Equal volume of RBC lysis buffer

[30mM Tris, 5mM EDTA (pH 8. 00), 50mM NaCl] was added to the blood sample collected in EDTA vial and was frozen at -70°C for 3hrs. The sample was then freeze-thawed at 65°C resulting in RBC lysis. The RBC lysed sample was centrifuged at 10,000rpm for 10min at 4°C. The supernatant containing the lysed RBC was discarded. The WBC pellet was resuspended in equal volume of WBC lysis buffer [75mM NaCl, 2mM EDTA (pH 8. 00)] and homogenized. SDS and Proteinase K were added to the lysate at final concentrations of 2% and 150µg/ml respectively and incubated at 37°C for 8hrs. During this step, the SDS ruptures the WBCs and the proteins in the cells is digested by the action of proteinase K. Equal volume of tris-saturated phenol (pH 7. 5) was added to the sample and mixed gently. The sample was centrifuged at 10,000rpm for 10min at 4°C. The aqueous layer was collected and the organic layer containing phenol and denatured proteins was discarded. To the aqueous layer, equal volume of a mixture of tris-saturated phenol/chloroform/isoamyl alcohol (25:24:1) was added and mixed gently. The sample was centrifuged at 10,000rpm for 10 min at 4°C. The aqueous layer was collected and the organic layer containing carbohydrates and lipids was discarded. To this sample equal volume of chloroform/isoamyl alcohol (24:1) was added and mixed gently. The sample was centrifuged at 10,000rpm for 10min at 4°C. The aqueous layer was transferred into a fresh tube and the organic layer was discarded. To the sample, 1/10th volume of sodium acetate (3M, pH 5. 2) and twice the volume of chilled absolute ethanol were added and mixed gently. The precipitated lump of DNA was spooled out into a microfuge tube. The DNA was washed twice in 70% ethanol and once with 100% alcohol. The pellet was then air dried and re-suspended in 1X TE buffer (pH 8. 0). The DNA samples were stored in –20°C until further use.

DNA QUANTIFICATION

The quality and quantity of genomic DNA was analyzed in a spectrophotometer (BioSpec-1601, Shimadzu). The ratio of absorbance at 260 nm and 280 nm (A260/A280) was used to estimate the purity of the DNA. A ratio between 1. 7-1. 9 was considered as good quality DNA without protein contamination. The absorption of 1 OD (A260) is equivalent to approximately 50μg/ml of double stranded DNA. Hence the concentration of DNA in each blood sample was calculated using the following formula: Concentration of DNA (mg/ml or ng/ul) = 50mg/ml × OD A260 × Dilution Factor. Using the calculated DNA concentration, working stocks of DNA samples for PCR amplifications were made to final concentration of 20ng/ul.

SNP SELECTION

The SNP for genotyping was selected based on the functionality (cSNP, SNP in promoter region, splice sites, 3'UTR), tagging status, extent of Linkage Disequilibrium (LD), minor allele frequency > 0. 10 in other populations.

GENOTYPING BY SEQUENCING

Genotyping was performed by direct sequencing for SNP where the variant was amplified by PCR amplifying. The sequencing primers were purchased as crude oligonucleotides from Sigma-Genosys and the sequences are shown. All primers were resuspended in sterile nuclease free water at stock concentrations of 100pmol/μl and stored at –20°C. The primers were diluted to working concentrations of 20pmol/μl. PCR was carried out for all the samples in Eppendorf

mastercycler (EP Gradient) and Applied Biosystems Veriti Thermal cycler. The final volume of each PCR reaction was 10µl. Around 50-100ng of genomic DNA was used for each PCR reaction. The PCR reaction mixture consisted of 1X PCR buffer (NEB, Inc. , USA), 200µM of each deoxynucleoside triphosphate (dNTP), 2pmol of each primer, and 0. 5 Units of Taq Polymerase (NEB, Inc. , USA). This PCR product was further used for sequencing reaction using Applied Biosystems PRISM Big Dye Terminator v3. 1 cycle sequencing kit. The 10µl reaction was carried out with 50-100ng PCR product, 0. 5µl of the ready reaction mix, 20pmol of the forward or the reverse primer and 1X reaction Buffer (Applied Biosystems). The thermocycling conditions were 25 cycles of 96°C for 30sec and 60°C for 4min with a thermal ramp rate of 1°/second

SEQUENCING CLEANUP

The post sequencing PCR reaction product was transferred to a 1. 5ml microfuge tube. To the sample 10µl of sterile distilled water and 2µl of 125mM EDTA, 2µl NaOAc (3M) and 50µl absolute ethanol were added. The tubes were mixed on a vortex mixer briefly. The tubes were incubated at room temperature for 15min to precipitate the extension products. The samples were centrifuged at 12,000rpm for 20min at room temperature. The supernatant was carefully aspirated. To the pellet 300µl of 70% ethanol was added, and vortexed briefly. The samples were then centrifuged at 10,000rpm for 10min at room temperature and the supernatant was aspirated. The above step with 70% ethanol was repeated. The pellet was then air-dried.

ELECTROPHORESIS OF THE SEQUENCING SAMPLE

Formamide (10µl) was added to the dried pellet, mixed well, denatured at 95ºC for 10min and snap chilled. The samples were then loaded in the 3730 Applied Biosystems PRISM DNA analyzer. The sample was resolved through the POP 7 polymer and the sequencing data normalized using the matrix standard. Sequence analysis was done using the Applied Biosystems sequence scanner V. 1. 1.

STATISTICAL ANALYSIS

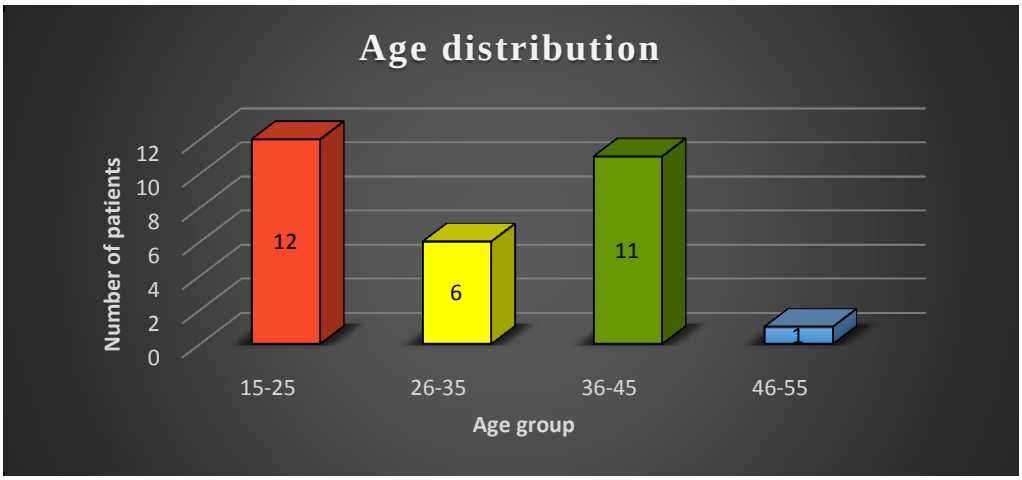
Genotype and allelic frequencies were computed and were checked for deviation from Hardy-Weinberg equilibrium (http://ihg2. helmholtzmuenchen. de/cgi-bin/hw/ hwa1. pl). Case-control genetic comparisons were performed using the chi-square test and allelic odds ratios (OR), and 95% confidence intervals (CI) were calculated by Fisher's exact test (two-tailed). All statistical analyses were performed using the Graph Pad Prism 5. 01, San Diego, CA, USA. We considered p value of <0. 05 as significant.

RESULTS

The study population were those diagnosed with relapsing and remitting multiple sclerosis (RRMS) fulfilling the Mc Donald's criteria, attending the neuromuscular and Multiple sclerosis clinic and those receiving the in-patient services of SCTIMST, Trivandrum. A total of 31subjects satisfied the criteria and were included in the study. Sixty healthy individuals who were not the relatives of the study subjects were taken as controls. The study was conducted between January 2015 and December 2015. The results of the descriptive analysis of the subjects are as follows: Demographic data

The age-wise distribution of the subjects showed a predominant clustering between 15-45 year age group with the highest number in the 15-25 age group (38%) followed by 36-45 age group (35. 5%). The mean age of the study population was 31. 1 years. The mean age for males and females were 32. 43 and 30. 7(+/-11. 4) years respectively.

Fig: 1 Age wise distribution of the patients



The sex-wise distribution showed 77. 4% of the study population to be females. Females between the age group 15-25 years has the maximum prevalence of the disease constituting approximately one third of the study population. However, among the males the maximum prevalence was observed between 36-45 years which constituted around 13% of the study population.

Fig: 2 Sex wise distribution of patients

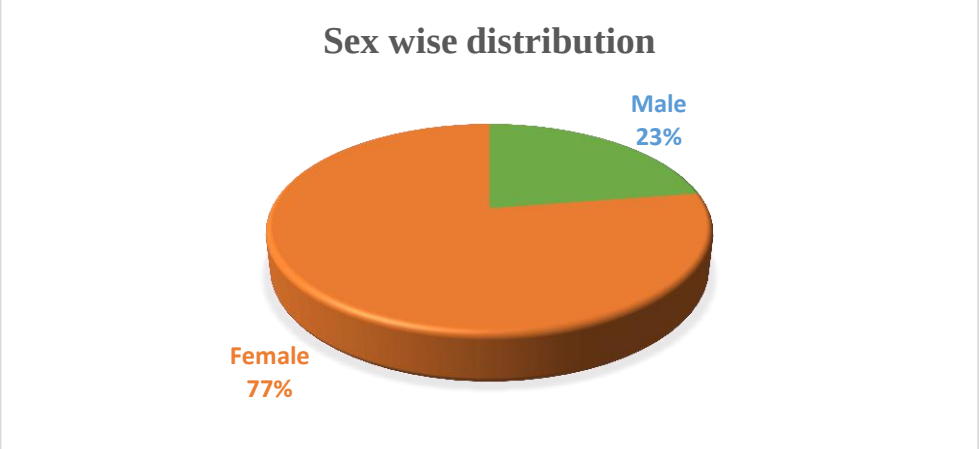


Table: 1 Age and sex wise distribution

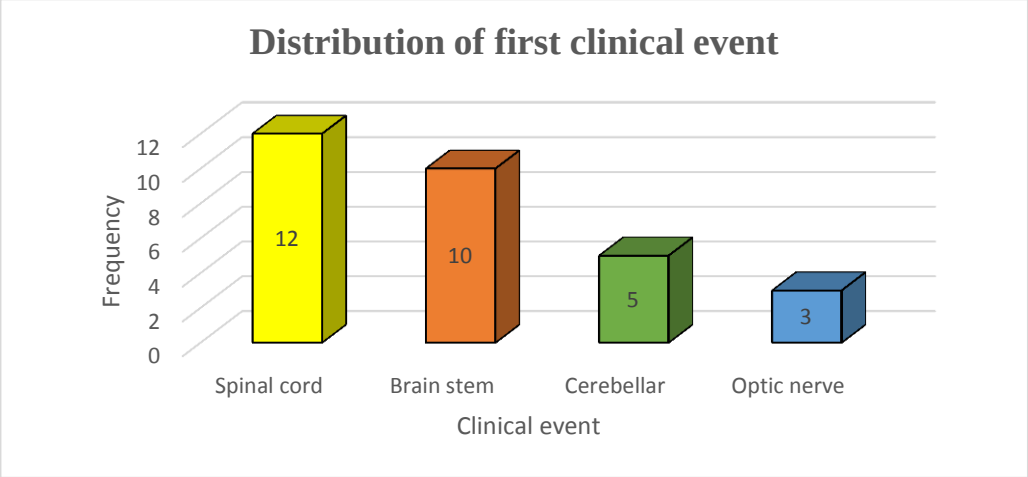
Age group (years)	Male N (%)	Female N (%)	Total
16-25	2(6. 4)	10(32. 2)	12(38. 7)
26-35	1(3. 2)	5(16. 1)	6(19. 4)
36-45	4(12. 9)	7(22. 5)	11(35. 5)
46-55	0	1(3. 2)	1(3. 2)
56-65	0	1(3. 2)	1(3. 2)
Total	7(22. 5)	24(77. 4)	31(100)

Clinical Characteristics of the study population

The clinical details analyzed included the first clinical event, number of relapses in the first year, total number of relapses, the site of involvement in the neuraxis and the EDSS score.

The most common site of involvement of the neuraxis was spinal cord occurring in 38% of the patients. The other sites involved were brainstem, cerebellum and optic nerve which constituted 32%, 16% and 10% respectively. None of the patients had relapses with overt cognitive presentation in the study.

Fig: 3 Distribution based on first clinical event



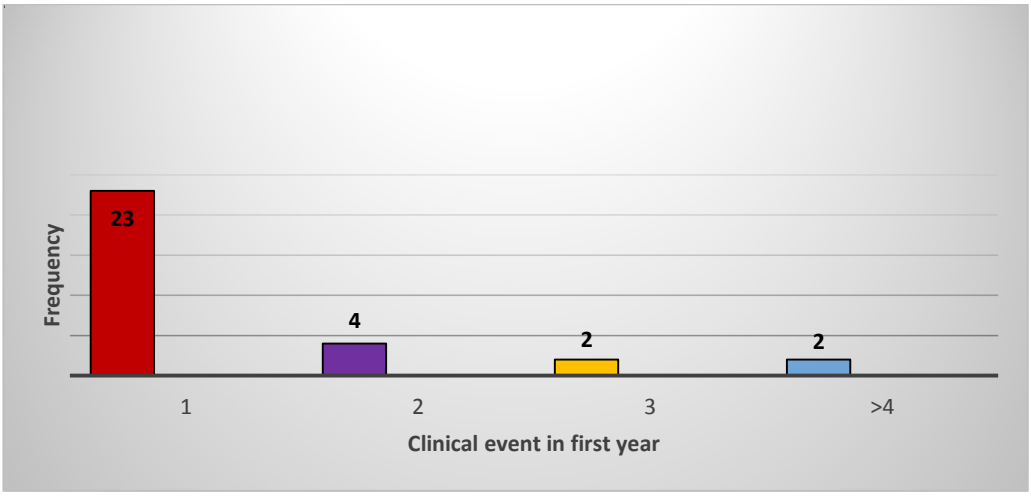
The total number of neurological events ranged from one event to as many as 8 events. The mean number of total attacks in the study was 3. 87.

Table: 2 Total number of attacks

Events	Minimum	Maximum	Mean	Standard deviation
Total number of attacks	1	8	3. 87	2. 277

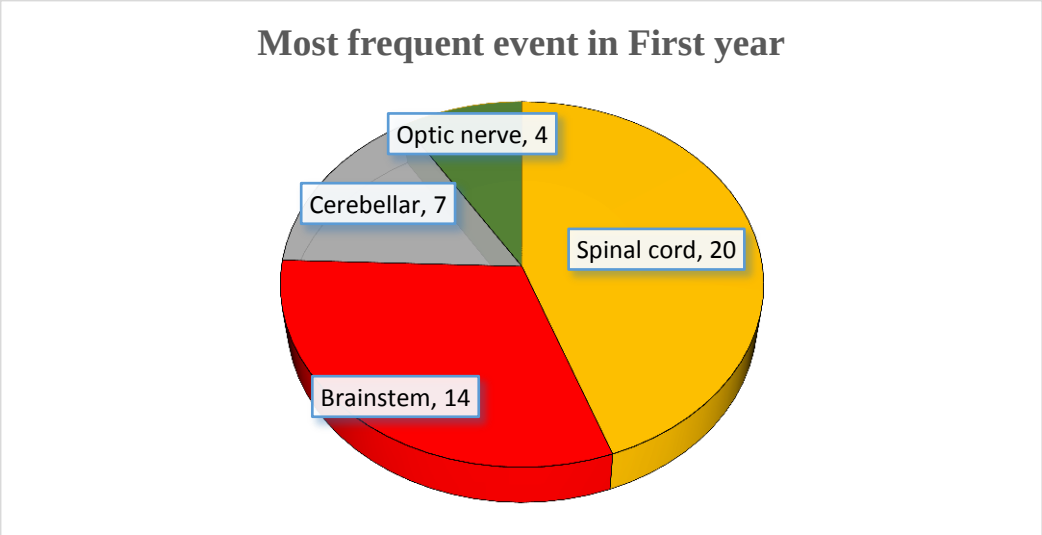
Following the index event 23(75%) did not experience a second event within the first year. However 4 patients (13%) had a second attack in the same year itself and 2 patients (6. 4%) reported more than 4 events in the first year.

