# ASSOCIATION OF MULTIPLE SCLEROSIS WITH LILRA3

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OCTOBER 1, 2022
MSc MOLECULAR BIOMEDICINE
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# Abstract

**Background**

Multiple Sclerosis (MS) is an autoimmune disease affecting the CNS with multifactorial aetiology while the genetic susceptibility to MS is only partially explained. The soluble leukocyte immunoglobulin-like receptor A3 (LILRA3, 19q13) has been associated with MS as it carries a 6.7kb deletion which acts as a risk factor for MS in German and Spanish but not in Polish patients. Interestingly, other autoimmune diseases such as neuropsychiatric lupus erythematosus affect the CNS while at early stages the differentiation of MS from other autoimmune diseases affecting the CNS is difficult.

**Aims**

We aim to correlate the genotype frequency of LILRA3 with MS in a Greek patient cohort. In addition, we aim to compare these findings against a patient cohort presenting with systemic autoimmune diseases with CNS involvement who do not fulfil the criteria for MS diagnosis. Furthermore, we will correlate the LILRA3 genotype frequencies with clinical, laboratory and imaging evidence for the two patient cohorts.

**Methods**

200 consecutive patients with undiagnosed first untreated CNS demyelinating episode, admitted to the Neurology Department, Aeginition Hospital, National and Kapodistrian University of Athens, have undergone full clinical, laboratory and imaging evaluation. All patients have been revaluated at 6 and 12 months. At 12 months, a final diagnosis has been attributed to each patient according to previously established criteria for MS or CNS autoimmune disease (non-MS). Using PCR typing, deficiency of LILRA3 was examined in 400 HC, 157 MS and 40 CNS systemic autoimmune patients of Caucasian Greek origin.

**Results**

Heterozygous LILRA3 genotypes were less prevalent in MS patients (3.9%) than in blood donors (7.3%; P=0.037) and CNS autoimmune patients (5.2%, p=0.610). LILRA3 heterozygosity was associated with lower white blood cell counts (6,697 vs 8,348 per μl) in all patients cohorts combined, a finding which was also verified upon further stratification of the MS cohort into WT/WT and WT/DEL whereby lower WBCs counts were observed in the heterozygous strata (6,796 vs 8,619, p=0.037).

**Conclusion**

LILRA3 deficiency was found to confer protection for MS in the Greek population and hence the wild-type variant is likely risk factor for MS.

# Contents

# Introduction

## Multiple Sclerosis

Multiple sclerosis (MS) is characterised by chronic neuroinflammation, demyelination, disruption of the blood-brain barrier (BBB) and axonal injury, and it is therefore rendered both as an autoimmune disease and a neurodegenerative disease of the central nervous system (CNS) (1,2). The hallmark of the disease is demyelination; the immune system becomes reactive towards myelin on axons, thereby inhibiting the saltatory movement of the action potential along the CNS, affecting communication within/between the brain and spinal cord. Damaged neurons form multiple lesions in the CNS, mainly in the white matter where axons reside, and for that reason they affect functions such as vision, movement, touch, cognition and emotion (3). Even though there is not a definitive cause for MS, genetic factors such as sex (prevalence 3:1 female:male), polymorphisms in the HLA gene and lifestyle/environmental factors such as latitude, smoking, and previous exposure to viruses (EBV, measles, etc) have been shown to play roles in MS progression.

## MS disease courses

MS is characterised by variable disease courses based on firstly, active/inactive demyelinating lesions that disseminate in the CNS in terms of space and time (3) and secondly, the progression rate of the disease. The manifestation of the disease begins with an initial attack termed as clinically isolated syndrome (CIS) described by neurological deficits that last more than 24 hours and can be visualised with the help of magnetic resonance imaging (MRI) (4). In order for CIS to progress to the relapsing-remitting (RRMS) stage, further relapse incidences have to occur followed by periods of remittance. During relapses, the patient may present optic neuritis with symptoms such as peripheral visual loss and photophobia, myelitis with symptoms such as severe pain and acute urinary retention and other clinical presentation of MS (3). During remittance, some or all the symptoms retract, and the re-myelination process begins.

Eventually, some patients with RRMS will experience worsening of their symptoms and increased neurological dysfunction and thereby will transition to a secondary progressive MS (SPMS) course (4). However, the minority of MS patients are diagnosed with primary progressive MS (PPMS), which is characterised by increased neurological dysfunction from the onset of the disease, without a distinict CIS. It is also important to mention that the accurate diagnosis of MS was largely enabled by the advances in MRI. Through this imaging modality, demyelinating lesions can be monitored in time and space. In addition, the presented symptoms can be confidently attributed to the right disease and decrease the risk of mis-diagnosis and hence mis-treatment. Lastly, with the use of MRI, the effectiveness of the administered disease modifying treatment for MS can be monitored on an individual basis to enable for better treatment but also to facilitate future research (5).

## Risk factors

The sequential events that lead to demyelinating lesions and eventually to neurodegeneration are a result of complex interplay between genetic and environmental factors. The onset of RRMS is observed in young adults between the ages of 20 and 40 whereas the average age of PPMS diagnosis is 40 (3). Moreover, there seems to be a prevalence of the disease in females with a 3:1 female to male ratio established in recent years (6). Epidemiological evidence suggest that MS is more prevalent in individuals of European decent, with the frequency of the diseases in western countries being 1:1000 individuals (3).

Environmental factors such as low Vitamin D, smoking tobacco and previous exposure to virus such as EBV were shown to be associated with earlier SPMS onset. More specifically, as highlighted by Rotstein and Montalban (7), a post hoc analysis of the BENEFIT clinical trial – in which patients received interferon β-1b treatment or placebo- low vitamin D levels were associated with brain volume loss, relapse rate and 4-year disability progression. Furthermore, some suggest that lack of UV-B exposure in areas with higher latitude may be the reason behind the increased prevalence of MS. However, the definitive mechanism of action behind the correlation of low vitamin D and increased incidences of MS is unknown (3).

Another environmental factor that has implications in MS progression is tobacco smoking. As demonstrated by Wingerchuk (8), smoking has been reported to impose a greater risk of conversion from RRMS to SPMS but also increases the rate of conversion from CIS to RRMS. It was also shown that the risk of MS increases in a dose-dependent manner in relation to smoking and that passive smoking has similar effects on MS risk (3). The above association has not been linked to a molecular mechanism as of yet but the toxic smoke components may be the trigger.

In addition, prior infection by EBV has been proposed as the etiological agent for MS (9,10) however this is subjected to ongoing debate in the scientific community. It is also suggested that following an EBV infection, the immune system becomes augmented therefore creating a “fertile field” for MS due to the lowered threshold for the activation of auto-reactive T cells (11). Nevertheless, both humoral and cell-mediated immunity are believed to play roles in linking EBV infection and MS. Future research is needed to determine if there is a definitive causality between the two events

As MS is a complex trait disease, genetic factors can also largely influence the prevalence of MS, with familial MS being approximately 13% for all MS types (3). Genome-wise association studies (GWAS) facilitated the understanding of genetic determinants for MS (12). Briefly, polymorphisms in several genes such as: HLA class I and II, IL2, IL7R, TNF and in genes involved in vitamin D metabolism (3), were identified to be linked with MS. However, how this genetic variation affects the clinical features of MS remains unclear.

