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Microchimerism and multiple sclerosis: a study on the impact of the sex of offspring on clinical, radiological, and paraclinical features of maternal disease

A new point of view for the sex differences in Neurological disease

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ABSTRACT

Background. Multiple sclerosis (MS) is a chronic autoimmune disorder characterised by inflammation and neurodegeneration and representing one of the most common causes of neurologic disability among young adults. Over the last 40 years, several authors have confirmed the existence of fetal cells in maternal blood and their pregnancy-related origin, demonstrating that pregnancy may establish a long-term, low-grade chimeric state in women. The biological and clinical significance of fetal microchimeric cells (fMCs) in maternal health is largely unknown, although a role in autoimmune diseases have been hypothesised.

Aims. The overarching aim of my PhD dissertation was to investigate the role of the sex of offspring, considered an indirect marker of fMCs, in clinical, paraclinical, and radiological MS features.

Methods. During my PhD course, I conducted two retrospective studies and one prospective study on female MS patients. proceeding from literature data on the association between pregnancy and microchimerism, subjects were classified according to their pregnancy status and sex of offspring as follow: (a) subjects with history of at least a male pregnancy and supposedly carrying XY microchimeric cells (XYp), (b) subjects with history of only female pregnancy and supposedly carrying XX microchimeric cells, but not XY microchimeric cells (XXp), and (c) nulliparous subjects supposedly without microchimeric cells (NLp).

In the first project, I obtained information on pregnancy history for a population of 354 MS female patients, including 87 nulliparous subjects (NLp), 188 subjects with history of at least a male pregnancy (XYp), and 79 subjects with history of only female pregnancy

(XXp). Medical records were used to collect clinical, radiological, and paraclinical data at onset, diagnosis, and last clinical follow-up for this large cohort of patients.
In the second study I selected a subgroup of 54 patients from the previous cohort, including 26 NLp, 8 XXp, and 20 XYp. I processed their magnetic resonance imaging (MRI) scan using the Lesion Segmentation Tool toolbox and FreeSurfer software to obtain quantitative data on white matter, cortical, and subcortical areas. Additional clinical, radiological, and paraclinical data at onset, diagnosis, and last clinical follow-up were also collected using medical records and telephone interview.
Finally, in the third project, I enrolled 43 patients in a prospective study. The study is still on-going and the population recruited so far includes 18 NLp, 19 XYp, and 6 XXp. All patients were classified according to their obstetric history and underwent a blood test analysis to determine the microchimeric group amplifying Y chromosome-specific sequences. Each patients underwent a baseline visit to collect clinical data, an MRI scan, and an optical coherence tomography (OCT) scan.

Results. The first study showed that, at disease onset, NLp were younger than XYp and XXp and that the same group reported a longer disease duration when attending the first visit at MS Centre. In addition, data showed that NLp had less frequently a pyramidal onset when compared to XXp. Comparing XYp and XXp patients, I observed that XXp had higher ARR, a higher disability after 3 and 5 years of disease duration, and more severe ambulation scores at EDSS at 3 years of disease duration.
In the second project, I observed that NLp had lower brain volumes in several cortical areas, as well as in some subcortical and white matter volumes. More specifically, comparing NLp and XXp, I found that the former group had larger 4^{th} ventricle and smaller right pallidum and left enthorinal volumes. NLp also reported lower thickness in left paracentral cortex, left precuneus cortex, and right lateral occipital cortex when compared with XXp. A similar trend was observed comparing NLp and XYp: NLp group had lower thickness in left paracentral cortex, left pericalcarine cortex, and right paracentral cortex. Interestingly, at the comparison between XYp and XXp, I observed that the thickness was higher in XYp in the left cuneus cortex, left pericalcarine cortex, and left insula, while XXp had a higher thickness in the right lateral occipital cortex.
In the last project, preliminary data showed that the risk of MS onset in post-partum was higher in XYp when compared to XXp. I also found that XXp patients had higher spine

lesion load at diagnosis and registered higher ARR, while XYp had more frequently brainstem involvement at onset, presented more frequently with progressive MS phenotype at last clinical follow-up, and reached lower scores at PASAT. OCT revealed that, despite having a similar age and disease duration, XXp patients had lower RNFL and GCIPL thickness when compared with XYp, although the difference was not significant. I also found similar trends in XYp and XXp when these groups were compared to NLp. However, the RNFL and GCIPL was again higher in XYp when compared to NLp, while non-significant differences were detected between NLp and XXp. Overall, my results support the hypothesis that XY and XX fMCs could differently modulate the inflammatory and degenerative processes underlying MS.

Discussion. The results reported in this thesis demonstrated that the sex of offspring could influence disease features in MS. Being most of the changes occurring during pregnancy not different in male and female pregnancies, the results obtained from two retrospective studies and the preliminary findings of the prospective study suggested that (1) fMCs could be one of the pregnancy-related factors modulating the disease onset and course, and (2) the sex chromosome of fMCs could play a role on the biological processes underlying MS.