Fig: 4 Number of clinical events in first year



It was observed that spinal cord involvement constituted 43% which was the most frequent clinical manifestation in the study followed by brain stem and cerebellar symptoms which constituted 14 and 7 percent respectively. Optic nerve involvement (6%) was also observed in the study. However involvement of other areas neuraxis was not observed in the study.

Fig: 5 The most frequent event in the first year



The interval between the clinical attacks did not reveal any difference in the study population with almost equal number of attacks as depicted in the figure 6 except for those between 3-6 months interval. The EDSS of the study population ranged from 0 to 6 with a mean EDSS of 1. 95. Median EDSS was 2.

Fig: 6 Interval between clinical events

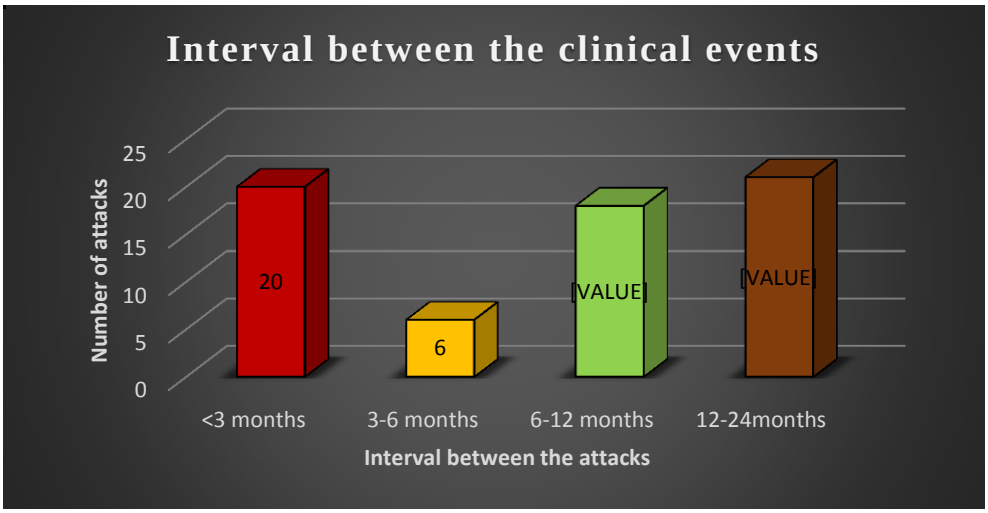


Table: 3 EDSS Score of the patients

	Minimum	Maximum	Mean	SD
EDSS Score	0	6	1. 95	1. 792

The distribution of MS lesions on MR images was studied and all patients in the study demonstrated a lesion in the MR images. Periventricular lesions of more than 10 numbers was observed in 60% of the patients and 35% was found to have 1-5 lesions in the periventricular region typical of MS. Juxta cortical lesions were observed in 87%. Brain stem lesions were noted in 68% of all the MRI which constituted the in MRI. Cerebellar lesions were noted in 11 (35. 5%) and optic nerve lesions in 4 (13%) patients. T1 black holes were observed in 25% of the MRIs studied. Cerebral atrophy was documented in 58%. Confluent spinal cord hyper intensity involving the cervico-thoracic region was the most common spinal imaging finding noted 38. 7% in the study population and 25% of the MRI did not reveal any lesion in the spinal cord.

Fig: 7 MRI distribution of lesions

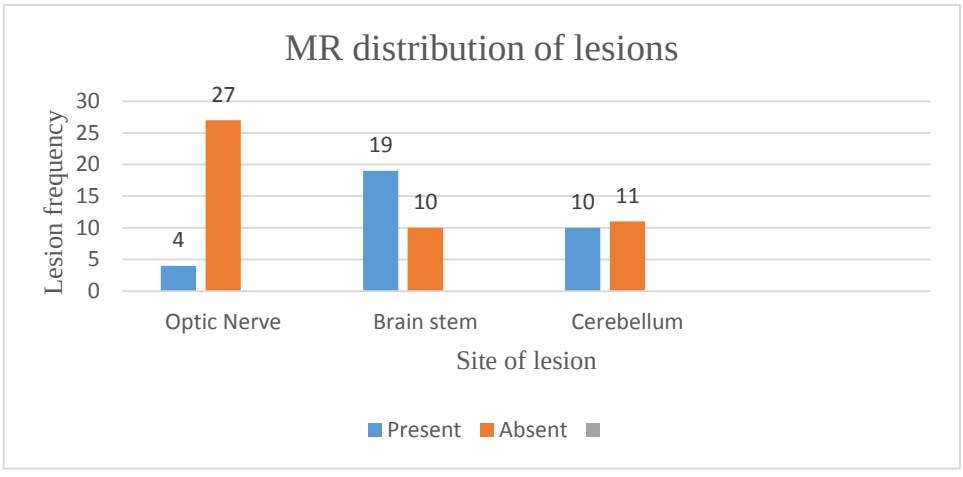


Fig: 8 Distribution of Spinal cord lesions

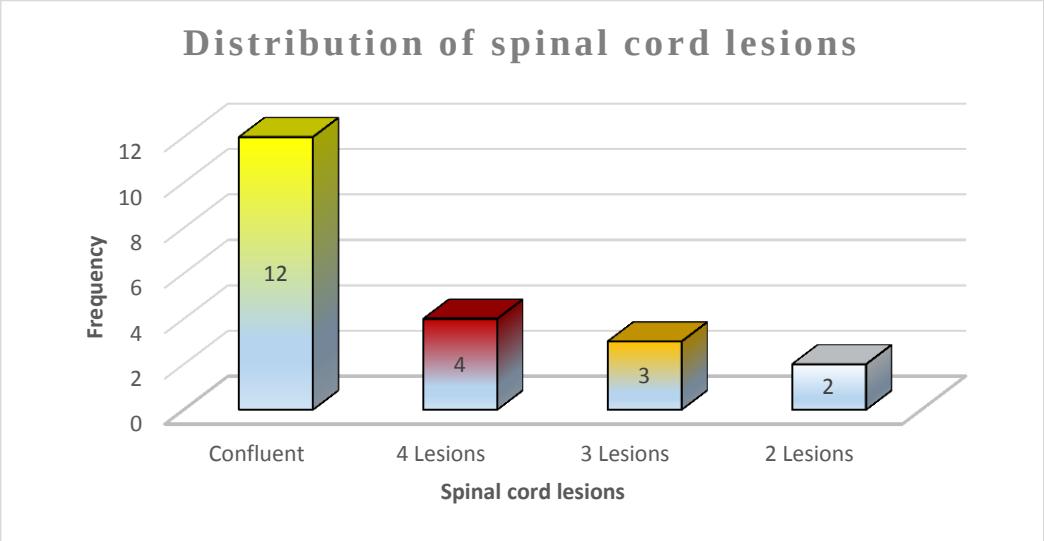


Fig: 9 Distribution of Periventricular lesions

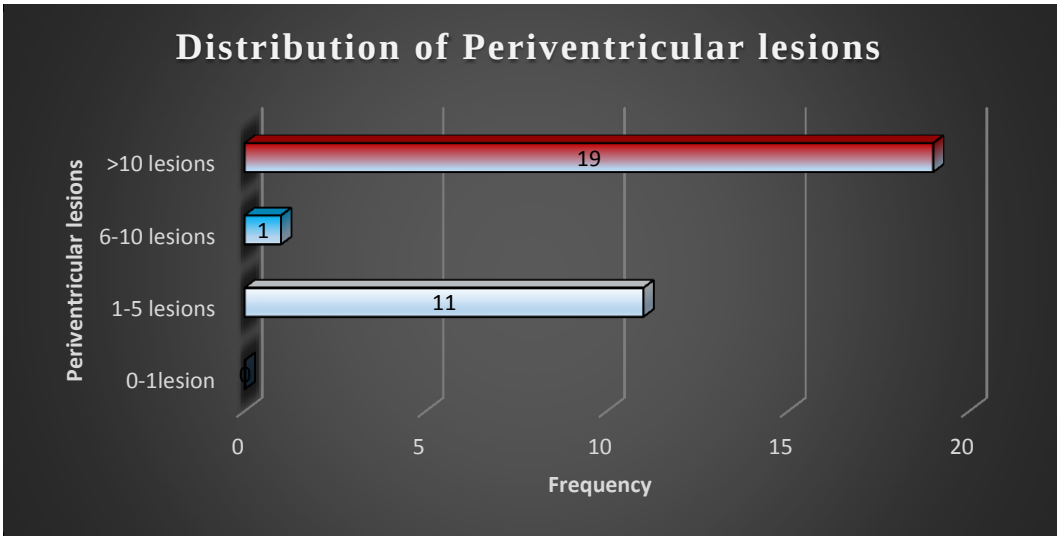


Fig: 10 Juxta cortical lesions

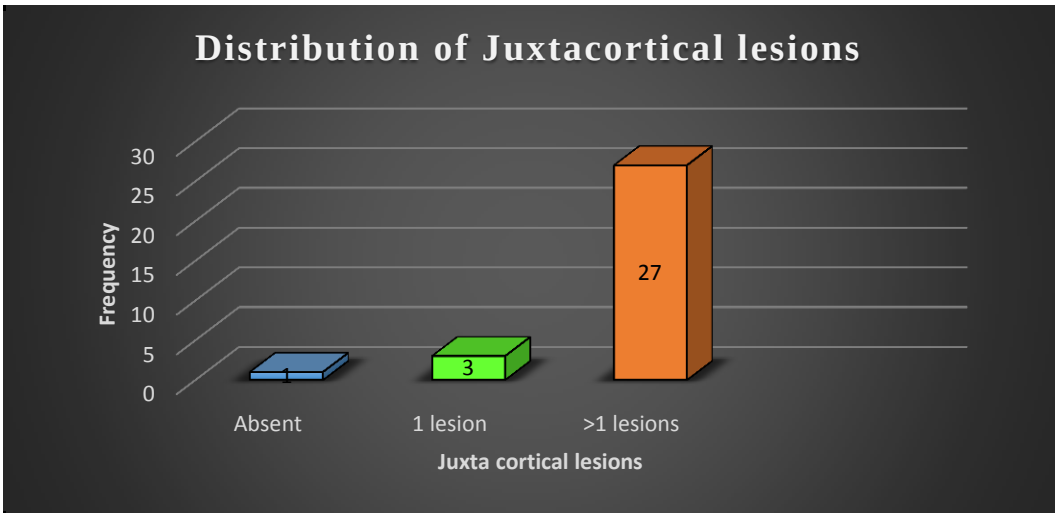


Fig. 11 Atrophy and Black holes:

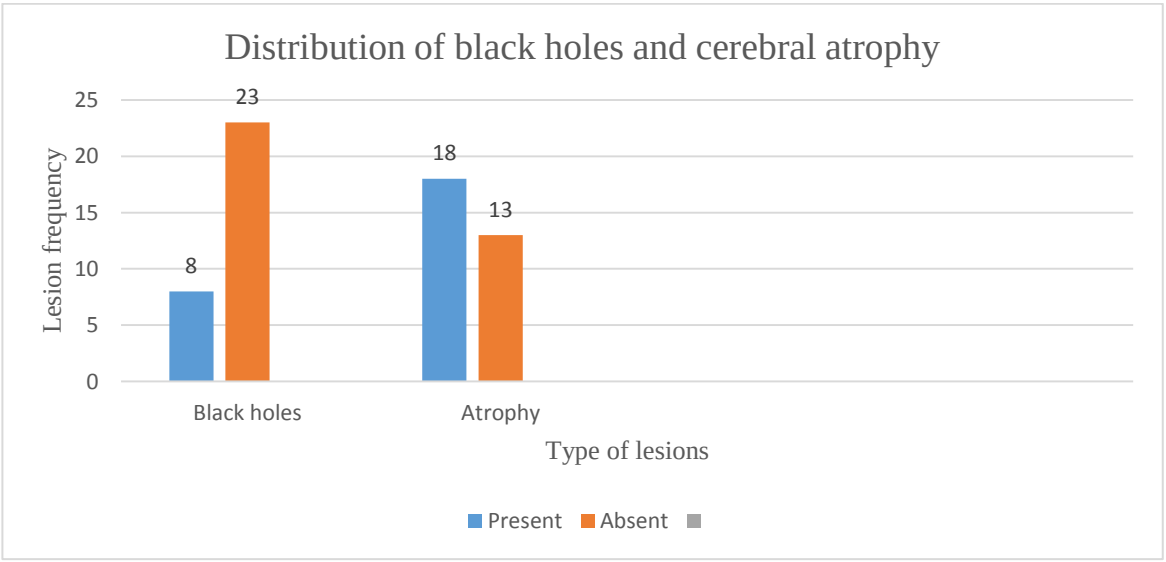
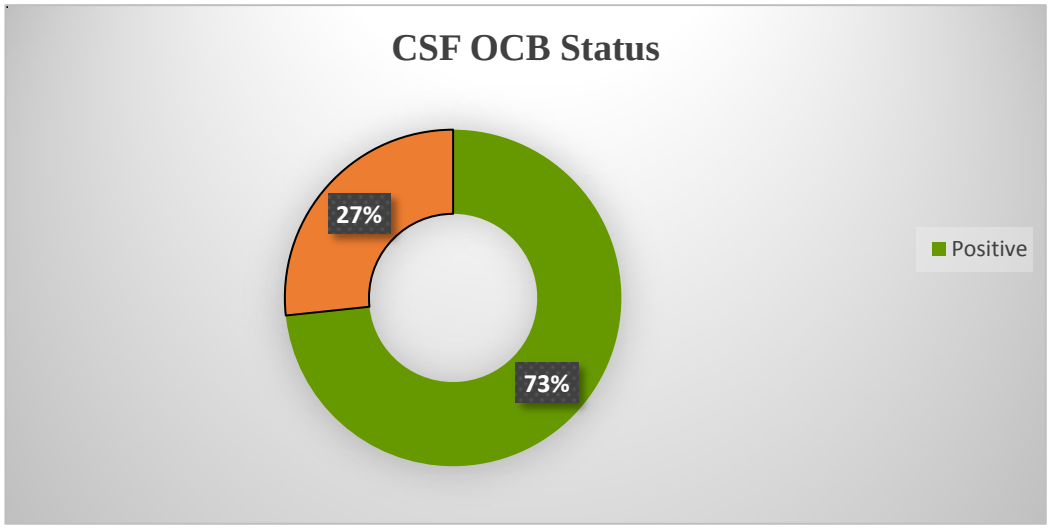


Fig: 12 CSF OCB status of the patients



CSF OCB positivity was observed in 71% of the subjects diagnosed with MS. CSF Ig G index was increased in only 16 % of the subjects. However CSF study was not done in one patient.