Overall, the understanding that MS is a complex trait disease was enabled by the advances in biotechnology and bioinformatics (12). Identifying environmental and genetic factors that affect MS occurrence and progression helped the diagnosis of MS, the development of pharmacological that lead to the increase in life expectancy of MS patients.

## Pathophysiology

In MS, focal lesions are observed in areas where there is breakdown of the BBB which is responsible for maintaining the integrity of the CNS and isolating it from the peripheral circulation so that the neuronal microenvironment remains intact (13). Endothelial cells (ECs) and their basement membrane (BM) are the major structural characteristics of the BBB. ECs form a continuum enabled by tight junctions (TJs) and lack fenestration, in contrary to the endothelium of peripheral tissues. In this way, selective permeability of soluble molecules and restrictive access of immune cells into the brain is ensured (14). Pericytes are also surrounded by a BM and act as the first defense mechanism if the BBB is compromised (13). Foot processes of astrocytes extend to the walls of ECs and can instruct the maintenance of the BBB through paracrine interactions with ECs (15).

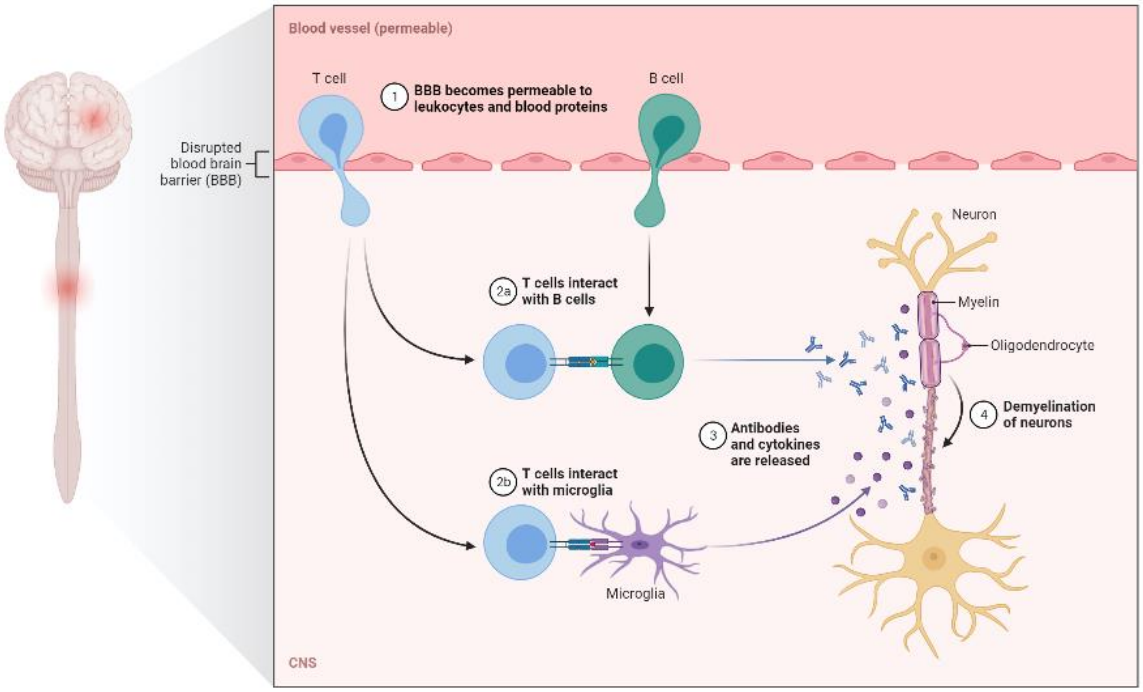


Figure 1- Multiple sclerosis pathophysiology. Due to reasons that are not fully elucidated, the Blood-Brain barrier (BBB) becomes permeable to leukocytes that are able to undergo diapedeses (step 1) into the Central Nervous system (CNS). There, (step 2a, 2b) T cells interact with B cells or microglia to release antibodies and cytokines that will eventually cause the demyelination of neurons (step 3), leading to the symptomatology of MS. Figure created in Biorender.com.

The BBB is responsible for retaining cells of the immune system within the capillaries and prohibiting their exit in the CNS. However, the BBB is not impenetrable as once hypothesized, and immune cells can migrate into the CNS, which is characteristic of MS. T cell transmigration or

diapedesis according to the British Society for Immunology, is defined as “the process by which T lymphocytes migrate across venular blood vessel walls to enter various tissues and organs”. Diapedesis is observed mostly at inflammatory sites and is governed by receptor-ligand interactions between ECs and lymphocytes in terms of adhesion molecules and chemokines. Initially, lymphocytes from the flowing blood form low-affinity interactions on ECs via selectins and their ligands. In this way, through bind-release events they can roll along the endothelium until leukocyte activation is triggered. This process is mediated by the engagement of chemokine receptors on ECs and GPCRs on leukocytes (1).

Activation of T cells results in integrin remodelling and the tight adhesion of leukocytes to endothelial cells mediated by VLA4 –VCAM1 and LFA1—ICAM1 high affinity binding. Leukocytes then flatten and undergo diapedesis through the BBB which is mediated by the degradation of the BM by matrix metalloproteinases (MMPs) secreted by T cells. Diapedesis can occur either paracellularly or transcellularly i.e. through adjacent ECs or through pores on a single EC respectively (figure 1, step 1) (1). Monocytes and neutrophils enter the CNS through the BBB in a similar manner. Effectively, introduction of inflammatory cells to the CNS leads to neuroinflammation. In MS, chronic neuroinflammation leads to neurodegeneration.

The understanding of molecular mechanisms in MS came from the MS animal model EAE (experimental autoimmune encephalomyelitis) which has allowed for better prognosis and treatment of the disease. In MS, aberrant T cell activation (both $CD4^+$ and $CD8^+$) leads to demyelination which is believed to be occurring due to suppressed Treg function. Moreover, $T_H17$ cells are also actively proliferating, and act on oligodendrocytes thereby impairing the re-myelination process during MS remission (3). It was also shown that during their transmigration through the BBB, $T_H1$ cells upregulate CCR5 and CXCR3 chemokines receptors upon their activation, $T_H2$ cells similarly upregulate CCR3 while RANTES is also overexpressed by inflammatory cells (14).

The modulation of chemokine receptors enables lymphocyte diapedesis and increased permeability of the BBB. What is more, B cells also appear to play a role in MS as they can access the CNS through the BBB and produce brain reactive antibodies (figure 1, step 2a and 3)which can block neurotransmitter binding, enhance receptor activity and block ion channels through cytokine secretion (16). Studies on MS patients showed that B cells secrete pro-inflammatory cytokines such as IL-6 and TNF leading to the recruitment of more $T_H1$ and $T_H17$ cells (3). Activated astrocytes and microglia (figure 1, step 2b and 3) facilitate disease progression by releasing cytokines and chemokines such as TNF which can in turn directly kill oligodendrocytes. Overall, release of cytokines and chemokines can promote the degradation of the BBB which renders it more permeable to a larger number of cells. In addition, these secreted molecules can have chemo-attractive action by recruiting B cells and instructing them to produce anti-myelin antibodies, and macrophages to degrade myelin (17). All these mechanisms eventually lead to neuroinflammation and lesions in multiple areas of the brain of MS

patients, impairing multiple functions such as vision, movement, touch, cognition and emotion (figure 1, step 3).

## Making the diagnosis for MS

While there is currently no one tool available that can definitively point to an MS diagnosis, there are several strategies in place to help physicians make the diagnosis. Collectively, to receive a diagnosis for MS there have to be evidence of damage in at least two separate areas of the CNS (dissemination in space), the damage should have been occurred at different time points (dissemination in time), and any other possible diagnosis must be ruled out. More specifically, in 2017, the revised McDonald criteria (Figure 2) were published by the International Panel on the Diagnosis of MS, and are a set of clinical, laboratory and imaging criteria in order to facilitate MS diagnosis. These include cerebrospinal fluid analysis to accelerate the diagnosis process and MRI (18,19).

| Clinical Presentation | Additional Data Needed for Diagnosis |
|---|---|
| ≥ 2 clinical attacks and objective evidence of ≥ 2 lesions | None |
| ≥ 2 clinical attacks and objective evidence of 1 lesion | DIS: an additional attack implicating a different CNS site OR by MRI[a] |
| 1 clinical attack and objective clinical evidence of ≥ 2 lesions | DIT: an additional clinical attack OR by MRI[b]<br>**OR**<br>CSF-specific oligoclonal bands |
| 1 clinical attack and objective evidence of 1 lesion | DIS: an additional clinical attack implicating a different CNS site OR by MRI[a]<br>**OR**<br>DIT: an additional clinical attack OR by MRI[b]<br>**OR**<br>CSF-specific oligoclonal bands |

Adapted from Thompson AJ, Banwell BL, Barkhof F, et al. Diagnosis of multiple sclerosis: 2017 revisions of the McDonald criteria. *Lancet Neurol.* 2018;17:162–73.
Abbreviations: CNS, central nervous system; CSF, cerebrospinal fluid; DIS, disseminated in space; DIT, disseminated in time; MRI, magnetic resonance imaging.
[a]DIS by MRI: new lesions on follow-up imaging or both gadolinium-enhancing and non-enhancing lesions on single MRI.
[b]DIS by MRI: ≥ 1 symptomatic or asymptomatic lesion in ≥ 2 areas including cortical/juxtacortical, periventricular, infratentorial, or spinal.

*Figure 2- 2017 McDonald Criteria for the diagnosis of Multiple Sclerosis*

## Differential diagnosis

Though MS is considered the cornerstone of CNS demyelinating disorders, several clinical entities are important MS mimickers and need to be taken into consideration in the differential diagnosis (18,20,21). Among those, systemic autoimmune diseases (SADs), neuromyelitis optica spectrum disease (NMOSD), and myelin oligodendrocyte glycoprotein antibody-associated disease (MOGAD)

are the most common. Less frequently, neurosarcoidosis, neuro-Behçet disease, and chronic lymphocytic inflammation with pontine perivascular enhancement responsive to steroids (CLIPPERS) may present with MS reminiscent manifestations. Despite a distinct pathogenetic background, atherosclerotic small vessel disease or even cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), Fabry disease, and Susac's syndrome can also present with MS-resembling features (18).

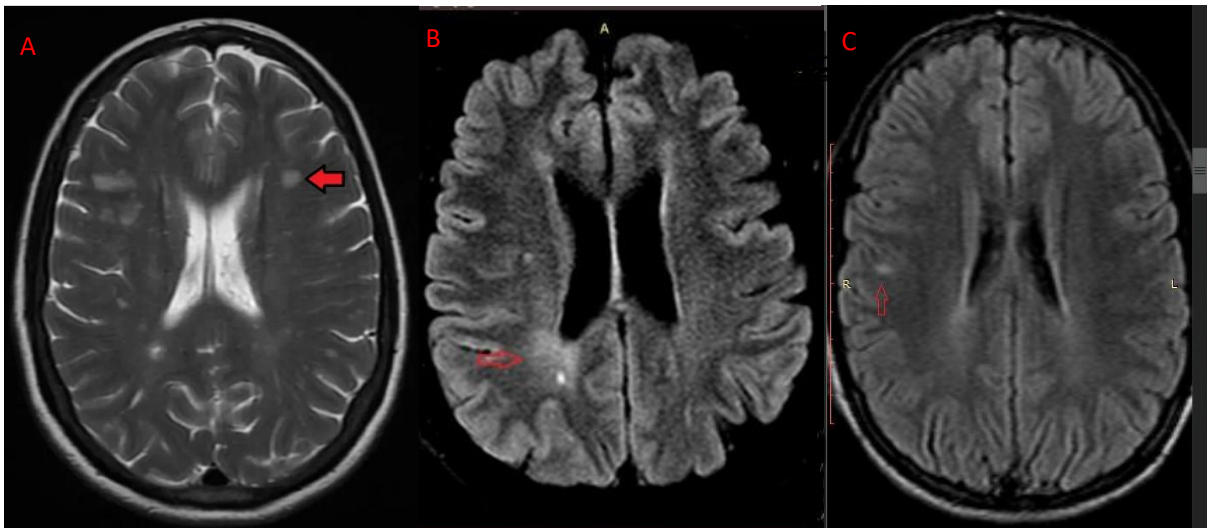


*Figure 3- MRI images of patients with MS or CNS autoimmune diseases. A: White matter lesions in brain of MS patient (T2 MRI). B: White matter lesions with gadolinium enhancement, brain of SLE patient (FLAIR MRI), C: White matter lesions with blear margins in brain of SS patient (FLAIR).*

SADs, mainly systemic lupus erythematosus (SLE), Sjögren's syndrome (SS), Systemic lupus erythematosus (SLE) and antiphospholipid syndrome (APS) have been previously shown to invariably affect the CNS, with transverse myelitis, optic neuritis, and white matter hyper-intensities (WMH) in CNS magnetic resonance imaging (MRI) (Figure 3) being the main MS-like manifestations (22–27).