Fig: 13 CSF IgG Index

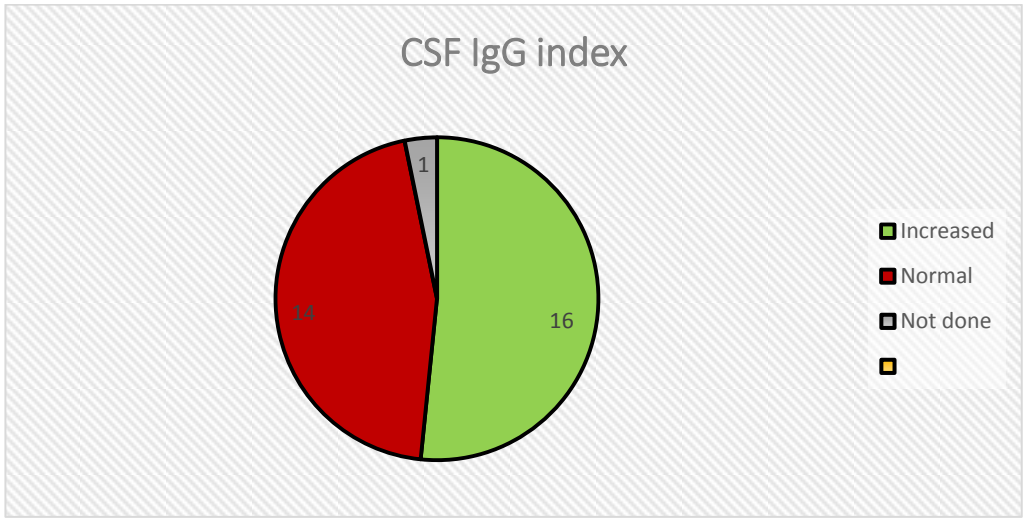
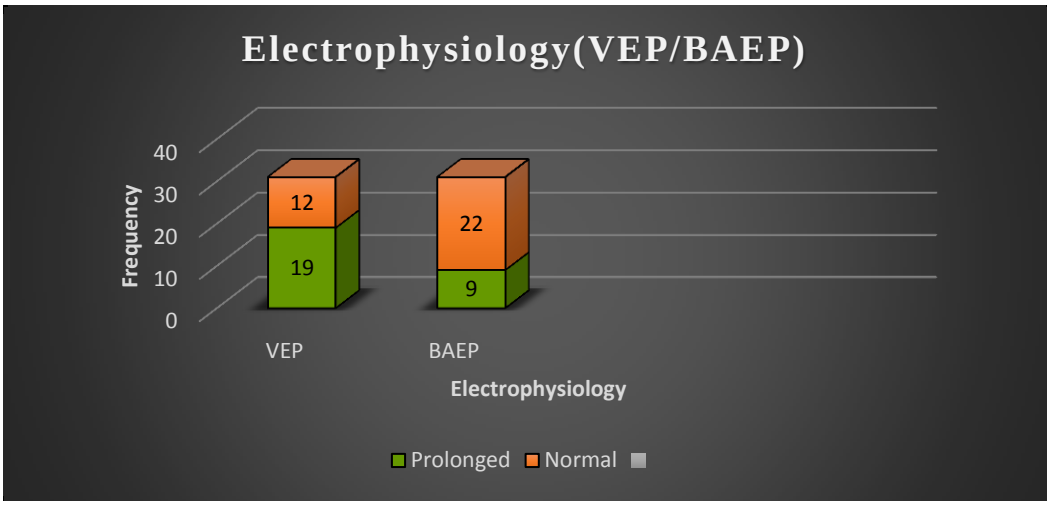


Fig: 14 Electrophysiological profile



Electrophysiological data revealed prolonged VEP and BAEP in 61% and 29% respectively.

Table: 4 Case control Association

SNP		CC	CT	TT	p-value	C	T	p-value
rs3135388	**Cases**	24	0	0	0. 43	48	0	0. 47
		1	0	0		1	0	
	Controls	56	4	0		116	4	
		0. 94	0. 06	0		0. 97	0. 03	

Out of 60 controls sequenced for genotyping, we could find out 56 patients homozygous for C allele and four were heterozygous for CT and none were homozygous for T allele. All 24 MS patients screened were homozygous for C allele.

Functional Prediction score for **rs3135388**

Functional Category	**Prediction Tool**	**Prediction Result**	**Prediction Detail**		**FS score**
			rs3135388.C	rs3135388.T	
Transcriptional regulation	TFSearch	Changed	ADR1 P AML-1a	NF-Y GATA-1 GATA-2	0. 5

The functional prediction score for rs3135388 in the study was 0. 5 Functional prediction for SNP (rs3135388) is done with *in silico* database F-SNP, which provides integrated information about the deleterious effects of SNPs with respect to functional category i. e., protein coding, splicing regulation, transcriptional regulation, and post-translation. Prediction details exposed entirely different transcription factor binding for respective alleles as shown in table above.

Fig: 15 Genotype analysis:

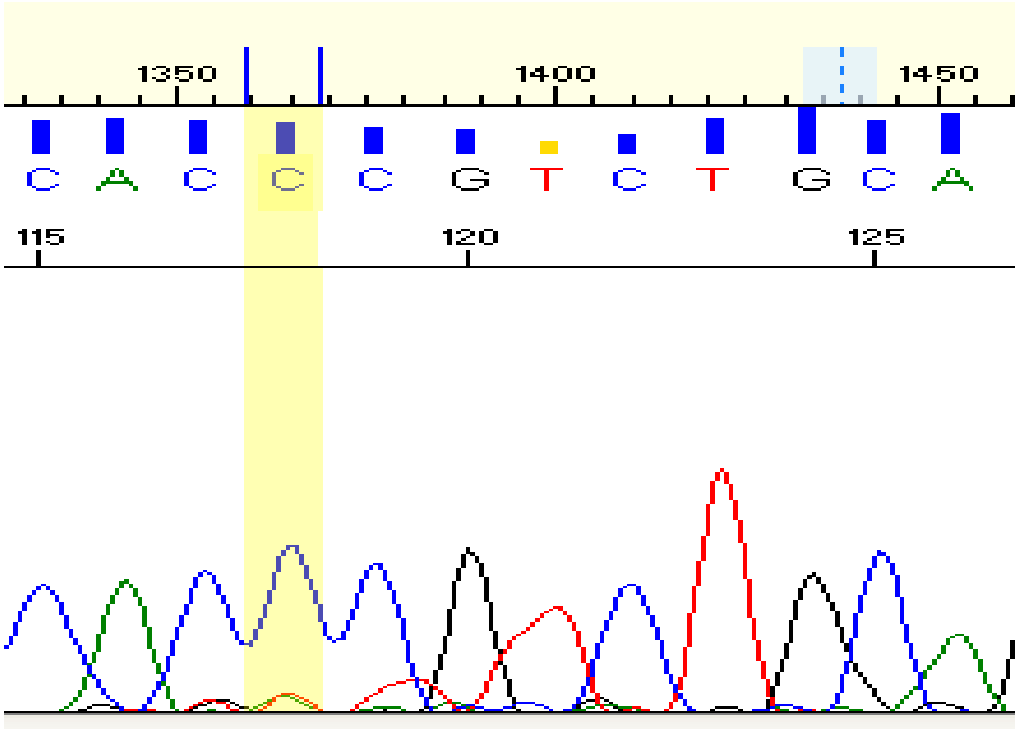


Fig 16. Depicting CC genotype

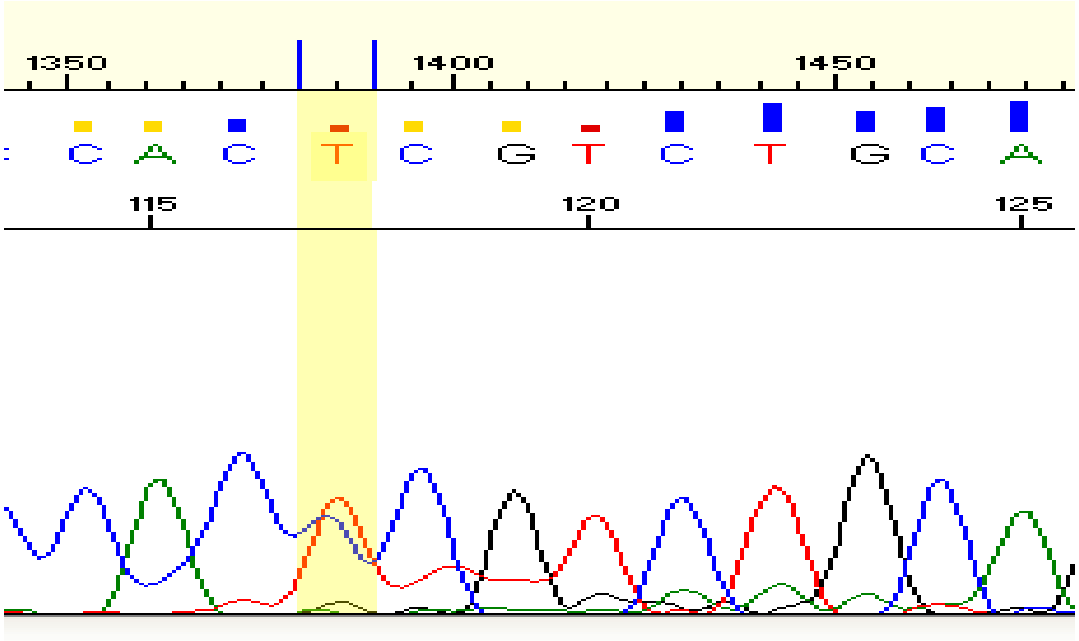
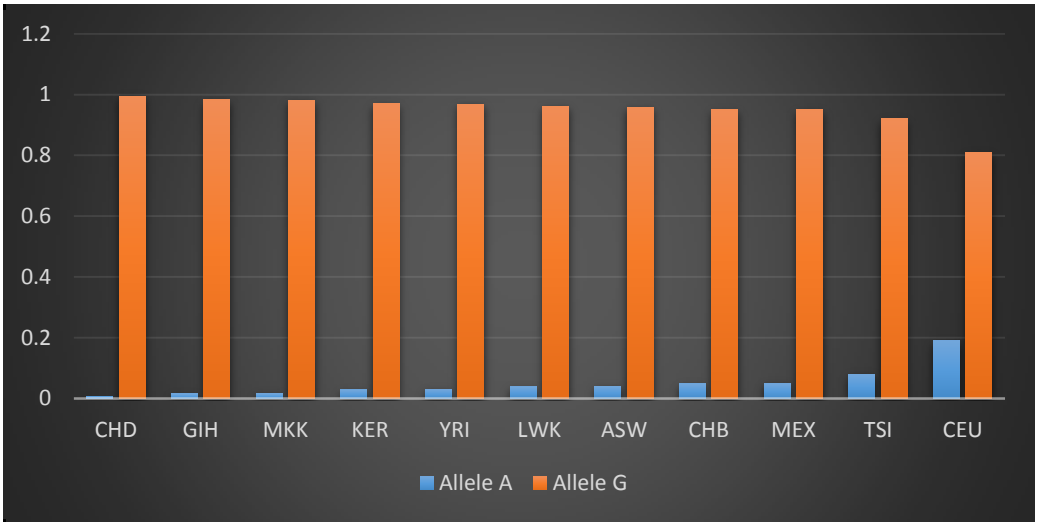


Fig: 17 Allele frequency of different population

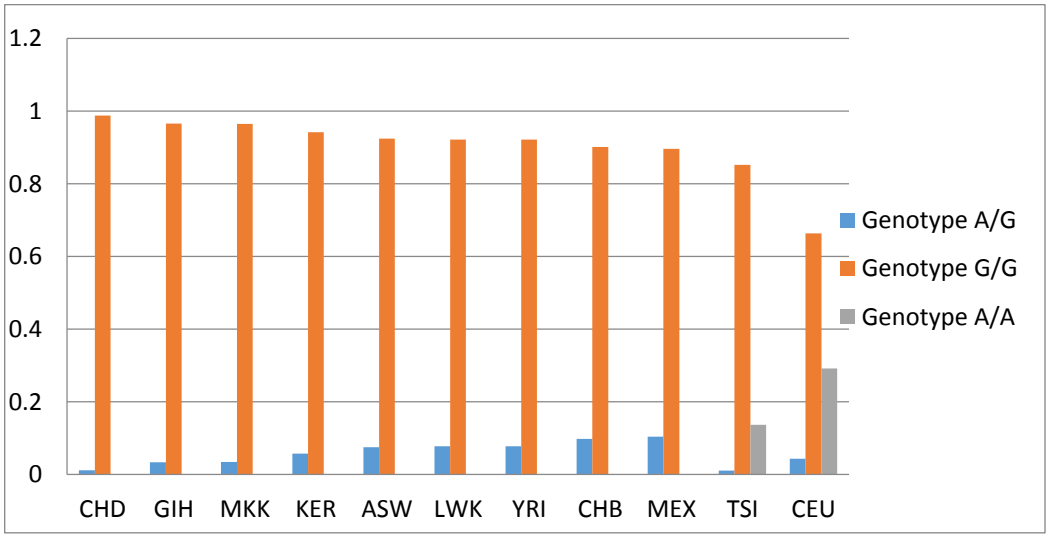


The A and G allele frequency of the study population was compared with that of the other global population and it was observed that Caucasians had high allele frequency so also the the SNP polymorphism. The study population was compared with the Guajarati Indians and was observed that allele frequency was low in them also. Similar results were observed when genotype analysis was done. This can be interpreted in such a way that the genetics of Caucasians differ from that of south Indian population. The linkage disequilibrium of the South Indians were compared with that of the Caucasians and that also showed significant difference between the populations (Fig 19-24).