Despite international research efforts over the last years, distinction of these entities at both clinical and imaging level is challenging and the underlying pathogenetic mechanisms are obscure. Importantly, type I interferons (IFNs) and particularly IFN-β, have been for decades, the cornerstone for treating MS while at the same time it is also a well-recognized pathogenetic denominator in systemic autoimmune diseases with CNS involvement. Therefore, administration of type I IFNs in demyelinating CNS disorders in the presence of unsuspected underlying systemic autoimmunity would be detrimental. For these reasons, early biomarkers for the distinction of disease courses at an alternative therapeutic options should be sought (19,24,25,28–30).

MS diagnosis remains challenging while no biomarker exists so far to differentiate MS from other diseases mimicking this inflammatory demyelinating process. Atypical presentations need to be investigated further and more cautiously for the presence of "red flags" indicating other clinical entities like SADs. The possibility to establish MS diagnosis after a single attack according to the revised

McDonald criteria of 2017, increase the need for proper and thorough exclusion of alternative diagnosis (19) A recent study, presented in ECTRIMS 2018, found that MS diagnosis was questioned after re-evaluation for almost 1 in 5 patients (31). Auto-antibodies can raise the suspicion of an underlying autoimmune background, but are not pathognomonic (29). While ANA positivity seems to be a relatively common finding in MS patients, with a prevalence ranging from 20% to 33.3% according to different studies (24,32–35) a thorough study in biomarkers present during an initial clinical attack is not so far available.

## Treating multiple sclerosis

MS manifests through a plethora of symptoms and clinical features that vary between individuals, therefore imposing a hurdle for MS treatment. Great efforts in the last 25 years lead to the implementation of treatments that decrease the annual relapse rate (aRR) and increase the time for disability progression, improving the quality of life of the individual and increasing life expectancy with MS (36). However, the focus has been placed on treating RRMS which is the most frequently occurring MS type and accounts for the greater number of MS cases. In order to tackle MS, administered disease modifying treatments are coupled with symptomatic therapy to manage both the progression of the disease and the symptoms (Figure 4).

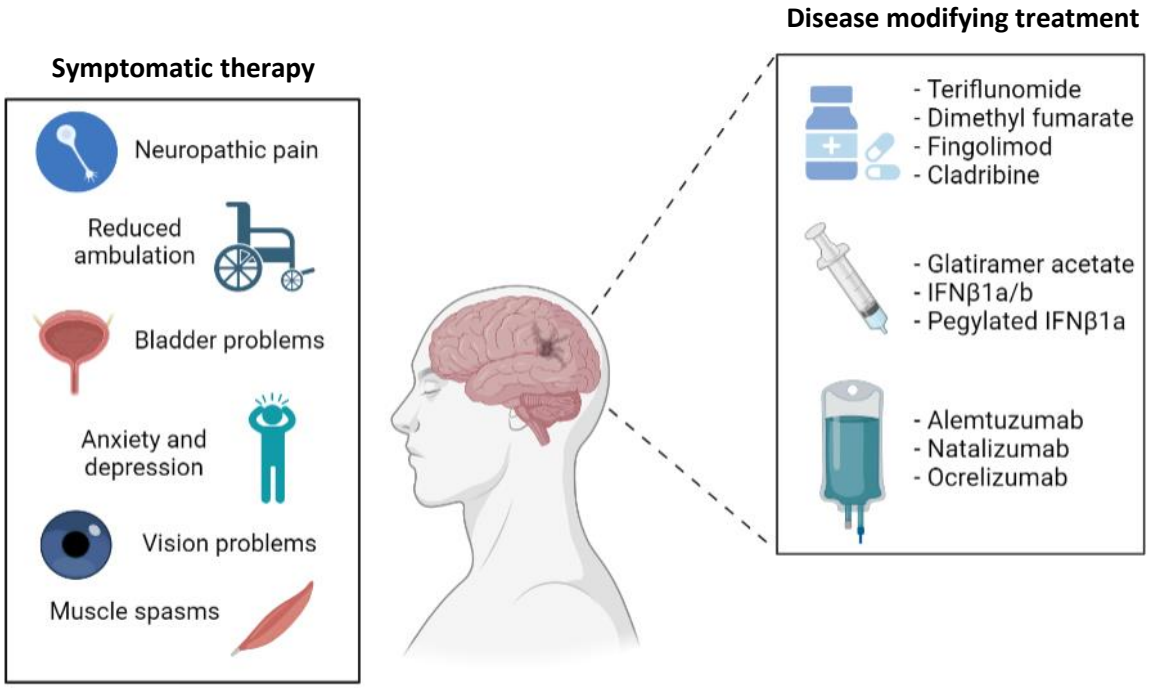


*Figure 4- Treating MS. Symptomatic therapy is utilised in order to manage symptoms (box on the left-hand side). Disease modifying treatments available for MS: oral drugs, injections and infusions (box on the right-hand side). Original figure made in Biorender.com (includes information from Filippi et al 2018 and "Nature milestones- Treatment of Multiple Sclerosis").*

Focusing on treatments for RRMS, the first therapies to obtain FDA approval in 1993 were IFNβ1a and IFNβ1b which have an overall anti-inflammatory effect. Treatment with glatiramer acetate was authorised a few years later and is also thought to have an anti-inflammatory activity. The above treatments are all injectables (subcutaneous or intramuscular) and all appear to have similar efficacy as they decrease aRR by one third (36). Moreover, adverse effects are rarely reported with these treatments with most common side-effects being flu-like symptoms and injection site reactions (3). Importantly, through this treatments the realisation that the course of MS can be modified came about and the term "disease-modifying therapy" was established (36).

In recent year, oral treatment for MS entered the market. Fingolimod, a SIP antagonist that restrains T cells in secondary lymph nodes, has been shown to decrease aRR by 50% while reports render it more effective than IFNβ injections (36). Moreover, teriflunomide, another oral drug for MS, inhibits actively proliferating T and B cells and in this way controls neuroinflammation. Teriflunomide was shown to have similar efficacy with IFNβ treatments (36). Dimethyl fumarate, an immunomodulatory oral drug, exhibited significant efficacy as demonstrated by the 50% reduction in aRR during its clinical trial (36). The efficacy rate of dimethyl fumarate was matched by that of cladribine which decreases lymphocyte count by leading them to apoptosis.

Overall, the determination of the treatment strategy is reached by patient-clinician communications, taking into account multiple factors. In particular, the frequency of relapses, the severity as well as the level of incapacitation of symptoms along with the clinical features and MRI scans are some of the factors that need to be addressed. Moreover, clinicians should account for the lifestyle of patients and if that interferes with the disease progression and potentially with the treatment course. Oftentimes, escalation treatment is the chosen strategy in which a therapy of moderate efficacy but low risk is administered first, followed by treatment of higher efficacy but also higher risks (3).

## Importance of biomarkers for MS

Over the previous decades, significant progress has been made in the understanding of the pathophysiology and immunological mechanisms underlying MS. Novel markers, therapies and treatment options have emerged, facilitating the diagnosis and treatment of MS. However, the need to treat MS patients in a timely manner and to tailor treatment to the individual is hindered by the lack of specific biomarkers. These biomarkers need to be rapid, cost-effective, non-invasive and easily measured. Currently, oligoclonal bands (OCBs) in the CSF, neutralising antibodies against IFNβ, and natalizumab are used in standard clinical practice. However, serum biomarkers thar are more accessible are yet to be discovered (37). Unlike CSF, peripheral blood and serum samples are easily obtainable in routine ambulatory care on numerous time points, and thus they would not only facilitate proper diagnosis, but also enable non-invasive monitoring of treatment response. To date, and despite the extensive search for biomarkers, no laboratory test has proven 100% specific of MS. While MRI is

currently the most valuable tool in differential diagnosis of MS, excessive reliance on radiological modalities may be the cause of diagnostic errors. Therefore, further research into disease-specific biomarkers is of utmost importance (38).

In addition, there is low availability of biomarkers that can differentiate between diseases with disseminating white matter lesions, primarily systemic diseases including connective tissue disorders, diverse vasculopathies e.g. genetic and infectious diseases have to be taken into account (38). For example, elevated erythrocyte sedimentation rate (ESR) and lymphopenia may be indicative of a systemic disease. However, positive antinuclear antibodies (ANA) tests have been observed in 22.5% to 30.4% of MS patients without any systemic autoimmune disease. However, in most cases of such nonspecific autoantibodies presence, ANA titres are usually not higher than 1:320 (39). One study in MS population has shown ANA positivity in as much as 51.0% and a positivity for either aCL or anti-β2GP1 antibodies was observed in 32.6% of participants (40). Although such non-specific prevalence of these autoantibodies is considerably high, the possibility of coexisting autoimmune disease should not be neglected, especially in NMOSD (41).

According to the latest revisions of McDonald criteria for MS, CSF examination and especially OCBs detection should be considered a standard diagnostic test. Although OCBs are not specific and are often present in other disorders e.g. connective tissue diseases, their presence is reported in approximately 90% of MS patients. Consequently, lack of CSF OCBs should raise the suspicion of disorder other than MS (42). The pattern of OCBs is also a valuable information, since the vast majority of MS patients show type 2 pattern, that is CSF OCBs positivity with no corresponding abnormality in serum. In some cases of MS type 3 OCBs can be detected, which means the IgG bands are present both in the CSF and serum with additional bands detected in the CSF. Identical CSF and serum OCBs constitute type 4 positivity and are usually detected in systemic inflammatory diseases or infections (43).

Furthermore, lack of disease-specific biomarkers may lead to MS misdiagnosis which in turn can increase morbidity as a consequence of psychological damage, risk associated with DMTs and corticosteroid use, or inadequate treatment (44).

## Leukocyte Immunoglobulin-like receptor A3

As it will be discussed in the next section, various studies have reported that the gene variants of *LILRA3*, encoded on subregion 19q13.4, are associated with MS. The transcribed Leukocyte immunoglobulin-like receptor subfamily A, member 3 (LILRA3), belongs to a family of 13 homologous immunoreceptors that are expressed on myeloid and lymphoid cells (45–47). LILR molecules play a modulatory role in innate and adaptive immune responses while they are classified as inhibitory, activating or soluble based on their transmembrane and cytoplasmic domain structures (48). Among the activating LILRs (LILRA) is LILRA3, which is the only member of the LILR family lacking

the transmembrane and cytoplasmic domains and therefore acting as a soluble immunoreceptor (48). LILRA3 binds non-classical and classical HLA class I molecules while its exact role in the immune system is yet not fully elucidated (49).

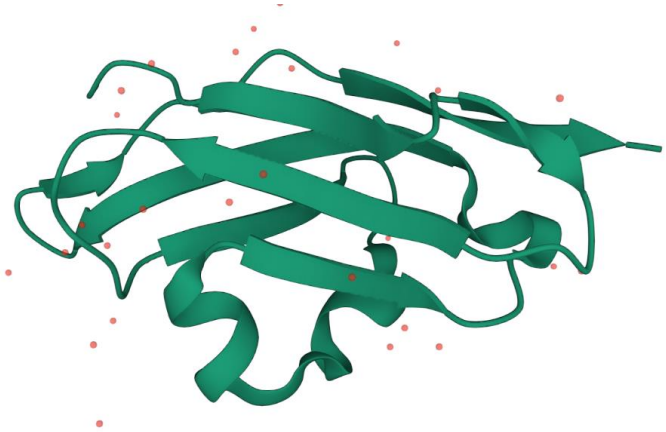

*Figure 5- LILRA3 protein structure. From rcsb.org (structure code: 3Q2C), (49).*

Structurally, LILRA3 contains four Ig-like domains (D1, D2, D3, D4), arranged in a beta sandwich. From a functional viewpoint, LILRA3 may greatly control the inhibition of the immune response induced by LILRB1, LILRB2, and other HLA-binding LILR molecules like LILRA1 (49). This implies that LILRA3 would play an important role in the strict inhibitory control between classical and non-classical HLAs. Thus, expression of LILRs with various affinities to different types of classical and non-classical HLAs on a certain tissue or cell seems make the delicate organization of the immune response in the human body possible (50).

Importantly, *LILRA3* full-length (WT) gene is not present in all haplotypes, a feature attributed to a 6.7 kbp deletion which removes the first seven of eight exons, creating the so-called "null allele", which is essentially not expressed (49). The frequency of this deletion varies among populations and it oscillates from 6% - 84% (51).

## LILR and Autoimmune diseases

LILR genes have been associated with various autoimmune diseases (summarised in figure 4). A low-expressing LILRB1 haplotype is significantly associated with rheumatoid arthritis (RA) susceptibility in HLA-DRB1 shared epitope negative subjects, possibly because of insufficient inhibitory signaling in leukocytes (52). Genome-wide association studies identified a SNP in LILRB as a risk factor for Takayasu's arteritis in Turkish and North American cohorts (53). In addition, a low-expression allele and cytoplasmically located SNP of LILRB4 are independently associated with an increased serum type I interferon activity in SLE patients (54).

Furthermore, LILRA1 also binds to HLA-B27, which is strongly associated with spondyloarthropathies (55). A unique functional SNP of LILRA2 is significantly associated with SLE

and microscopic polyangiitis (56). We have previously demonstrated that the functional LILRA3 gene variant increases susceptibility to Sjogren's Syndrome lymphoma development with young disease onset, suggesting a genetic predisposition to lymphoma development in Greek patients (57). Moreover, LILRA3 is correlated to increased disease activity and severity of systemic lupus erythematosus (SLE) as LILRA3 serum levels are elevated in Chinese SLE patients (58). Similar to LILRB1, LILRA5-expressing cells in synovial tissue are correlated with disease activity score in patients with RA (59).