CHD	Chinese in Metropolitan Denver, Colorado
GIH	Gujarati Indians in Houston, Texas
MKK	Maasai in Kinyawa, Kenya
YRI	Yoruba in Ibadan, Nigeria
LWK	Luhya in Webuye, Kenya
ASW	African ancestry in Southwest USA
CHB	Han Chinese in Beijing, China
MXL	Mexican ancestry in Los Angeles, California
TSI	Toscani in Italia
CEU	Utah residents with Northern and Western European ancestry from the CEPH collection

Population	Allele A	Allele G
CHD	0. 006	0. 994
GIH	0. 017	0. 983
MKK	0. 018	0. 982
KER	0. 029	0. 971
YRI	0. 031	0. 969
LWK	0. 039	0. 961
ASW	0. 041	0. 959
CHB	0. 049	0. 951
MEX	0. 05	0. 95
TSI	0. 08	0. 92
CEU	0. 19	0. 81

Fig: 18 Genotype Frequency of different world populations



Population	Genotype A/G	Genotype G/G	Genotype A/A
CHD	0. 012	0. 988	0
GIH	0. 034	0. 966	0
MKK	0. 035	0. 965	0
KER	0. 058	0. 942	0
ASW	0. 075	0. 925	0
LWK	0. 078	0. 922	0
YRI	0. 078	0. 922	0
CHB	0. 098	0. 902	0
MEX	0. 104	0. 896	0
TSI	0. 011	0. 852	0. 137
CEU	0. 044	0. 664	0. 292

Fig. 19 CEU


7.40 kb
Forward strand
Genes
(Comprehensive s...
HLA-DRA-001 >
protein coding
HLA-DRA-002 >
protein coding
All sequence
SNPs/indels
Variations
All HapMap
SNPs/indels
All HapMap - short variants (SNPs and indels)
32.410Mb
32.411Mb
32.412Mb
32.413Mb
32.414Mb
32.415Mb
32.416Mb
Variation Legend
Splice acceptor variant
Splice region variant
3 prime UTR variant
Downstream gene variant
Missense variant
Synonymous variant
Intron variant
Gene Legend
Protein Coding
protein coding
merged Ensembl/Havana
1000GENOMES:phase_3:CEU
LD (r2)
LD(r2): 1000GENOMES:phase_3:CEU (1000genomes:phase_3:eur) 112 SNPs
LD (d_prime)
LD(d_prime): 1000GENOMES:phase_3:CEU (1000genomes:phase_3:eur) 112 SNPs

Fig 20 CHB

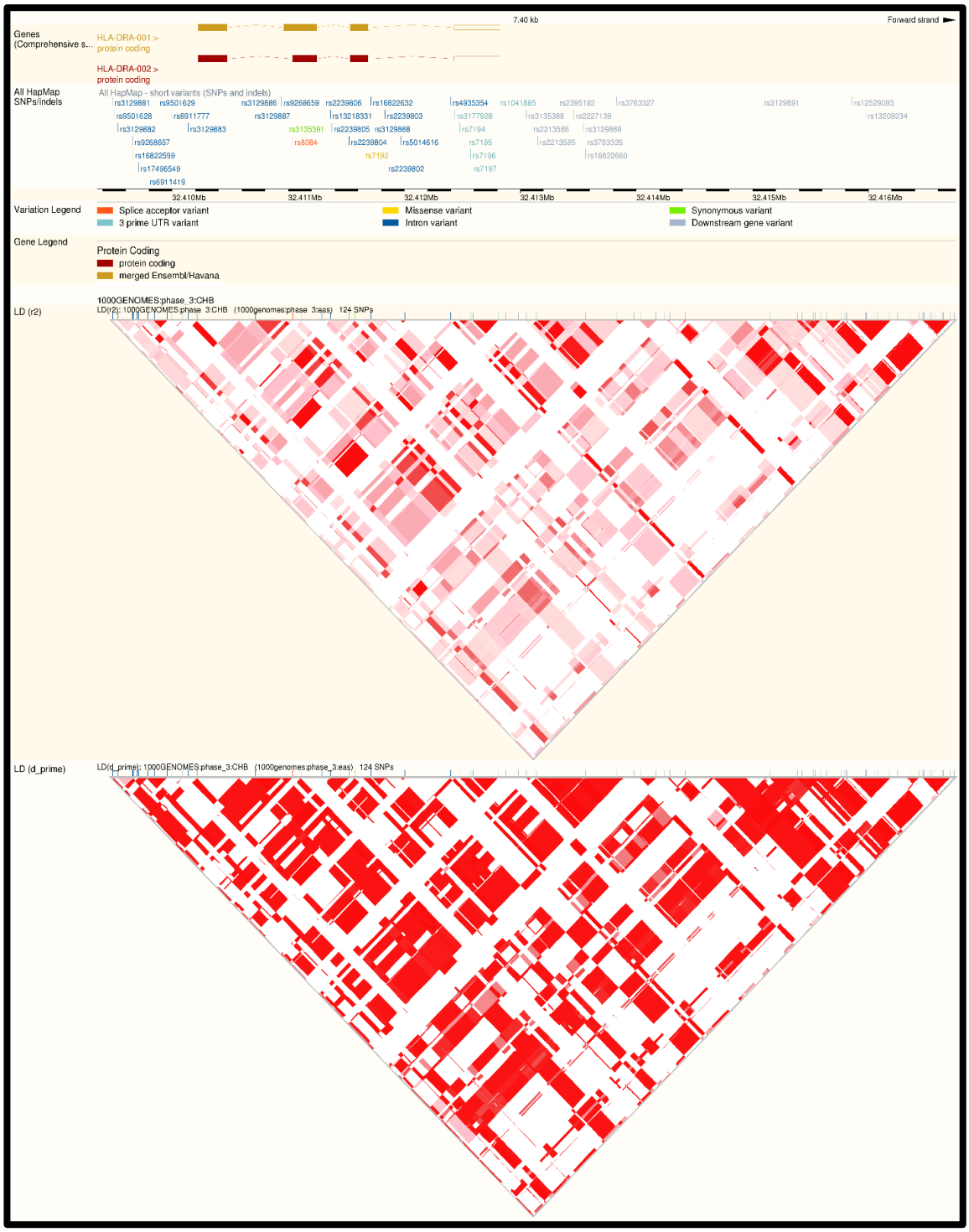

7.40 kb
Forward strand
Genes (Comprehensive s...
HLA-DRA-001 > protein coding
HLA-DRA-002 > protein coding
All HapMap SNPs/indels
All HapMap - short variants (SNPs and indels)
32.410Mb
32.411Mb
32.412Mb
32.413Mb
32.414Mb
32.415Mb
32.416Mb
Variation Legend
Splice acceptor variant
3 prime UTR variant
Missense variant
Intron variant
Synonymous variant
Downstream gene variant
Gene Legend
Protein Coding
protein coding
merged Ensembl/Havana
1000GENOMES:phase_3:CHB
LD (r2)
LD(r2): 1000GENOMES:phase_3:CHB (1000genomes:phase_3:eas) 124 SNPs
LD (d_prime)
LD(d_prime): 1000GENOMES:phase_3:CHB (1000genomes:phase_3:eas) 124 SNPs

Fig 21 GIH


7.40 kb
Forward strand
Genes (Comprehensive s...
HLA-DRA-001 > protein coding
HLA-DRA-002 > protein coding
All HapMap SNPs/indels
All HapMap - short variants (SNPs and indels)
32.410Mb
32.411Mb
32.412Mb
32.413Mb
32.414Mb
32.415Mb
32.416Mb
Variation Legend
Splice acceptor variant
3 prime UTR variant
Missense variant
Intron variant
Synonymous variant
Downstream gene variant
Gene Legend
Protein Coding
protein coding
merged Ensembl/Havana
1000GENOMES:phase_3:GIH
LD (r2)
LD(r2): 1000GENOMES:phase_3:GIH (1000genomes:phase_3:sas) 122 SNPs
LD (d_prime)
LD(d_prime): 1000GENOMES:phase_3:GIH (1000genomes:phase_3:sas) 122 SNPs

Fig 22 ITU


7.40 kb
Forward strand
Genes (Comprehensive s...
HLA-DRA-001 > protein coding
HLA-DRA-002 > protein coding
All HapMap SNPs/indels
All HapMap - short variants (SNPs and indels)
32.410Mb
32.411Mb
32.412Mb
32.413Mb
32.414Mb
32.415Mb
32.416Mb
Variation Legend
Splice acceptor variant
3 prime UTR variant
Missense variant
Intron variant
Synonymous variant
Downstream gene variant
Gene Legend
Protein Coding
protein coding
merged Ensembl/Havana
LD (r2)
1000GENOMES:phase_3:ITU
LD(r2): 1000GENOMES:phase_3:ITU (1000genomes:phase_3:sas) 128 SNPs
LD (d_prime)
LD(d_prime): 1000GENOMES:phase_3:ITU (1000genomes:phase_3:sas) 128 SNPs

Fig 23 TSI

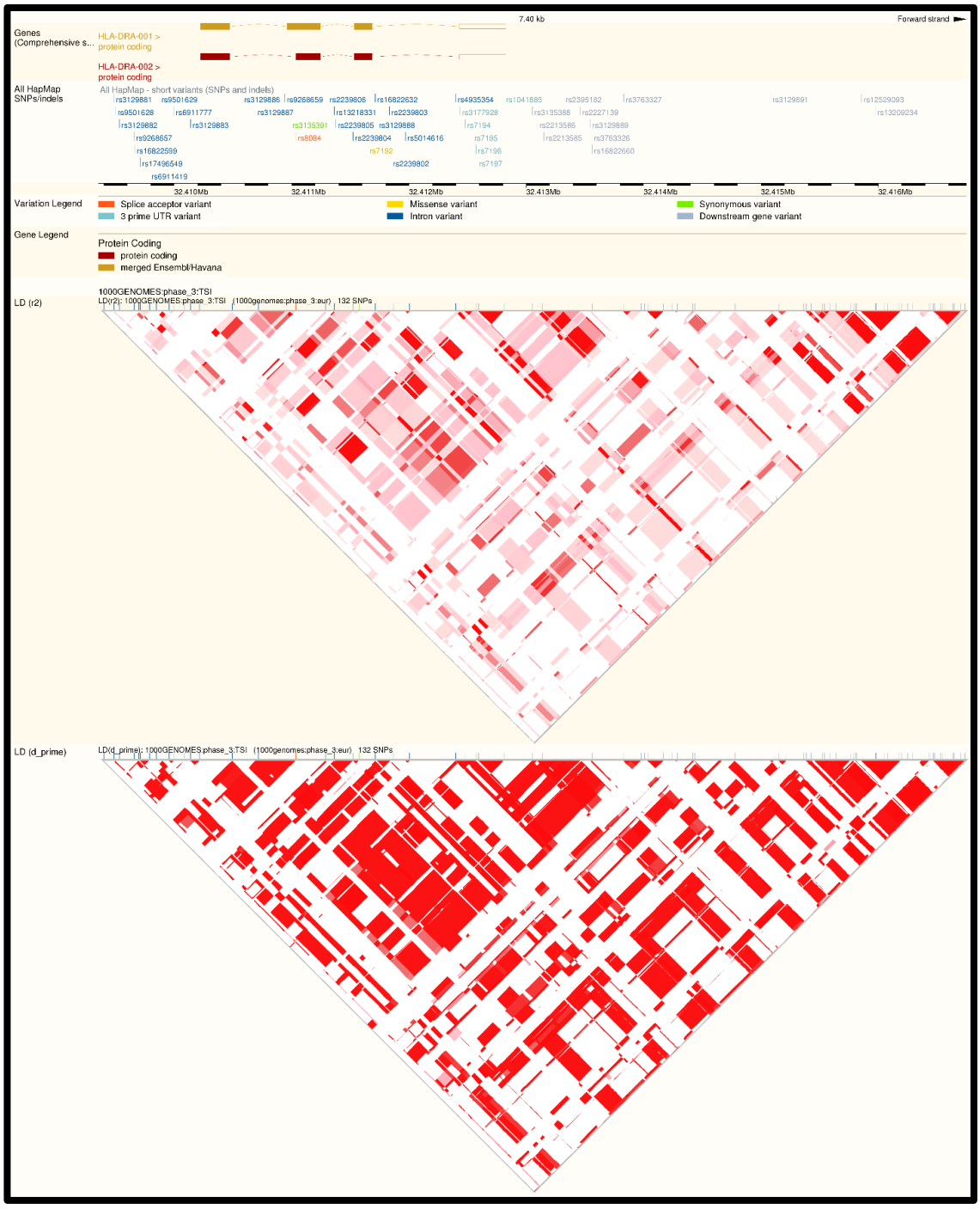

7.40 kb
Forward strand
Genes (Comprehensive s...
HLA-DRA-001 > protein coding
HLA-DRA-002 > protein coding
All HapMap SNPs/indels
All HapMap - short variants (SNPs and indels)
32.410Mb
32.411Mb
32.412Mb
32.413Mb
32.414Mb
32.415Mb
32.416Mb
Variation Legend
Splice acceptor variant
3 prime UTR variant
Missense variant
Intron variant
Synonymous variant
Downstream gene variant
Gene Legend
Protein Coding
protein coding
merged Ensembl/Havana
LD (r2)
1000GENOMES:phase_3:TSI
LD(r2): 1000GENOMES:phase_3:TSI (1000genomes:phase_3:eur) 132 SNPs
LD (d_prime)
LD(d_prime): 1000GENOMES:phase_3:TSI (1000genomes:phase_3:eur) 132 SNPs

Fig 24 YRI


7.40 kb
Forward strand
Genes (Comprehensive s...
HLA-DRA-001 > protein coding
HLA-DRA-002 > protein coding
All HapMap SNPs/indels
All HapMap - short variants (SNPs and indels)
32.410Mb
32.411Mb
32.412Mb
32.413Mb
32.414Mb
32.415Mb
32.416Mb
Variation Legend
Splice acceptor variant
3 prime UTR variant
Missense variant
Intron variant
Synonymous variant
Downstream gene variant
Gene Legend
Protein Coding
protein coding
merged Ensembl/Havana
LD (r2)
1000GENOMES:phase_3:YRI
LD(r2): 1000GENOMES:phase_3:YRI (1000genomes:phase_3:afr) 134 SNPs
LD (d_prime)
LD(d_prime): 1000GENOMES:phase_3:YRI (1000genomes:phase_3:afr) 134 SNPs

DISCUSSION

The present study looked at the genetic polymorphisms in RRMS patients compared to healthy controls in a south Indian cohort from January 2015 to December 2015. In the study we did not find an association between rs3135388 SNP polymorphism in any of the MS susceptibility.

The detailed demographic profile of the subjects revealed that there was clustering of cases between the ages 15- 45 years with the highest number of MS cases in the age group 15 – 25 years. The mean age of the study population was 31 years. The mean age in males and females were 32 years and 30 years respectively. The median age for males and females were 37 years and 26 years respectively. The lowest age of onset in the study population was 15years and the highest age of onset was 65 years. The female: male ratio was 3. 3, indicating that the females had 3 times higher risk for MS than males. The decade wise sex distribution revealed that between the age group 15 – 25 years this ratio was 5, indicating that at younger ages, the female predisposition for the disease is higher.

The demographic data observed in the study is comparable to the studies conducted worldwide. Majority of the studies conducted worldwide observed that the mean age of onset of RRMS is between 29 – 32 years. Along the same lines, a study of 940 patients in the Montreal Neurological Institute showed a mean age of onset of 30. 6 years and peak incidence at 25 years. It is also recognized that the onset can be well outside the mean age and as much as 5% of the MS cases can have their onset before the age of 18 years[31]. Tremlett et al 2006[63] reported that

approximately 3 – 12 % of incident cases can have their first symptom after the age of 50 years.

Sex and sex hormones affect the central nervous system and immune system differently and it is gauged that sexual dimorphism has a significant impact on the broad aspects of MS, such as susceptibility, disease course and radiological phenotypes. The current data reveals that women are more likely than men to get MS with a female to male ratio of 3. 3. The pooled data from Orton et al (2006)[64], Alonso et al (2008)[65] and Sudovnik et al (2009)[66] demonstrated a sex ratio of 3. 2: 1 with a clear female preponderance. Literature review reveals a dramatic shift in male: female ratio from the early 20th century to the present time which may be multifactorial. It can be postulated that women utilize health care more frequently now than in the early 20th century. The advent of MRI, drastically improved the ability to identify more cases with resultant improvement in case detection rates. The ability to detect the characteristic neurological changes and the ability to reach a diagnosis earlier in the disease course might have also influenced a higher detection rate.

There is a notion that overall women carry a more favorable prognosis than men which is supported by many clinical trials[61]. Beck et al 2003[67] in the optic neuritis treatment trial (ONTT) noted that female sex is a risk factor for conversion of clinically isolated syndrome to clinically definite MS (CDMS). Since MS is often diagnosed during the reproductive years, issues such as menstruation, fertility, pregnancy and breast feeding need to be clearly dealt with.

In the current study it was observed that the most common site of clinical presentation was spinal cord which accounted for 38% of the events, followed by brainstem, cerebellar and optic nerve involvement. The total number of neurological events ranged from one to as many as eight events in a single patient and mean number of total attacks in the study ranged between 3 and 4. Interval between clinical events did not reveal any significant association with what in the present study. Previous studies revealed that patients presenting with acute complete transverse myelitis(TM) have risk of 5 – 10% for progression to CDMS, though partial myelitis is a much more common clinical entity and attains more relevance with MS. Various studies have evaluated this issue of acute TM (partial) as an initial presentation in MS, and it was observed that 57 – 72% have cranial MRI consistent with MS. Subsequent follow up for 3 – 6 years revealed that 60 – 90 % of these patients developed MS. In patients with chronic progressive myelopathy, 60 – 70 % have cranial MRI consistent with MS in the absence of any clinical evidence of disease above the cord. What needs to be clarified is whether the remaining 30% have a disease other than MS or whether MS can manifest as a pure spinal cord syndrome alone (Morrissey et al 1993)[68].

Optic nerve is the most common site of involvement in the visual pathway. Bilateral simultaneous optic nerve involvement is rare in MS. It usually begins asymmetrically and is more severe in one eye. In the current study optic nerve involvement accounted to be approximately 10% which was asymmetric in presentation and this correlates well with the standard global statistics[31]. In the ONTT trial, 15% developed recurrent optic nerve involvement, either ipsilateral or

contralateral, within 6 – 24 months after the initial attack. Homonymous field defects can be seen in MS due to involvement of optic radiation though it is uncommon. The reported risk of progression to CDMS ranges from 15 – 75% in patients with initial presentation as optic neuritis. In a large population study (Rodriguez et al 1995)[69], 39% with isolated optic nerve involvement progressed to CDMS by 10 years of follow up, 49% by 20 years and 54 % by 30 years.

In this study, pyramidal involvement was noted in 70% of the cases. Corticospinal tract involvement in MS can manifest with different presentations. Paraparesis occurs most frequently, the next common presentation being hemiparesis occasionally sparing the face. Compared to the Western statistics the data in the south Indian population is comparable. Cerebellar involvement accounted for approximately 16% in the present study and cerebellar pathway impairment lead to gait imbalance, limb incoordination and slurred speech in the current study population. Bladder dysfunction was noted in approximately 50% of the patients in the study and it is comparable with studies conducted elsewhere in India and globally. The extent of sphincter dysfunction correlates with the degree of motor impairment in lower extremities. The most common urinary complaint reported in the study was urinary urgency. Constipation is a common feature which may be due to spinal cord involvement, decreased motility and dietary issues and has been reported in 50% of the study population[31]. No sexual dysfunction was reported in the present study population though the global data reveals it to be approximately 40-80% and it is a frequently overlooked problem. Cognitive problems also affect patients with MS[31]. Neuropsychological testing have demonstrated, approximately

34-65% of patients with MS having cognitive impairment. A longitudinal study by Penny et al 2010[70], reported 29% of early PPMS are cognitively impaired. In the current study none of the patients reported any cognitive impairment. It has been recognized that low physical disability can co-exist with significant cognitive impairment.

The median EDSS of the subjects in this study was 2. All the subjects were ambulant and independent of activities of daily living which could partially explain the preserved cognitive domains in the study patients. However in early MS, cognitive impairment is an infrequent symptom and subtle impairment can be detected only by sensitive cognitive batteries such as minimal assessment of cognition in multiple scleorosis (MACFIMS)[62]. There were two patients who reported paroxysmal symptoms which is characteristic of demyelinating disorders. There were no reports of Lhermitte's phenomenon, Uhthoff's phenomenon in the present study.

Overall, it can be summed that the clinical presentation in the study population were largely similar to those in the West. It was observed previously that the most frequent initial clinical presentation in the South Indian population is a severe myelopathy. Optic nerve involvement was also noted, but less frequent, though it has been reported that opticospinal form of MS is more common in Asian population. Cerebellar and brain stem involvement seem to be more frequent than optic nerve involvement[31]. Another observation was less cerebral involvement. It has been frequently observed that among initial symptoms, impairment in sensory pathway or cranial nerve dysfunction (optic nerve) have a

favorable prognosis, whereas pyramidal, brainstem and cerebellar involvement carry a bad prognosis[31].

The rate of clinical progression of MS is variable and the most commonly used index of clinical disability is the EDSS. It uses numbers ranging from 0, for normal examination to 10, for death. This scale is nonlinear with extreme emphasis on the ambulatory capacity of the patient with score above 4[31]. In the current study the mean EDSS was 2. Although the scale takes account of disability associated with advanced MS, most people will never reach the advanced scores. A large study that looked at MS patients at a clinic in Ontario found that 51% people had an EDSS score of ≤5 and 88% had score < 7 after a mean duration of follow up of 5 years[72]. Most of the population have bimodal distribution of EDSS score with peak at values 1 and 6. In the present study the bimodal distribution was not demonstrated. Runmaker et al (1993)[71] observed in a cohort of 308 MS patients followed up for 25 years, 80% evolved into progressive phase by 25 years,65% reached EDSS 6 and 50% reached EDSS 6 within 16 years of onset and 11% died. Although universally used in clinical trial, EDSS has numerous serious limitations. Interrater and intrarater variation is high while using EDSS. EDSS ≥ 4 depends entirely on ability to walk but dementia, visual loss and hand weakness may pass undetected. An important implication of these facts is that other outcome measures should be used as well and minor changes in EDSS alone should not be overinterpreted. MS functional composite scale (MSFC) is a more recent tool to avoid the issues encountered with EDSS. MSFC consists of 3 parts. 1) PASAT (Paced auditory serial addition test) 2) 9-Hole peg test 3) Timed 25 foot walk. These 3 parameters take into

account cognition, upper extremity movements and lower extremity function in a given individual. A large database of 1844 MS cases were analyzed to evaluate the predictors of disability and it was concluded that it takes longer to reach landmarks of irreversible disability in younger females with RRMS, patients presenting with optic neuritis and patients with fewer relapses in first year of disease onset. They also stressed that these good prognostic variables held true for patients up to EDSS of 4. It did not seem to remain predictive of disability in patients past 4 to landmarks 6 and 7. Another large study (1976, 1987) conducted in Norway verified these results and concluded that probability of being alive after 15 years was 94. 5%. The probability of managing without a wheelchair was75. 8% and of walking without assistance was 60. 3%. They also showed that patients with PPMS had more than 7. 5 times higher risk of reaching EDSS of 6 than with RRMS patients[31].

In the present study the CSF OCB positivity was observed in 71% and Ig G index was elevated in approximately 50% of the patients. CSF alone neither makes nor excludes the diagnosis of MS. Determining the presence or absence of OCB is a valuable diagnostic test. Isoelectric focusing followed by immune blot is the preferred test representing excess antibody produced by one or more clones of plasma cells. OCB positivity has been demonstrated in 85-95% of clinically definite MS[31]. In the present study it is only 71%. The low value may be due to the small sample size selected. Increased IgG index also has a sensitivity of 70-90% which was found to be low in the present study. Presence of OCB in a patient with clinically isolated syndrome seems to confer a higher rate of conversion to clinically definite MS (CDMS)(Ferraro et al 2013)[73].

Evoked potentials (EPs) are central nervous system electrical events generated by peripheral stimulation of a sensory organ. Three most commonly used EPs are VEP, SSEP and BAEP. In the current study VEP P100 prolongation was detected in 61 % of the patients and BAEP was abnormal only in approximately 30 % of the patients[31]. From the study it can be pointed out that VEP is a better EP tool for the evaluation in MS than BAEP. In a review by Gronseth and Ashman 2000[74], for the evaluation of role of EPs in MS, it was revealed that only VEP can be considered as a useful tool to determine increased risk for MS. They noted that P100 prolongation was detected in over 90% of the patients even in the setting of complete restoration of vision. Likewise in a case of CDMS, BAEP was abnormal in 50-65% of patients. In our study also similar results were obtained.

MRI is the preferred imaging modality for making the diagnosis as well as for the longitudinal follow up of MS patients. More than 95% of patients with MS demonstrated FLAIR/ T2 abnormalities[31]. In brain typical MS lesions are located in periventricular white matter, near corpus callosum, deep white matter, cortical and gray matter structures along with and involvement of juxta cortical U fibers, cerebellum, middle cerebellar peduncle, posterior optic radiations[31]. The presence of periventricular lesions has been considered as a hallmark of MS based on the observations of Swanton et al[75] and has been included in the Mc Donald's MS criteria. The volume of periventricular lesions adjacent to posterior horn and body of lateral ventricle in FLAIR sequence are significantly associated with RRMS and CIS. In the present study periventricular lesions of more than 10 numbers was observed in approximately 60% of the patients and 35% was found to have 1-5

lesions in the periventricular region typical of MS. A recent study to assess the association between MR and pathologic findings from MS patients has confirmed that the amount of lesions located in the subcortical white matter and within the cortex is not negligible. Since these lesions often involve the U fibers and other intra- and interhemispheric associative pathways, their load significantly contribute to the presence and severity of cognitive impairment. Miki et al[76], using conventional T2-weighted MR imaging, studied 53 MS patients and observed one or more U fiber lesions in 53% of the MS cases. Approximately two thirds of these lesions were located in the frontal white matter and memory and executive functions were significantly more compromised in patients with multiple U fiber lesions. Moriarty et al[77] also observed that impaired ability on delayed memory retrieval tests correlated well with juxtacortical lesion load in 20 patients with MS. Our study confirms that cortical/subcortical lesions can be detected in the majority of MS patients (about 87% has more than one lesion in our series). It has been noted that the load of MS lesions located in the cortical/subcortical areas was more than 10 times higher in the cognitively impaired patients, indicating that the presence and extent of cortical/subcortical lesions are major contributing factors to cognitive impairment in MS[31].

MS lesions that appear hypointense on T1-weighted images are commonly known as T1 black holes. With greater recognition of the role of neurodegeneration in MS in recent years, more interest has developed in evaluating the formation and possible evolution of persistent black holes as a marker of axonal loss and tissue destruction[31]. T1-weighted black holes have been shown to correlate with disability

in some studies. Recent evidence proposes that assessing correlations between clinical disability and a combined measure of T2 and T1 lesion volume and brain atrophy such as the magnetic resonance disease severity scale may be more meaningful than correlating individual measures of MRI lesion activity alone. The relationship between T1 hypointensities and relapse rates is also consistent[31]. Another study demonstrated a lack of positive predictive value of subsequent relapses and occurrence of new black holes in RRMS patients. In another study Cid et al pointed out a correlation between T1 lesion hypointensity and poor recovery from exacerbations.

There are several studies that focused on brain atrophy showing its relevant clinical impact not only in the diagnostic phase but also in predicting subsequent disability prognosis. A recent study by MAGNIMS group with 261 MS with MR imaging at baseline and after 1-2 years and EDSS score at baseline and after 10 years, the whole brain and central atrophy were good predictors of EDSS at 10 years[78]. Jasperse and colleagues pointed out that central atrophy is related to a declinein ambulatory function but central and peripheral atrophy is associated with decline in more neurologically demanding tasks[79].

Several voxel based and surface based MR studies revealed strong relationship between gray matter involvement, but not with white matter involvement in the disability progression. The regional analysis of grey matter atrophy revealed that the thalamus and cerebellum were consistently related to clinical disability[31]. Thalamus is the earliest structure involved in the pathological process and thalamic atrophy correlates with changes in EDSS. In a recent study

conducted by Calabrese et al[80], cerebellar cortical atrophy with age and cortical lesion load was indicated among the predictive parameters of progression. In the same study they have pointed out that high cortical lesion load at baseline showed the worse clinical evolution and subsequently progression of cortical atrophy after 5 years[80].

The rs3135388 was screened in 60 healthy controls and 25 patients. The allele and genotype frequencies were assessed and no association was observed. The mutant allele frequency was very low in healthy control while it was completely absent in the patients. The risk allele seems to be functionally relevant as evident from the F-SNP score which indicates its role in transcriptional regulation.

The sample size of the present study is small. However, this seems sufficient for interpretation, as evident by the population frequency from the control data. The control data frequency compares with Gujarati Indians extracted from 1000 genome data. While comparing the allele and genotype frequencies in south Indian population with other global populations, it was observed that both allele and genotype frequencies significantly differed with Caucasian population. The risk allele was seen to be increased in the Caucasian population. This is further evident from the LD map view spanning 7. 6 kb region flanked with multiple SNPs in the region. The pattern of LD differs in Caucasians and Indian population. Therefore, we need to identify the SNPs that could be tagged with the risk SNP in our population to identify probable SNP marker for the DRB1 locus that could predispose to MS.

CONCLUSION

1. Relapsing remitting multiple sclerosis is three times more common in females with young females having maximum predisposition to the disease.
2. The most common clinical manifestation was myelopathy which occurred in 43% patients followed by brain stem, cerebellar and optic nerve dysfunction in 14, 7 and 6 percent respectively.
3. Pyramidal signs were observed in 70% and bladder dysfunction in 50%.
4. Median EDSS of this study group was 2.
5. Unmatched CSF oligo clonal bands were detected in 71% and Ig G index was elevated in approximately 50% of the patients
6. VEP P100 prolongation was detected in 61 % of the patients and BAEP was abnormal in approximately 30 % of the patients.
7. Periventricular lesions of more than 10 numbers was observed in approximately 60% of the patients and 35% were found to have 1-5 lesions in the periventricular region typical of MS. T1 black holes were seen in 25%, cerebral atrophy in 58%, confluent spinal cord lesions in 38% of the study population.
8. The clinical presentation and investigations in the study population were largely similar to that in the western population.
9. None of the RRMS patients or healthy controls in this study exhibited the rs3135388 gene polymorphism.

10. Both allele and genotype frequencies of tag SNP, rs3135388 significantly differed with Caucasian population with the risk allele increased in the Caucasian population.
11. The linkage disequilibrium map view spanning 7. 6 kb region flanked with multiple SNPs in the region demonstrate that both allele and genotype frequencies of tag SNP, rs3135388 in Indian population significantly differed with Caucasian population.
12. We need to identify the SNPs that could be tagged with the risk SNP in our population to identify probable SNP marker for the DRB1 locus that could predispose to multiple sclerosis.

LIMITATIONS

1. The sample size of the study was small. A larger sample size may give more information regarding the MS genotype and its susceptibility.
2. In HLA sequencing, only one tag SNP was included. A study may conducted including more SNPs to through light into the association between other SNP and MS susceptibility.
3. A follow up study may be more meaningful to assess the tag SNP in Indian population.

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श्री चित्रा तिरुनाल आयुर्विज्ञान और प्रौद्योगिकी संस्थान
तिरुवनन्तपुरम - 695 011, केरल, इंडिया
SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES AND TECHNOLOGY
THIRUVANANTHAPURAM - 695 011, INDIA
(An Institute of National importance under Govt. of India)

Institutional Ethics Committee

(IEC Regn No. ECR/189/Inst/KL/2013)

SCT/IEC/732/FEBRUARY -2015

20-04-2015

Dr. Arun
Senior Resident
Department of Neurology
SCTIMST, Thiruvananthapuram

Dear Dr. Arun,

The Institutional Ethics Committee reviewed and discussed your application to conduct the study entitled "ASSOCIATION OF HLA-DRB1*1501 TAGGING RS3135388 GENE POLYMORPHISM WITH MULTIPLE SCLEROSIS SUSCEPTIBILITY (IEC/732)" on 21st February, 2015.

The following documents were reviewed:

Original submission

1. *Covering letter addressed to the Chairperson, IEC, SCTIMST dated 27.01.2015.*
2. *TAC Clearance letter.*
3. *IEC Application Form.*
4. *Proposal.*
5. *Proforma.*
6. *Consent form in English and Malayalam.*
7. *CVs of the PI and Co-PI.*

Revised submission

8. *Covering letter addressed to the Chairman, IEC, SCTIMST dated 17.04.2015.*
9. *Modified IEC Application Form is submitted.*

तार : चित्रमेट | फोन | फाक्स | ई-मेइल
Grams : Chitramet | Phone : 2443152 | Fax : (91)471-2446433 2550728 | E-mail : sct. @sctimst.ker.nic.in

The following members of the Ethics Committee were present at the meeting held on 21st February, 2015 at G. Parthasarathi Board Room, AMCHSS, SCTIMST.