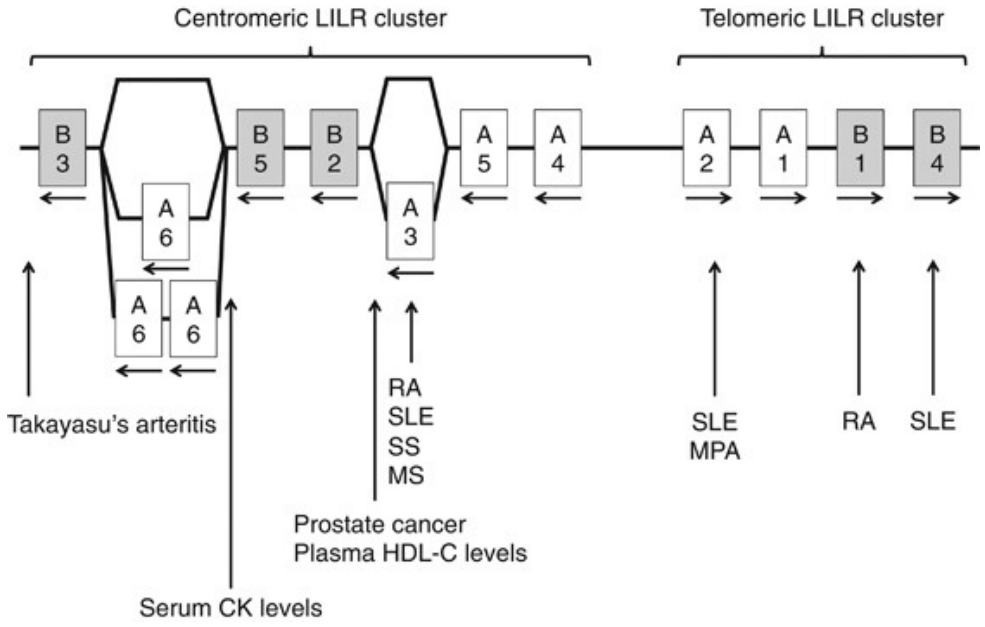


*Figure 6- Gene organization and genetic associations of the LILR genes. CK, creatine kinase; HDL-C, high-density lipoprotein cholesterol; SS, Sjögren's syndrome; MS, multiple sclerosis; MPA, microscopic polyangiitis (from 51).*

### LILRA3 and MS

LILRA3 gene polymorphisms have also been associated with MS. A study in a Spanish patient cohort showed that the risk of suffering MS is highest in individuals lacking LILRA3 completely in their genome (DEL/DEL), intermediate in heterozygotes (DEL/WT) and lowest in individuals having two full-length LILRA3 alleles (WT/WT) (46). In addition, a prior study in German patient cohorts was in accordance with these findings (45). However, a Polish study with MS patients found no associations of MS with LILRA3 (51).

## Aims

Given the limited mentions of LILRA3 in the available literature, especially in the context of MS in the Greek population, we aim to correlate the genotype frequency of LILRA3 with MS in a Greek patient cohort. In addition, we aim to compare these findings against a patient cohort presenting with systemic autoimmune diseases with CNS involvement who do not fulfil the criteria for MS diagnosis. Furthermore, we will correlate the LILRA3 genotype frequencies with clinical, laboratory and imaging evidence for the two patient cohorts.

# Methods

## Study design & patients

200 consecutive patients with undiagnosed first untreated CNS demyelinating episode, admitted to the Neurology Department, Aeginition Hospital, National and Kapodistrian University of Athens, from June 2020 to December 2021 have undergone full clinical (by both neurologist and rheumatologist specialist), laboratory and imaging evaluation. All patients have been revaluated at 6 and 12 months. At 12 months, a final diagnosis has been attributed to each patient according to previously established criteria for MS or CNS autoimmune disease (non-MS). Definition of the first CNS demyelinating episode is based on both clinical presentation and imaging findings of demyelinating lesions in brain and/or spine MRI studies, according to previously published criteria (2017 McDonald, MAGNIMS). Patients with recent (within one month) viral infection, treatment with systemic steroids (within 6 months) or immunosuppressives have been excluded from the study (61).

Peripheral blood has been collected at enrolment (baseline), at 6 and 12 months and serum was isolated and stored at -80 $^{o}$C. Cerebrospinal fluid (CSF) has been also collected through lumbar puncture and stored at -80 $^{o}$C at baseline. Prior to study enrolment, all patients signed a predefined and printed informed consent form aligned with all the requirements of the Declaration of Helsinki. Laboratory evaluation at baseline included full blood count, full chemistry panel, testing for chronic viral infections and B12/25 OH D3 levels, thyroid function tests, ACE levels, inflammatory markers/protein electrophoresis and urinalysis. Standard immunology testing has been performed including ANA, RF, anti-DNA, antiphospholipid and antithyroid antibody titers as well as complement levels. CSF obtained by lumbar puncture at baseline was tested for cell count, glucose and protein levels, IgG index, oligoclonal bands, as well viral infections. (61).

## Patient cohorts

Patients have been distributed into two cohorts according to the received diagnosis at 12 months, following joint neurology and rheumatology consultation: MS and CNS autoimmune disorders. For all patient cohorts matched whole blood, serum and CSF are available. Whole blood and sera are available for a healthy control (HC) cohort as well.

## Ethics statement

The study was approved by the Aeginition Hospital Ethical Committee and prior to study enrolment, all patients provided written informed consent, in accord with the requirements of the declaration of Helsinki.

## DNA Extraction

DNA isolation was performed with Quick-DNA™ Miniprep Plus Kit (ZymoResearch) according to manufacturer's instructions. Spectrophotometry (Biospec Nano, Japan) was utilized to confirm the quantity and quality of DNA samples.

## LILRA3 genotyping

The detection of LILRA3 gene variants was performed with PCR method, using for each sample in the same tube the following triad of primers: Forward 1 (FW1): 5'-GACTTGTAAGGGTTAAAAAGCCAA-3'/ Forward 2 (FW2): 5'-CATCTCGATCTGCCACTGACAC-3'/ REVERSE(R): 5'-GACAGCAGATTCTAAAACAGTGG-3'. The product size resulting from combination of FW1-R is 150 bp, representative of the complete LILRA3 gene, while the combination of FW2-R primers results in a 241 bp product, not including FW1-R target site, leading to LILRA3 deletion (absence of first seven exons). (57).

## Statistical Analysis

Continuous and categorical variables were assessed by Mann–Whitney and chi-square tests, respectively. Statistical analysis was performed using the IBM® SPSS® software platform and GraphPad Prism® software. Differences were considered significant when p-value was $<0.05$.

# Results

## Demographics, clinical, laboratory, and imaging characteristics of the study cohort

Table 1 summarizes the demographical, clinical, laboratory, and imaging characteristics of the study cohort. Among 197 patients included in the final study sample, 148 (75.1%) were females and their age was (mean ± SD): 30.6 ± 9.2 years. Out of the 197 patients, 157 received a diagnosis for MS whereas 40 patients were classified in the CNS autoimmune group. Among the MS patient cohort, 95.4% were RRMS patients, 1.5% SPMS patients and 2.3% PPMS patients. The onset syndrome was sensory in 52.7% of the patients, visual in 26.3% and motor in 21.0% of MS patients. In the CNS autoimmune cohort, the onset syndrome was sensory in 29.8% of patients, visual in 26.3% and motor in 21.0% of the patients. The EDSS score (mean ± SD) was 2.1 ± 1.3 and 1.9 ± 1.4 in the MS and CNS autoimmune cohorts respectively.

## LILRA3 allele and genotype frequencies

Table 2 summarizes the allele and genotype frequencies in HC, MS and CNS autoimmune cohorts. The LILRA3 wild-type allele (W) is more frequently present in all three cohorts; 92.7% in HCs, 96.1% in MS patients and 94.7% in CNS autoimmune patients. The LILRA3 W/W (wild-type/wild-type) genotype was recorded in 86.0% of HCs, 92.4% of MS patients and 87.5% of CNS

autoimmune patients. Remarkably, the D/D (deletion/deletion) genotype was not identified in any of the three cohorts.

## LILRA3 Deleted variant confers protection for MS.

Following LILRA3 genotype and allele association analyses, utilizing the SHEsis software platform (62,63), it was evident that the WT/WT genotype and the WT allele overall predominate in the MS patient cohort (Figure 6). Upon age and sex adjustment between the HC and MS cohorts, deletion of LILRA3 has a statistically significant OR of OR: 0.507, (95% CI: 0.267 ~0.961) with a Fisher's p value of 0.034 calculated from Fisher's exact test. Analysis of genotypes showed that homozygosity for the wild-type allele tended to be more common in MS patients than in controls (p value = 0.037). According to this trend, the risk of suffering MS is highest in individuals presenting only the wild-type allele of LILRA3 in their genome and lower in individuals carrying at least one LILRA3 deleted allele.

*Table 1- Demographics, clinical, laboratory, and imaging characteristics of the study cohort.*

| | | **MS (n=157)**<br>**mean ± SD or N (%)** | **CNS autoimmune (n=40)**<br>**mean ± SD or N (%)** |
|---|---|---|---|
| **Female** | | 116/157 (73.9%) | 32/40 (80%) |
| **Age at disease onset** | | 30.3 ± 10.1 | 45.3 ± 13.8 |
| | | **Type of MS**<br>RRMS: 95.4%<br>SPMS: 1.5%<br>PPMS: 2.3% | N/A |
| **First symptom:** | Sensory | 52.7% | 29.8% |
| | Visual | 26.3% | 32.4% |
| | Motor | 21.0% | 37.8% |
| **EDSS score** | | 2.1 ± 1.3 | 1.9 ± 1.4 |

*Table 2- LILRA3 allele and genotype frequencies for healthy controls (HCs), Multiple Sclerosis (MS), and CNS autoimmune patient cohorts. W: wild-type allele, D: Deleted allele.*

| | | HC (n=380) | MS (n=157) | CNS autoimmune ( n=40) |
|---|---|---|---|---|
| **Allele frequency** | W | 92.7% | 96.1% | 94.7% |
| | D | 7.3% | 3.9% | 5.2% |
| **Genotype Frequency** | W/W | 86.0% | 92.4% | 87.5% |
| | W/D | 14.0% | 7.6% | 12.5% |
| | D/D | 0% | 0% | 0% |

Furthermore, no statistical differences were observed in LILRA3 genotype frequencies between the CNS autoimmune cohort and HCs (p=0.61), while also the frequency distribution between the two cohorts was not significant (Wild type variant: p=0.877, OR 0.933 [0.388~2.243] and Deleted variant: p=0.491, OR 0.694 [0.244~1.971]). In addition, between the age-sex adjusted MS and CNS autoimmune cohorts no differences were identified in terms of genotype distribution (p=0.525), or in terms of allele distribution (Wild type variant: p=0.407, OR 1.562 [0.59~4.133], and Deleted variant: p=0.533, OR 0.73 [0.228~2.328]). (Figure 7)