SL. No.	Member Name	Highest Degree	Gender	Scientific /Non Scientific	Affiliation with Institution(s)
1.	Justice Gopinathan. P.S	BSc. LLB	Male	Legal Expert (Chairperson)	No
2.	Dr. J. M. Tharakan	MD	Male	Clinician (Cardiologist)	Yes
3.	Shri. O.S. Neelakandan Nair	BE	Male	Engineer	Yes
4.	Dr. Meenu Hariharan	DM	Female	Clinician (Gastro-Enterologist)	No
5.	Dr. R V G Menon	PhD	Male	Lay Person	No
6.	Dr. Rema M. N	MD	Female	Pharmacologist	No
7.	Dr. Kala Kesavan. P	MD	Female	Pharmacologist	No
8.	Dr. Mala Ramanathan	MSc, PhD, MA	Female	Ethicist/Social Scientist (Member Secretary)	Yes

IEC Decision

The IEC approved the conduct of the study in the present form.

Remarks:

The Institutional Ethics Committee expects to be informed about the progress of the study, any SAE occurring in the course of the study, any changes in the protocol and patient information/informed consent and asks to be provided a copy of the final report.

There was no member of the study team who participated in voting / decision making process. The ethics committee is organized and operated according to the requirements of Good Clinical Practice and the requirements of the Indian Council of Medical Research (ICMR).

Sincerely,

Mala Ramanathan
Member Secretary, IEC

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Proforma to the study of association of HLA-DRB1*1501 tagging rs3135388 gene polymorphism with multiple sclerosis

1. PATIENT DETAILS

1.1 Name of the patient _____

1.2 Hospital Number_____

1.3 Age of the patient _____

1.4 Sex_____1.Male 2.Female

1.5 Occupation_____

1.6 If outpatient or inpatient ---------- ---------If outpatient date seen in OPD----------

1.7 If inpatient, date of admission. ----------

1.8 Phone No 1:----------

1.9 Phone No 2:----------

2. CLINICAL EVALUATION

2.1. Clinical Data:

2.1.a Age of onset of the symptom_____

2.1.b First clinical symptom_____

2.1.c Number of attacks_____

2.1.d Interval between the attacks_____

2.1.e Current attack_____If yes, details of the attack_____

1.Pyramidal 2.Cerebellar 3.Brain stem 4.sensory 5.Bladder/bowel 6.Visual 7.Cerebral

2.1.f Details of previous attacks_____

Attacks (month and year)	**Pyramidal**	**Cerebellar**	**Brain stem**	**Sensory**	**Bladder/ bowel**	**Visual**	**Cerebral**	**Treatment give**	**Resolved /not**

2.1.g Family History:

2.2. FUNCTIONAL STATUS SCORE:

A. Pyramidal Functions:

0 = Normal

1 = Abnormal signs without disability

2 = Minimal disability

3 = Mild or moderate paraparesis or hemiparesis, or severe monoparesis

4 = Marked paraparesis or hemiparesis, moderate quadriparesis, or monoplegia

5 = Paraplegia, hemiplegia, or marked quadriparesis

6 = Quadriplegia

B. Cerebellar Functions:

0 = Normal

1 = Abnormal signs without disability

2 = Mild ataxia

3 = Moderate truncal or limb ataxia

4 = Severe ataxia all limbs

5 = Unable to perform coordinated movements due to ataxia

C. Brainstem functions

0 = Normal

1 = Signs only

2 = Moderate nystagmus or other mild disability

3 = Severe nystagmus, marked extra ocular weakness, or moderate disability of other cranial nerves

4 = Marked dysarthria or other marked disability

5 = Inability to swallow or speak

D. Sensory functions

0 = Normal

1 = Vibration or figure-writing decrease only, in 1 or 2 limbs

2 = Mild decrease in touch or pain or position sense, and/or moderate decrease in vibration in 1 or 2 limbs, or vibratory decrease alone in 3 or 4 limbs

3 = Moderate decrease in touch or pain or position sense, and/or essentially lost vibration in 1 or 2 limbs, or mild decrease in touch or pain, and/or moderate decrease in all proprioceptive tests in 3 or 4 limbs

4 = Marked decrease in touch or pain or loss of proprioception, alone or combined, in 1 or 2 limbs or moderate decrease in touch or pain and/or severe proprioceptive decrease in more than 2 limbs

5 = Loss (essentially) of sensation in 1 or 2 limbs or moderate decrease in touch or pain and/or loss of proprioception for most of the body below the head

6 = Sensation essentially lost below the head

E. Bowel and bladder functions

0 = Normal

1 = Mild urinary hesitancy, urgency, or retention

2 = Moderate hesitancy, urgency, retention of bowel or bladder, or rare urinary incontinence

3 = Frequent urinary incontinence

4 = In need of almost constant catheterization

5 = Loss of bladder function

6 = Loss of bowel and bladder function

F. Visual (or optic) functions

0 = Normal

1 = Scotoma with visual acuity (corrected) better than 20/30

2 = Worse eye with scotoma with maximal visual acuity (corrected) of 20/30 to 20/59 3 = Worse eye with large scotoma, or moderate decrease in fields, but with maximal visual acuity (corrected) of 20/60 to 20/99

4 = Worse eye with marked decrease of fields and maximal acuity (corrected) of 20/100 to 20/200; grade 3 plus maximal acuity of better eye of 20/60 or less

5 = Worse eye with maximal visual acuity (corrected) less than 20/200; grade 4 plus maximal acuity of better eye of 20/60 or less

6 = Grade 5 plus maximal visual acuity of better eye of 20/60 or less

G. Cerebral (or mental) functions

0 = Normal

1 = Mood alteration only (does not affect EDSS score)

2 = Mild decrease in mentation

3 = Moderate decrease in mentation

4 = Marked decrease in mentation

5 = Chronic brain syndrome—severe or incompetent

	Functional Status (FS) Score							
		0	1	2	3	4	5	6
A	Pyramidal functions							
B	Cerebellar functions							-
C	Brainstem functions							-
D	Sensory functions							
E	Bowel and bladder functions							
F	Visual functions							
G	Cerebral functions							-

2.3. Cognitive Function Assessment:

2.2. a PASAT:

2.4. Kurtzke Expanded Disability Status Score (EDSS)

0.0 = Normal neurologic exam [all grade 0 in functional status (FS)]

1.0 = No disability, minimal signs in one FS (i.e., grade 1)

1.5 = No disability, minimal signs in more than one FS (more than one grade 1)

2.0 = Minimal disability in one FS (one FS grade 2, others 0 or 1)

2.5 = Minimal disability in two FS (two FS grade 2, others 0 or 1)

3.0 = Moderate disability in one FS (one FS grade 3, others 0 or 1) or mild disability in three or four FS (three/four FS grade 2, others 0 or 1) though fully ambulatory

3.5 = Fully ambulatory but with moderate disability in one FS (one grade 3) and one or two FS grade 2; or two FS grade 3; or five FS grade 2 (others 0 or 1)

4.0 = Ambulatory without aid or rest for 500 m

4.5 = Ambulatory without aid or rest for 300 m

5.0 = *Ambulatory without aid or rest for 200 m*

5.5 = Ambulatory without aid or rest for 100 m

6.0 = Unilateral assistance required to walk about 100 m with or without resting

6.5 = Constant bilateral assistance required to walk about 20 m without resting

7.0 = Unable to walk beyond about 5 m even with aid; essentially restricted to wheelchair; wheels self and transfers alone

7.5 = Unable to take more than a few steps; restricted to wheelchair; may need aid to transfer

8.0 = Essentially restricted to bed or chair or perambulated in wheelchair, but out of bed most of day; retains many self-care functions; generally has effective use of arms

8.5 = Essentially restricted to bed much of the day; has some effective use of arm(s); retains some self-care functions

9.0 = Helpless bed patient; can communicate and eat

9.5 = Totally helpless bed patient; unable to communicate or eat

10.0 = Death due to MS

3. INVESTIGATIONS:

3.1. MRI Brain (Mac Donald's Criteria)

Dissemination in Space:

>/= 1 T2 Lesions in at least 2 of 4 Areas of the CNS:

1. Periventricular
2. Juxtacortical
3. Infratentorial
4. Spinal cord

Dissemination in time:

1. A new T2 and/or gadolinium-enhancing lesion(s) on follow-up MRI, with reference to a baseline scan, irrespective of the timing of the baseline MRI.

2. Simultaneous presence of asymptomatic gadolinium-enhancing and non enhancing lesions at any time.

3.1.e MRI based Severity Scale:

3.2. CSF Study:

Total cells Sugar Protein OCBs
Ig G Index

3.3. VEP: P_{100} Wave prolongation-_____1.Present 2.Absent.

3.4. BAEP:

3.5. SNP analysis:

4. TREATMENT AT DISCHARGE:

4.1 Current disease modifying agent patient is using-_____1.Interferon beta-1a 2.Interferon beta-1b 3.Glatiramer acetate 4.Natalizumab 5. Mitoxantrone 6.Fingolimod 7.Diethyl fumarate 8. Teriflunamide

4.2 Previously tried disease modifying agents_____1.Interferon beta-1a 2.Interferon beta-1b 3.Glatiramer acetate 4.Natalizumab 5. Mitoxantrone 6.Fingolimod 7.Diethyl fumarate 8. Teriflunamide.

5. OUTCOME:

5.1 Date of discharge_____

5.2 Condition at discharge_____

5.3 Date of new event_____

5.4 Final diagnosis_____

5.5 Complications in the Hospital: _____ 1. Pneumonia 2. MI 3. DVT 4. UTI 5. Bedsore 6. Pulmonary embolism 7. LVF 8.None

TABLE 4: The 2010 McDonald Criteria for Diagnosis of MS

Clinical Presentation	Additional Data Needed for MS Diagnosis
≥2 attacks[a]; objective clinical evidence of ≥2 lesions or objective clinical evidence of 1 lesion with reasonable historical evidence of a prior attack[b]	None[c]
≥2 attacks[a]; objective clinical evidence of 1 lesion	Dissemination in space, demonstrated by: ≥1 T2 lesion in at least 2 of 4 MS-typical regions of the CNS (periventricular, juxtacortical, infratentorial, or spinal cord)[d]; or Await a further clinical attack[a] implicating a different CNS site
1 attack[a]; objective clinical evidence of ≥2 lesions	Dissemination in time, demonstrated by: Simultaneous presence of asymptomatic gadolinium-enhancing and nonenhancing lesions at any time; or A new T2 and/or gadolinium-enhancing lesion(s) on follow-up MRI, irrespective of its timing with reference to a baseline scan; or Await a second clinical attack[a]
1 attack[a]; objective clinical evidence of 1 lesion (clinically isolated syndrome)	Dissemination in space and time, demonstrated by: For DIS: ≥1 T2 lesion in at least 2 of 4 MS-typical regions of the CNS (periventricular, juxtacortical, infratentorial, or spinal cord)[d]; or Await a second clinical attack[a] implicating a different CNS site; and For DIT: Simultaneous presence of asymptomatic gadolinium-enhancing and nonenhancing lesions at any time; or A new T2 and/or gadolinium-enhancing lesion(s) on follow-up MRI, irrespective of its timing with reference to a baseline scan; or Await a second clinical attack[a]
Insidious neurological progression suggestive of MS (PPMS)	1 year of disease progression (retrospectively or prospectively determined) plus 2 of 3 of the following criteria[d]: 1. Evidence for DIS in the brain based on ≥1 T2 lesions in the MS-characteristic (periventricular, juxtacortical, or infratentorial) regions 2. Evidence for DIS in the spinal cord based on ≥2 T2 lesions in the cord 3. Positive CSF (isoelectric focusing evidence of oligoclonal bands and/or elevated IgG index)

If the Criteria are fulfilled and there is no better explanation for the clinical presentation, the diagnosis is "MS"; if suspicious, but the Criteria are not completely met, the diagnosis is "possible MS"; if another diagnosis arises during the evaluation that better explains the clinical presentation, then the diagnosis is "not MS."

[a]An attack (relapse; exacerbation) is defined as patient-reported or objectively observed events typical of an acute inflammatory demyelinating event in the CNS, current or historical, with duration of at least 24 hours, in the absence of fever or infection. It should be documented by contemporaneous neurological examination, but some historical events with symptoms and evolution characteristic for MS, but for which no objective neurological findings are documented, can provide reasonable evidence of a prior demyelinating event. Reports of paroxysmal symptoms (historical or current) should, however, consist of multiple episodes occurring over not less than 24 hours. Before a definite diagnosis of MS can be made, at least 1 attack must be corroborated by findings on neurological examination, visual evoked potential response in patients reporting prior visual disturbance, or MRI consistent with demyelination in the area of the CNS implicated in the historical report of neurological symptoms.

[b]Clinical diagnosis based on objective clinical findings for 2 attacks is most secure. Reasonable historical evidence for 1 past attack, in the absence of documented objective neurological findings, can include historical events with symptoms and evolution characteristics for a prior inflammatory demyelinating event; at least 1 attack, however, must be supported by objective findings.

[c]No additional tests are required. However, it is desirable that any diagnosis of MS be made with access to imaging based on these Criteria. If imaging or other tests (for instance, CSF) are undertaken and are negative, extreme caution needs to be taken before making a diagnosis of MS, and alternative diagnoses must be considered. There must be no better explanation for the clinical presentation, and objective evidence must be present to support a diagnosis of MS.

[d]Gadolinium-enhancing lesions are not required; symptomatic lesions are excluded from consideration in subjects with brainstem or spinal cord syndromes.

MS = multiple sclerosis; CNS = central nervous system; MRI = magnetic resonance imaging; DIS = dissemination in space; DIT = dissemination in time; PPMS = primary progressive multiple sclerosis; CSF = cerebrospinal fluid; IgG = immunoglobulin G.

FUNCTIONAL STATUS SCORE:

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vibration in 1 or 2 limbs, or mild decrease in touch or pain, and/or moderate decrease in all proprioceptive tests in 3 or 4 limbs

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	Functional Status (FS) Score							
		0	1	2	3	4	5	6
A	Pyramidal functions							
B	Cerebellar functions							-
C	Brainstem functions							-
D	Sensory functions							
E	Bowel and bladder functions							
F	Visual functions							
G	Cerebral functions							-

Kurtzke Expanded Disability Status Score (EDSS)

0.0 = Normal neurologic exam [all grade 0 in functional status (FS)]

1.0 = No disability, minimal signs in one FS (i.e., grade 1)

1.5 = No disability, minimal signs in more than one FS (more than one grade 1)

2.0 = Minimal disability in one FS (one FS grade 2, others 0 or 1)

2.5 = Minimal disability in two FS (two FS grade 2, others 0 or 1)

3.0 = Moderate disability in one FS (one FS grade 3, others 0 or 1) or mild disability in three or four FS (three/four FS grade 2, others 0 or 1) though fully ambulatory

3.5 = Fully ambulatory but with moderate disability in one FS (one grade 3) and one or two FS grade 2; or two FS grade 3; or five FS grade 2 (others 0 or 1)

4.0 = Ambulatory without aid or rest for 500 m

4.5 = Ambulatory without aid or rest for 300 m

5.0 = *Ambulatory without aid or rest for 200 m*

5.5 = Ambulatory without aid or rest for 100 m

6.0 = Unilateral assistance required to walk about 100 m with or without resting

6.5 = Constant bilateral assistance required to walk about 20 m without resting

7.0 = Unable to walk beyond about 5 m even with aid; essentially restricted to wheelchair; wheels self and transfers alone

7.5 = Unable to take more than a few steps; restricted to wheelchair; may need aid to transfer

8.0 = Essentially restricted to bed or chair or perambulated in wheelchair, but out of bed most of day; retains many self-care functions; generally has effective use of arms

8.5 = Essentially restricted to bed much of the day; has some effective use of arm(s); retains some self-care functions

9.0 = Helpless bed patient; can communicate and eat

9.5 = Totally helpless bed patient; unable to communicate or eat

10.0 = Death due to MS

CONSENT FORM (Subjects)

Sree Chitra Tirunal Institute of Medical Science and Technology Thiruvananthapuram, Kerala-695011

TITLE OF STUDY: "Association of HLA-DRB1*1501 tagging rs3135388 gene polymorphism with multiple sclerosis."