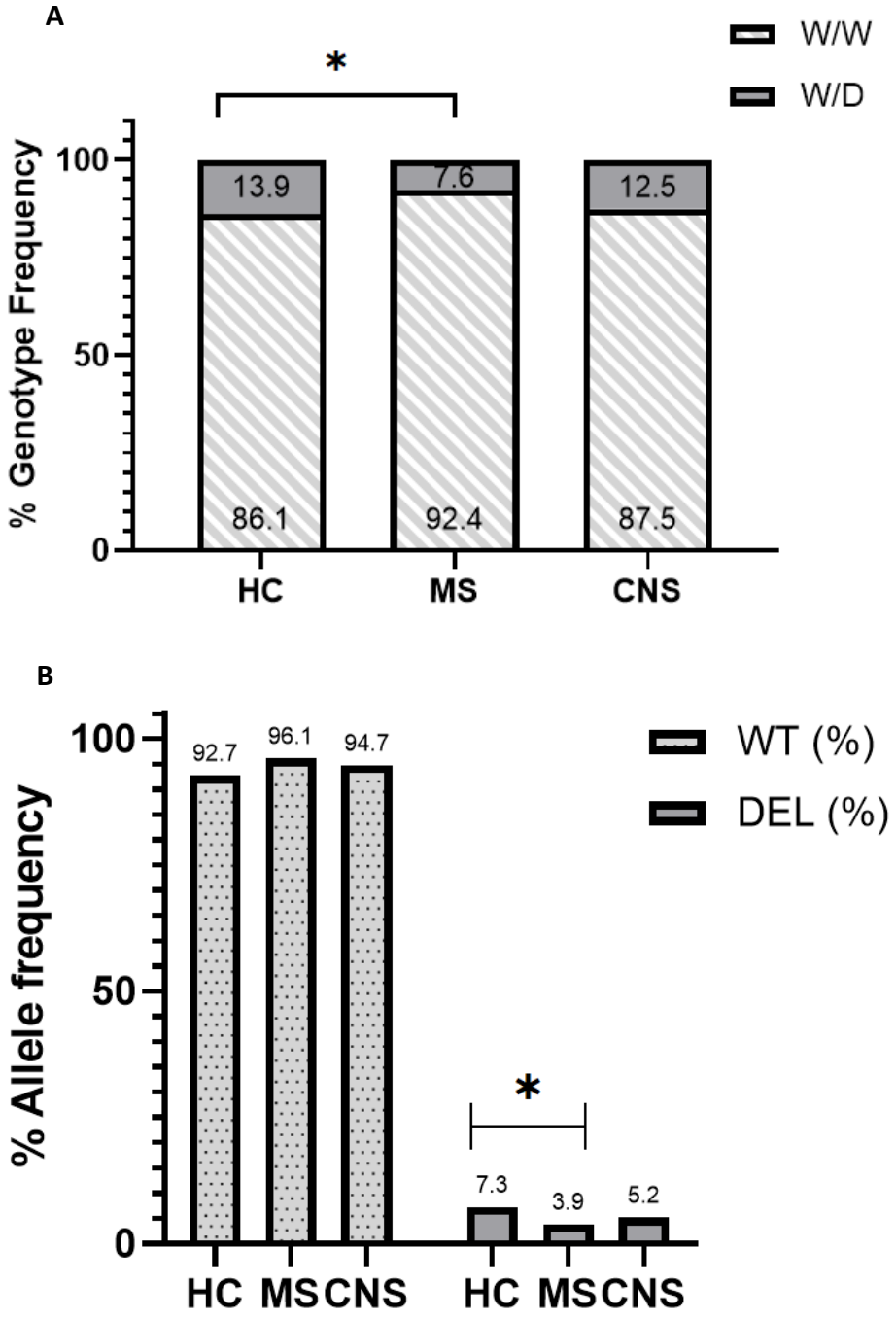


*Figure 7- A. Genotype and B. allele frequencies of LILRA3 among the three cohorts. HC: healthy control, MS: multiple sclerosis, CNS: systemic autoimmune patient cohort with CNS involvement, W/W: wild-type/wild-type, W/D: wild-type/Deletion, W: wild-type, D: deletion.*

## Patients (MS or CNS autoimmune) Heterozygous for LILRA3 have lower White Blood cell counts

Following stratification of patients according to genotypes (W/W or W/D), association with clinical, imaging and laboratory parameters was performed utilising Graphpad Prism and SPSS software (Table 3). Among all the parameters, LILRA3 heterozygosity was associated with lower white blood (WBC) cell counts (6,697 vs 8,348 per μl), a finding which was also verified upon further stratification of the MS cohort into WT/WT and WT/DEL whereby lower WBCs counts were observed in the heterozygous strata (6,796 vs 8,619, p=0.037, data not shown). Moreover, lymphocytes were also lower among LILRA3 heterozygous patients (2,653 vs 3,369 per μl), but this difference was marginally significant (p=0.063). Regarding MRI findings, first symptom at disease onset, EDSS score and CSF findings, no significant differences were observed between the two strata.

*Table 3- Clinical, imaging and laboratory features in LILRA3 homozygous (wild-type/wild-type) vs LILRA3 heterozygous(wild-type/deletion) patients.*

| | | W/W (n=178) | W/D (n=17) | p value |
|---|---|---|---|---|
| **Demographics** | **Female Sex (%)** | 72% | 80% | 0.135 |
| | **Age at disease onset (mean, std)** | 31.75 ± 10.61 | 31.65 ± 11.50 | 0.672 |
| **EDSS** | | 2.06 ± 1.27 | 2.10 ± 1.27 | 0.765 |
| **MRI findings (%)** | **Brain Involvement** | 49% | 41% | 0.072 |
| | **Spine Involvement** | 29% | 29% | >0.999 |
| **Final diagnosis** | **MS** | n=145 | n=12 | >0.999 |
| | **CNS autoimmune** | n=33 | n=5 | >0.999 |
| **First Symptom at Disease Onset (%)** | **Motor** | 13% | 29% | >0.999 |
| | **Sensory** | 34% | 23% | >0.999 |
| | **Visual** | 37% | 47% | >0.999 |
| **CSF findings (%)** | **Positive T2 OCBs** | 25% | 33% | >0.999 |
| | **High IgG Index** | 11% | 20% | >0.999 |
| **Laboratory Parameters (per µl)** | **WBC** | 8348 ( ± 2,348) | 6697 ( ± 1,588) | **0.023** |
| | **Lymphocytes** | 3369 ( ± 1,257) | 2653 ( ± 956) | 0.063 |

## Discussion

MS is an autoimmune disorder affecting the CNS, of multifactorial etiology. Family studies have shown strong genetic contributions. Linkage analyses have revealed several regions harboring risk genes including chromosome region 19q13. LILRA3 is one of the most interesting candidate genes, since LILRA3 are involved in the generation of immunological tolerance. There is an absence/presence of variability of the LILRA3 gene comprising several exons, thus incapacitating the gene function. In the present study, we examined the association of LILRA3 deletion with MS. Using PCR typing, deficiency of LILRA3 was examined in 400 HC, 157 MS and 40 CNS systemic autoimmune patients of Caucasian Greek origin.

Heterozygous LILRA3 genotypes were significantly less prevalent in MS patients (3.9%) than in blood donors (7.3%; P=0.037). LILRA3 deficiency was found to confer protection for MS in the Greek population and hence the wild-type variant is likely risk factor for autoimmune disorders. Remarkably, these findings are opposed to those from analogous studies in Spanish (46) and German (45) MS patient cohorts that reported the association of *LILRA3* deletion with the presence of MS. However, this contradiction is not surprising given that the deleted variant of *LILRA3* greatly oscillates among populations. Furthermore, as with many autoimmune disorders that are polygenic, the wild-type *LILRA3* variant might be one among the many contributory factors to disease susceptibility. It is clear that the functional *LILRA3* variant is not a prerequisite for the development of MS. In the future, it will be valuable to investigate LILRA3 protein levels in sera and CSF from MS and CNS autoimmune patients. It is speculated that upon protein quantification, patients who are homozygous (W/W) for *LILRA3* will have greater LILRA3 protein levels compared to those who are heterozygous (W/D), that is because the deleted variant is unable to transcribe a functional protein (64). Moreover, detection of LILRA3 in CSF collected from patients who at the time were naïve in terms of treatment, will give a clearer insight as to whether LILRA3 is secreted in the CNS upon inflammation generated by lymphocytes entering the CNS.

It was further shown that the heterozygous *LILRA3* genotype is associated with lower WBC counts in the two patient cohorts combined. While LILRA3 is secreted primarily by monocytes (65), it is unlikely that heterozygosity for *LILRA3* can affect white blood cell counts. To investigate this association further, qPCR for LILRA3 expression on isolated WBC populations such from whole peripheral blood can verify the LILRA3-expressing cell populations in MS and CNS autoimmune patients.

Overall, the deleted variant of *LILRA3* confers protection for MS in a Greek patient cohort, a finding not observed in the CNS autoimmune patient cohort. Further investigation will be crucial to unravel whether these findings are translated on a gene expression and protein expression level.

## Acknowledgements

I would like to firstly thank my supervisor, Prof Clio Mavragani, for allowing me to complete my Master thesis in her lab, for providing me with the appropriate guidance and feedback throughout this time and for allowing me to witness her dedication in research, which is admirable. I would also like to thank our lab manager, Dr Charalampos Skarlis, for his patience and excellent communication skills.

Thanks should also go to the incredible neurology team of Aeginition hospital, Assoc. Prof. Maria-Eleftheria Evangelopoulos, Dr. Dimitirs Karathanasis and Dr. Vasilis Papapodopoulos for the meticulous collection of clinical information and laboratory samples and for being overall a great team to collaborate with.

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