Principal investigator: Dr. Arun K

Principal Co-investigators: Dr C. Sarada

Dr Moinak Banerjee

Introduction:

This is an important form. Please read it carefully. It tells you what you need to know about this study. If you agree to take part in this research study, you need to sign this form. Your signature means that you have been told about study and what the risks are. Your signature on this form also means that you want to take part in this study.

Why is this study being done?

Multiple sclerosis (MS) is an autoimmune disease of the central nervous system (CNS) leading to demyelination, axonal damage, and progressive neurological disability. It has been proved that HLA-DRB1*1501 has the strongest association with MS, with a consistent influence within the cohort. Within the HLA-DRB1 gene, the rs3135388 SNPs have been demonstrated as the most strongly associated with MS. Only a few genetic association studies have been published that address this gene polymorphism in relation to MS. In the present study, the HLA-DRB1*1501 allele rs3135388 gene polymorphism will be investigated in relation to MS susceptibility, disability and potential gender differences in the South Indian population.

You are invited to participate in this study as a subject if you are having relapsing and remitting Multiple sclerosis.

How many people will take part in the study?

The plan is to include 160 patients with relapsing and remitting Multiple sclerosis

based on the Mc Donald's criteria and 160 healthy volunteers.

What will happen in the study?

Your participation will require:

- Donation of about 10 cc of blood to be drawn from a vein in your arm. The procedure will take about 15 minutes.
- Blood samples will be sent to Rajiv Gandhi Centre for Biotechnology, Trivandrum for genetic analysis. Because the genetic tests in this study are not used for regular medical care, you will not be told about the results of the test(s). The test results will not be put in your medical record either.

How long will I be in the study?

You will be in the study for a minimum of 2 years though blood donation is a one time event.

Will any biological sample(s) be stored and used in the future by Rajiv Gandhi Centre for Biotechnology, Trivandrum?

No

What are the risks of the study?

There may be the minor pain associated with the needle stick required during blood drawing. There is also light chance of bruising at the site of needle puncture.

Are there benefits to taking part in this study?

You will receive no direct benefit from participation in this study.

What other choices do I have if I don't take part in this study?

The study is only being done to gather scientific information. You may choose not to take part in this study.

What are the costs of the tests and the procedures?

You will not need to pay for any tests or procedures, which are done just as a part of the research study.

Who can answer my questions?

You may talk to Dr. Arun K at any time about any question you have on this study. You may contact Dr. Arun K by calling him at the phone number: 08281232084.

Will I lose my rights if I do not take part in this study?

Taking part in this research study is your decision. You do not have to take part in this study if you are unwilling. Your medical care in SCTIMST now or in the future will not be affected whether or not you take part in this study.

You will be told of important new findings that may happen, if you choose to have that information.

You do not give up any of your rights by taking part in this study.

What about confidentiality?

Data from this study may be published. However, your name and other identifying information will not be sent outside of SCTIMST.

Declaration

I had an opportunity to have my questions answered. I have been given a copy of this form. I agree to take part in this study.

_____ _____ _____

(Date) (Signed and printed name of Participant) (Clinic) number)

_____ _____

(Date) (Signed and printed name of Individual obtaining consent)

_____ _____

(Date) (Signed and printed name of witness)

CONSENT FORM (CONTROLS)

Principle Investigator: - Dr. Arun K

Principle Co-Investigator:- Dr.C.Sarada,

Dr.Moinak Banerjee

Hospital: Sree Chitra Tirunal Institute for Medical sciences and Technology

Title of the Study:

Association of HLA-DRB1*1501 tagging rs3135388 gene polymorphism with multiple sclerosis**.**

Introduction:

You are being requested to participate in a study on " Association of HLA-DRB1*1501 tagging rs3135388 and gene polymorphism with multiple sclerosis". This study aims to have 160 number of patients with multiple sclerosis and equal number healthy control individuals.

If you take part what will you have to do?

The study will require consent only at the time of enrolment. You will be subjected to withdraw about 10 ml of blood for detection of HLA-DRB1*1501 tagging rs3135388 gene polymorphism in the blood. The participation in the study is purely voluntary. These results appear to be very promising in future applications in clinical tests, to complement or replace classical HLA typing.

Will you have to pay for the investigations?

All the investigations done for the purpose of the study will be done free of cost. No additional follow up visits are required.

Will your personal details be kept confidential?

The results of this study will be published in a medical journal but you will not be identified by name in any publication or presentation of results. However, your medical notes may be reviewed by people associated with the study, without your additional permission.

If you have any further questions, please ask Dr.Arun K (Tel: 08281232084) email: drkarun@sctimst.ac.in

This test for detection of SNP is not currently included in the diagnostic test for multiple sclerosis but only for research purpose.

Participant's name:

Date of Birth / Age (in years):

Declaration:

I_____, Son/daughter of _____ declare that I have read the above information provided to me regarding the study: "Association of HLA-DRB1*1501 tagging rs3135388 and rs3135391 gene polymorphism with multiple sclerosis". and have clarified any doubts that I had. [ ]

• I also understand that participation in this study is entirely voluntary and that I am free to withdraw permission to continue to participate at any time without affecting my usual treatment or my legal rights [ ]

• I understand that the study staff and institutional ethics committee members will not need permission to look at health records even if I withdraw from the trial. I agree to this access [ ]

• I understand that the participant's identity will not be revealed in any information released to third parties or published [ ]

• I received a copy of this signed consent form [ ]

Name:

Signature:

Date:

Name of witness:

Signature:

Date:

I attest that the requirements for informed consent for the medical research project described in this form have been satisfied. I have discussed the research project with the participant and explained to him or her in non-technical terms all of the information contained in this informed consent form, including any risks and adverse reactions that may reasonably be expected to occur. I further certify that I encouraged the participant to ask questions and that all questions asked were answered.

_____ _____

Name and Signature of Person Obtaining Consent

സ്വമേധയായുള്ള സമ്മതപത്രം (സബ്ജക്ട്സ്)

ശ്രീചിത്തിരതിരുനാൾ ഇൻസ്റ്റിറ്റ്യൂട്ട് ഫോർ മെഡിക്കൽ സയൻസ് ആന്റ് ടെക്നോളജി, തിരുവനന്തപുരം , കേരളം -695011.

പഠന വിഷയം :

മൾട്ടിപ്പിൾ സ്ക്ലീറോസിസും HLA –DRB1 *1501 ന്റെ സൂചകമായ rs 3135388ഉം തമ്മിലുള്ള ബന്ധം .

പ്രധാന ഗവേഷകൻ: ഡോ.അരുൺ.കെ

ഇത് ഒരു പ്രധാനപ്പെട്ട ഫോറം ആണ് . ദയവായി ഇത് ശ്രദ്ധയോടെ വായിക്കുക .ഈ പഠനത്തെക്കുറിച്ച് നിങ്ങൾ അറിയേണ്ടതെല്ലാം ഇതു വെളിപ്പെടുത്തുന്നു. ഈ ഗവേഷണ പഠനത്തിൽ പങ്കുചേരുവാൻ നിങ്ങൾക്ക് സമ്മതമാണെങ്കിൽ ഈ ഫോറത്തിൽ നിങ്ങൾ ഒപ്പിടേണ്ടതാണ്. ഈ പഠനത്തെപ്പറ്റിയും ഇതിലുണ്ടാകുന്ന റിസ്ക്കുകളെപ്പറ്റിയും നിങ്ങൾക്കു പറഞ്ഞു തന്നിരിക്കുന്നു എന്നതാണ് നിങ്ങളുടെ ഒപ്പ് വ്യക്തമാക്കുന്നത് . നിങ്ങൾ ഈ പഠനത്തിൽ പങ്കെടുക്കുവാൻ ആഗ്രഹിക്കുന്നു എന്നും ഈ ഫോറത്തിലുള്ള നിങ്ങളുടെ ഒപ്പ് അർത്ഥമാക്കുന്നു.

ഈ പഠനം എന്തിനു വേണ്ടിയാണ് നടത്തുന്നത് ?

മൾട്ടിപ്പിൾ സ്ക്ലീറോസിസ് കേന്ദ്ര നാഡീവ്യവസ്ഥയുടെ ഒരു സ്വപ്രതിരോധരോഗമാണ്. ഇത് മൈലിൻ ആവരണം ദ്രവിച്ചു പോകൽ, ആക്സോൺ നാശം , ഭയങ്കരമായ നാഡീവൈകല്യം എന്നിവയ്ക്കു കാരണമാകുന്നു. മൾട്ടിപ്പിൾ സ്ക്ലീറോസിസ് രോഗികളെ നല്ല പോലെ സ്വാധീനിക്കുന്ന HLA –DRB1 *1501 മൾട്ടിപ്പിൾ സ്ക്ലീറോസിസുമായി ശക്തമായി ബന്ധപ്പെട്ടിരിക്കുന്നുവെന്ന് തെളിയിക്കപ്പെട്ടിട്ടുണ്ട്. HLA –DRB1 ജീനിൽ തന്നെ rs3135388 മൾട്ടിപ്പിൾ സ്ക്ലീറോസുമായി ശക്തമായി ബന്ധപ്പെട്ടിരിക്കുന്നതെന്ന് വ്യക്തമായിട്ടുണ്ട്. മൾട്ടിപ്പിൾ സ്ക്ലീറോസിസുമായി ബന്ധപ്പെട്ട ഈ ജീൻ പോളിമോർഫിസത്തെപ്പറ്റി വളരെ കുറച്ചു ജനിതക പഠനകുറിപ്പുകളെ പ്രസിദ്ധീകരിച്ചിട്ടുള്ളു. ഇപ്പോഴത്തെ ഈ പഠനത്തിൽ ദക്ഷിണേന്ത്യയിലെ മൾട്ടിപ്പിൾ സ്ക്ലീറോസിസ് വരാൻ സാധ്യതയുള്ളതും, വൈകല്യമുള്ളതുമായരോഗികളിൽ HLA –DRB1 *1501 ന്റെ സൂചകമായ rs 3135388ന്റെ പങ്ക് എന്ത് എന്ന് കണ്ടെത്തുന്നതാണ് . പുരുഷനിലാണോ, സ്ത്രീയിലാണോ ഇതുകൂടുതലെന്നു തെളിയിക്കുന്നതാണ്.

നിങ്ങൾക്ക് വന്നും പോയും കൊണ്ടിരിക്കുന്ന മൾട്ടിപ്പിൾ സ്ക്ലീറോസിസ് ഉണ്ടെങ്കിൽ നിങ്ങളെ ഈ പഠനത്തിൽ ഒരു പഠനവിഷയവുമായി പങ്കെടുക്കുവാനായി ക്ഷണിച്ചുകൊള്ളുന്നു.

ഈ പഠനത്തിൽ എത്ര പേർ പങ്കെടുക്കും?

മെക്ഡൊണാൾഡിന്റെ മാനദണ്ഡപ്രകാരമുള്ള ഇടവിട്ടു വരുന്ന മൾട്ടിപ്പിൾ സ്ക്ലീറോസിസുള്ള 160 രോഗികളെയും, 160 ആരോഗ്യവാനാമാരായ വളണ്ടിയർമാരെയും ഉൾക്കൊള്ളിക്കുവാനാണ് ഉദ്ദേശിക്കുന്നത്.

ഈ പഠനത്തിൽ എന്താണ് നടക്കുന്നത് ?

നിങ്ങൾ പങ്കെടുക്കുകയാണെങ്കിൽ ചെയ്യുന്നത്

- കൈയിലെവെയിനിൽ നിന്നും ഏകദേശം 10cc രക്തംദാനം ചെയ്യണം. ഇതിന് ഏകദേശം 15 മിനുട്ട് സമയമെടുക്കും.

രക്തസാമ്പിളുകൾ ജനിതക പരിശോധനയ്ക്കായി തിരുവന്തപുരത്തെ രാജീവ് ഗാന്ധിസെന്റർ ഫോർ ബയോടെക്ക്നോളജിയിലേക്കയക്കും എന്തെന്നാൽ ഈ പഠനത്തിനാവശ്യമായ ജനിതക പരിശോധനകൾ സാധാരണമെഡിക്കൽ ടെസ്റ്റുകളിൽ ചെയ്യാറില്ല. ആ ടെസ്റ്റുകളുടെ റിസൽട്ട് നിങ്ങളെ അറിയിക്കുകയില്ല. ടെസ്റ്റിന്റെ ഫലങ്ങൾ നിങ്ങളുടെ മെഡിക്കൽ റെക്കോർഡുകളിലും വയ്ക്കുന്നത് അല്ല.

ഈ പഠനത്തിൽ എത്രകാലം ഞാൻ ഉണ്ടായിരിക്കണം ?

രക്തദാനം ഒരൊറ്റ തവണ ചെയ്താൽ മതിയെങ്കിലും നിങ്ങൾ ഈ പഠനത്തിൽ കുറഞ്ഞ് 2 വർഷമെങ്കിലും ഉണ്ടായിരിക്കേണ്ടതാണ്.

തിരുവന്തപുരത്തെ രാജീവ്ഗാന്ധി സെന്റർ ഫോർ ബയോടെക്നോളജിയിൽ ഏതെങ്കിലും ജൈവീകസാമ്പിളുകൾ സൂക്ഷിക്കുകയോ ഭാവിയിൽ ഉപയോഗിക്കുകയോ ചെയ്യുമോ ?

ഇല്ല.

ഈ പഠനത്തിലെ റിസ്ക്കുകൾ എന്തൊക്കെയാണ് ?

രക്തം എടുക്കുവാനായി സൂചികുത്തുമ്പോൾ ഒരു ചെറിയ വേദന അനുഭവപ്പെന്നതായിരിക്കും. സൂചികുത്തിയ ഇടത്ത് നിറവ്യത്യാസം ഉണ്ടാകാൻ നേരിയ സാധ്യതയുണ്ട്.

ഈ പഠനത്തിൽ പങ്കെടുക്കുന്നതുകൊണ്ട് എന്തെങ്കിലും ലാഭമുണ്ടോ?

ഈ പഠനത്തിൽ പങ്കെടുക്കുന്നതുകൊണ്ട് പ്രത്യക്ഷമായ ഒരു ലാഭവും നിങ്ങൾക്ക് ലഭിക്കുന്നതല്ല.

ഈ പഠനത്തിൽ പങ്കെടുക്കുന്നതുകൊണ്ട് മറ്റ് എന്ത് ചോയ്സ് ആണ് എനിക്കു ലഭിക്കുക ?

ശാസ്ത്രീയവിവരങ്ങൾ ശേഖരിക്കുവാൻ മാത്രമായാണ് ഈ പഠനം നടത്തുന്നത്. നിങ്ങൾക്ക് ഈ പഠനത്തിൽ പങ്കെടുക്കാതിരിക്കാം എന്നു തീരുമാനിക്കാം.

പരിശോധനകൾക്കും മറ്റും എന്തു ചിലവുവരും?

നിങ്ങൾ പരിശോധനകൾക്കും മറ്റും ഒന്നും ചിലവാക്കേണ്ടതില്ല എന്തെന്നാൽ ഇതെല്ലാം ഗവേഷണ പഠനത്തിന്റെ ഭാഗമായി ചെയ്യുന്നതാണ്. ഒരു നാഡിപരിശോധനയും, രക്തമെടുക്കലും രക്തസാമ്പിളിന്റെ ജനിതകപരിശോധനയുമാണ് ഇതിൽ ഉൾപ്പെടുന്നത്.

എന്റെ ചോദ്യത്തിനുള്ള ഉത്തരം ആരു നല്കും ?

ഈ പഠനവുമായി ബന്ധപ്പെട്ട ഏത് ചോദ്യവും നിങ്ങൾക്ക് എപ്പോൾ വേണമെങ്കിലും ഡോ. അരുൺ. കെ യോടു ചോദിക്കാം.08281232084 എന്ന ഫോൺ നമ്പറിൽ വിളിച്ച് നിങ്ങൾക്ക് ഡോ. അരുൺ.കെ യെ ബന്ധപ്പെടാം.

ഈ പഠനത്തിൽ പങ്കെടുക്കുന്നില്ലെങ്കിൽ എന്റെ അവകാശങ്ങൾ എനിക്കു നഷ്ടമാകുമോ ?

ഈ ഗവേഷണപഠനത്തിൽ പങ്കെടുക്കുക എന്നത് നിങ്ങളുടെ തീരുമാനമാണ്. നിങ്ങൾക്ക് ഇഷ്ടമില്ലെങ്കിൽ ഈ പഠനത്തിൽ പങ്കെടുക്കാതിരിക്കാം. ഈ പഠനത്തിൽ നിങ്ങൾ പങ്കെടുത്താലും ഇല്ലെങ്കിലും എസ്. സി. ടി. ഐ. എം. എസ്. ടി.യിലെ ചികിത്സ ഇപ്പോഴോ വരും കാലങ്ങളിലോ ഒരു വിധത്തിനും ബാധിക്കപ്പെടുകയില്ല.

നിങ്ങൾക്ക് ഈ പഠനവുമായി ബന്ധപ്പെട്ട് കിട്ടാനിടയുള്ള പ്രധാനപ്പെട്ട പുതിയ കണ്ടെത്തലുകളെപ്പറ്റി അറിയുവാൻ ആഗ്രഹമുണ്ടെങ്കിൽ അറിയിക്കുന്നതാണ്.

ഈ പഠനത്തിൽ പങ്കെടുക്കാത്തതുകൊണ്ട് നിങ്ങളുടെ അവകാശങ്ങളൊന്നും ഉപേക്ഷിക്കേണ്ടി വരികയില്ല

വിശ്വാസ്യത എത്രത്തോളമുണ്ടായിരിക്കും ?

ഈ പഠനത്തിൽ നിന്നും ലഭിക്കുന്ന കുറിപ്പുകൾ പ്രസിദ്ധീകരിക്കുവാൻ സാധ്യതയുണ്ട്. എന്നിതുന്നാലും നിങ്ങളുടെ പേരോ മറ്റു തിരിച്ചറിയൽ വിവരങ്ങളോ എസ്. സി. ടി. ഐ. എം. എസ്. ടി. യിൽ നിന്നും പുറത്തേക്കു പോകുന്നതല്ല.

പ്രഖ്യാപനം

എന്റെ ചോദ്യങ്ങൾക്ക് ഉത്തരംലഭിക്കുവാനുള്ള ഒരു അവസരം എനിക്കുണ്ടായി. ഈ ഫോറത്തിന്റെ ഒരു കോപ്പി എനിക്കു നല്കിയിട്ടുണ്ട്. ഈ പഠനത്തിൽ പങ്കെടുക്കുവാൻ എനുക്കുസമ്മതമാണ്.

(തിയ്യതി) (പങ്കെടുക്കുന്ന ആളിന്റെ പേരും ഒപ്പും) (ക്ലിനിക്ക്)

(തിയ്യതി) (സമ്മതപത്രം വാങ്ങന്ന ആളിന്റെ പേരും ഒപ്പും)

(തിയ്യതി) (സാക്ഷിയുടെ പേരും ഒപ്പും)

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9 Narapureddy, Bayapareddy, Naveen KH, Pallavi Madithati, Rajiv Singh, and Pirabu RA. "Socio-demographic profile and health care seeking behaviour of rural geriatric population of Allahabad district of UP: A cross sectional study", International Journal of Medical Science and Public Health, 2012.
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sl. No	Hospital number	Age	Sex	Age of onset of the symptom	First clinical symptom	Number of attacks	Interval between the attacks	Current attack
1	373814	24	2	20	6	4	june 2012,august 2012, august 2013, nov 2013	nil
2	294591	18	2	14	7	5	April 2009,nov 2011,nov 2013,april 2014, april 2016	7
3	391631	21	1	20	6	2	6 months	8
4	404935	27	2	12	4	7	2001, 2004, 2006, 2007, 2009, 2010,2015	6
5	245906	36	2	20	7	8	sept2005, dec 2005, june 2006, feb 2007, 2010,feb 2011,dec 2014	nil
6	387450	45	2	43	7	4	May 2014, july 2014, oct 2014, nov 2014	nil
7	186205	34	2	32	6	4	July 2000, 2002, April 2003, jan 2014	nil
8	375621	26	2	20	6	2	August 2011, Feb 2014	7
9	299688	46	2	46	7	1	0	7
10	411655	37	1	29	7	3	2008, 2011, 2015	7
11	412404	22	2	21	7	3	Jan 2015, March 2016, May 2016	7
12	412405	18	2	18	6	1	0	6
13	412052	40	2	29	7	3	2005,2013, 2016	6
14	301422	24	2	17	6	8	2009, 2009, 2009,2009, 2009, 2009, Dec2014, Aug 2015	6
15	390396	26	2	15	5	7	2007, 2010, 2011, 2012, 2014 (may and aug), dec 2015	7
16	391157	40	1	35	7	3	2010, 2012, 2014	6
17	197969	31	1	20	7	7	Nov 2001, Oct 2002, jan 2010, july 2010, nov 2010, feb 2011, may 2013	7
18	389527	21	2	18	7	1	Nov-14	7
19	350988	17	2	13	5	6	Aug 2011, Sept 2011, Aug 2012, Sept 2012, Oct 2012, feb 2013	6
20	404327	41	1	41	6	1	Jan-15	6
21	415541	44	2	38	4	4	March 2010, 2012, 2014, 2016	7
22	415905	38	2	34	8	3	2012, 2014, April 2016	7
23	394889	23	2	20	6	4	Dec2013, Jan 2014, March 2015, Feb 2016	4

24	404587	65	2	65	5	2	July 2015, Oct 2015	7
25	371112	37	2	29	5	6	June 2005, May 2006,August 2007, Dec2009, Aug 2012, May 2013	8
26	352423	16	2	16	5	1	2012	
27	400231	42	1	30	6	4	2004, 2006,2012, 2014	NIL
28	312671	28	2	28	7	1	0	7
29	239382	39	2	46	6	7	April 2005, December 2005, 2006, December 2008, July 2012, October 2012	4
30	326783	15	1	14	4	2	May 2010, March 2011	nil
31	378112	25	2	23	7	6	Aug 2013, Oct 2013,Nov 2013, Nov 2014, Dec 2014, April 2015	NIL

	MALE-1			
	FEMALE-2			
	CEREBRAL-3			
	OPTIC NERVE-4			
	CEREBELLAR-5			
	BRAINSTEM-6			
	SPINAL CORD-7			
	COGNITIVE -9			
	CSF OCB POSITIVE- 10			
	CSF OCB NEGATIVE- 11			
	IgG INDEX INCREASED-12			
	IgG INDEX DECREASED-13			
	VEP PROLONGED-14			
	BAEP PROLONGED 15			
	AVONEX-A			
	GLATIRAMER-G			
	IVMP-MP			
	MMF-M			
	REBIFF-R			
	DIMETHYL FUMARATE-DMF			
	CORELATIONS TO BE STATISTICALLY EVALUATED:			
	1	Age of onset of the disease?		
	2	Mean disease duration?		
	3	Genotype and alleles in MS carriers and controls. AA homo/ hetero,GA/ GG- Also the gender distribution with genotype?		
	4	Genotype and age of onset of first symptom?		
	5	Genotype and number of relapses?		
	6	Genotype and site predeliction in brain?		
	7	Genotype and the functional disability by EDSS?		
	8	Genotype and MRI Brain T2 load?		

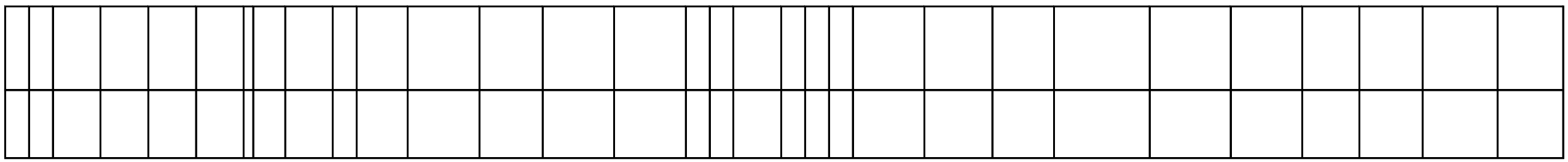
		Genotype and MRI Spine T2 load and number of segments affected?		
	9	Genotype and CSF Protein levels?		
	10	Genotype and OCB Positivity?		
	11	Genotype and elevated IgG index?		
	12	Genotype and electrophysiological corelation(VEP)?		
	13	SNP and MS severity- any association?ie EDSS		
	14	DRB1 association with MS susceptibility?		
	15	EDSS and number of spinal cord focal lesions?		
	Normal IgG Index-0.3-0.7 , Increased-1			
	Juxtacortical-1, >1-->2			
	Optic nerve lesions-1,>1			
	Black Holes- pesent, absent			
	Brainstem lesions-present, Absent			
	Cerebellar lesions- Present, Absent			
	Spinal cord lesions-1, >1, Confluent			

sl. No	Details of previous attacks	EDSS	Periventricular	JUXTACORTICAL	optic nerve lesions	Black holes	Atrophy	Cerebellar lesions	Brainstem lesions
1	june & aug 2012-5+6, AUG 2013-4, Nov 2013-5+7	3	19	2	Absent	Present	Present	present	present
2	7,4,4,5+7	3.5	16		Absent	Absent	Present	Absent	Absent
3	6	0	19	2	Absent	Absent	Absent	Absent	Absent
4	2001-4, 2004-7, 2006-7, 2007-4+5, 2009-7, 2010-7,2015-6	2	19	2	Absent	Absent	Absent	present	present
5	2005-7, 2005-7, 2006-7, 2007-7, 2010-4, 2011- 4, 2014-7	1	19	2	Present	Absent	Present	present	present
6	May 2014-7, july 2014-8, oct 2014-6, nov 2014-6	1.5	17	2	Absent	Absent	Absent	present	present
7	July 2000- 5+6, 2002-4, April 2003-4, jan 2014-6	0	17	1	Present	Absent	Present	Absent	present
8	Aug2012-6, 2014-7	2.5	19	2	Present	Absent	Present	present	present
9	7	2	19	2	Absent	Present	Absent	Absent	present
10	2008-7, 2011-7, 2015-7	3.5	17	2	Absent	Present	Present	present	present
11	jan 2015-7, March 2016-7, May 2016-7	1	17	2	Absent	Absent	Absent	present	present
12	6	0	19	2	Absent	Absent	Present	Absent	present
13	2005-7, 2013-7, 2016-6	3	19	2	Absent	Absent	Absent	Absent	Absent
14	2009-6, 2009-7, 2009-5,2009-7, 2009-6, 2009-4, Dec2014-7, Aug 2015-6	3	19	2	Absent	Absent	Absent	present	present
15	2007-5, 2010-7, 2011-4, 2012-4, 2014(May)-7, 2014(Aug)- 3 , Dec 2015- 7	2	19	2	Absent	Present	Absent	Absent	Absent
16	2010-7, 2012-4, 2014-6	0	17	2	Absent	Absent	Absent	Absent	present
17	Nov 2001-7, Oct 2002-7, Jan 2010-4+5, July 2010-5, Nov 2010-9, Feb 2011-5, May 2013-	5	19	2	Present	Absent	present	present	present
18	7	0	17	2	Absent	Present	Present	Absent	Absent
19	Aug 2011-5+6, Sept 2011-6, Aug 2012-6, Sept 2012-6, Oct 2012-	2.5	19	2	Absent	Absent	Present	Absent	Absent
20	jan 2015-6	0	17	1	Absent	Absent	Present	Absent	Absent
21	March 2010-4,2012-4, 2014-6, 2016-7	5	18	2	Absent	Present	Present	Absent	present
22	2012-8, 2014, April 2016-7	1.5	19	2	Absent	Absent	Absent	Absent	present
23	Dec2013-6, Jan 2014-7, March 2015-6, Feb 2016-4		17	2	Absent	Absent	Absent	Absent	present

24	July 2015-5, Oct 2015-7	0	19	2	Absent	Absent	Present	Absent	Absent
25	June 2005-5, May 2006-5,August 2007-7, Dec2009-8, Aug 2012-8, May 2013-8	6	19	2	Absent	Present	Present	Absent	Absent
26	2012-5	0	17	1	Absent	Absent	Absent	Absent	Absent
27	2004-6, 2006-5,2012-6, 2014-2 GTCS	4.5	19	2	Absent	Present	Present	Absent	present
28	7	0	19	2	Absent	Absent	Absent	Absent	Present
29	April 2005-6,Dec 2005-7, 2006-4, Dec 2008-6, July 2012-7, Oct 2012-4	0	19	2	Absent	Absent	Present	present	present
30	May 2010- 4, March 2011-6	0	19	2	Absent	Absent	present	Present	Present
31	Aug 2013-7, Oct 2013-7,Nov 2013-7, Nov 2014-7, Dec 2014-6, April 2015-7	2	17	2	Absent	Absent	Present	Absent	present

sl. No	spinal cord lesions
1	4
2	Absent
3	2+ CONFLUENT
4	CONFLUENT
5	CONFLUENT
6	CONFLUENT
7	Absent
8	CONFLUENT
9	CONFLUENT
10	CONFLUENT
11	3
12	Absent
13	CONFLUENT
14	CONFLUENT
15	1
16	2
17	CONFLUENT
18	4
19	Absent
20	1
21	CONFLUENT
22	4
23	3

24	1
25	Absent
26	CONFLUENT
27	Absent
28	CONFLUENT
29	Absent
30	3
31	4



sl. No	CSF Protein	CSF Sugar	OCB	IgG Index	VEP	BAEP	SNP	Current disease modifying agent patient is	Previously tried disease modifying agents
1	58	63	10	1	14	15		G	A
2	28.4	71	11	1	14	NORMAL		R	A
3	83	68	10	1	14	15		DMF	A
4	42	61	10	1	NORMAL	NORMAL		A	MP
5	42	71	10	1	NORMAL	NORMAL		G	G
6	68	123	10		NORMAL	NORMAL		A	A
7	39	43	11		14	15		A	A
8	32	`122	10	1	14	15		A	MP
9	38	66	10		14	15		MP	nil
10	40	77	10		NORMAL	NORMAL		G	G
11	18	102	10		NORMAL	NORMAL		MP	MP
12	21	82	10	1	NORMAL	NORMAL		A	MP
13	34	86	11		NORMAL	NORMAL		MP	MP
14	56	51	11		14	15		A	
15	54	61	10		14	NORMAL		G	MP
16	36	127	10		NORMAL	NORMAL		MP	A
17	34	65	11	1	14	NORMAL		R	MP, METHOTREXATE
18	34	104	10	1	14	15		G	A
19	45	76	10	1	14	NORMAL		G	MP
20	41	64	10	1	NORMAL	NORMAL		A	MP
21	58	108	10	1	14	NORMAL		A	
22	Unsuccessful CSF				NORMAL	NORMAL		A	
23	21	59	10	1	14	NORMAL		R	R,MP