Conclusion. The hypothesis that a small percentage of cells with an XX or XY genotype could, through the expressions of sexual chromosome genes, regulate the maternal immune system and the repair mechanisms activated in the mother is fascinating and lends a fresh perspective to the sexual differences in neurological diseases. My findings suggested that XX and XY fMCs could, although marginally and likely interacting with other factors, be involved in MS inflammation and axonal degeneration, influence the immune system activation, and induce mechanism of repair. Discovering whether the presence of fetal *non-self* chimeric cells, and their chromosomes, may play a role in the modulation of the nervous system and its pathology is surely one of the more interesting challenges for the future.

CHAPTER I

Multiple sclerosis

1.1. Multiple sclerosis: epidemiological data

Multiple sclerosis (MS) is an autoimmune-mediated inflammatory and degenerative disorder of the central nervous system (CNS). In the disease, multifocal demyelination driven by inflammatory processes and neuronal degeneration alter the nervous conduction and cause the occurrence of neurological symptoms and the development of clinical disability.

Described for the first time in 1938 by Robert Carswell e Jean Cruveilhier, the disease is one of the most common immune-mediated disease of CNS and a leading cause of disability in young adults in Europe and United States (Klineova and Lublin, 2018).
In 2020, the Multiple Sclerosis International Federation (MSIF) and the World Health Organization (WHO), collecting data from 115 Countries which covered 87% of the world population, estimated that the global prevalence of the disease is 36 cases/100,000 people (MSIF 2020). The report registered a consistent increase of 0.5 million cases from 2013 (MSIF 2013), a trend that was only partially explained by improvements in diagnosis, extension of life expectancy for people with MS, and the growth of global population (MSIF 2020). In addition, the Atlas confirmed the remarkable variation in disease prevalence and incidence across different geographical areas (***Figure 1.1***), reporting higher figures for Europe (143 per 100,000) and North America (118 per 100,000) and lower frequencies for South-East Asia (9), Africa (9), and the Western Pacific (5). This geographic distribution suggests that the frequency of MS varies throughout the world, a condition likely due to the influence of genetic and environmental factors on the pathological processes underlying MS (MSIF 2020).

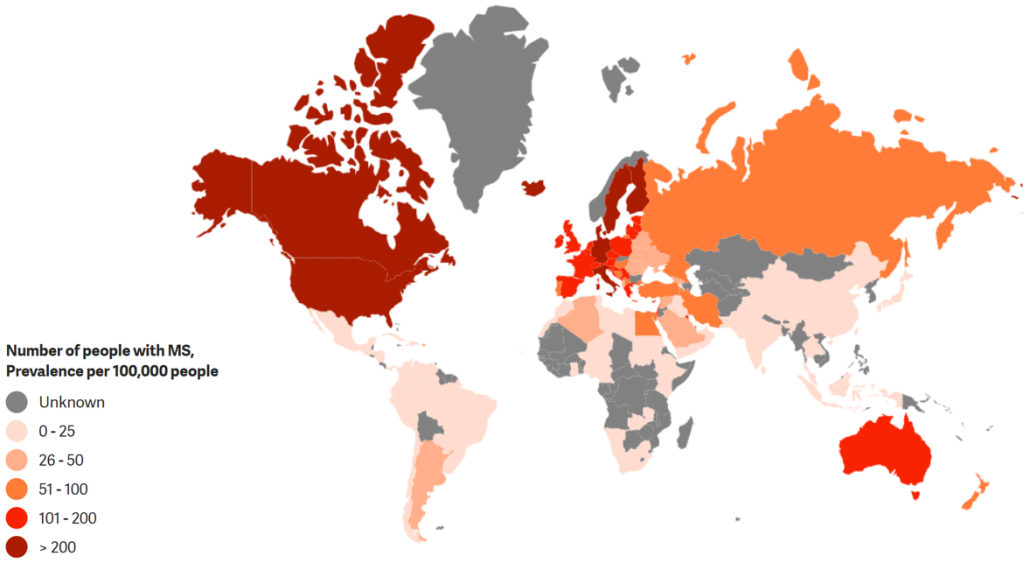


Figure 1.1. *Number of people with MS - prevalence per 100,000 people.* From MSIF, Atlas of Multiple Sclerosis. 2013.

The average age at disease onset is 32 years, ranging from 30 to 33 years, without geographical variations. Nonetheless, approximately 2–10% of individuals with MS experience their first episode as a child, with a global prevalence of at least 30,000 patients under the age of 18 years (Narula, 2016; The Multiple Sclerosis International Federation (MSIF), 2020). In a recent systematic review and meta-analysis, Yan et al. estimated that the global incidence and prevalence of pediatric MS were 0.87 cases/100,000 individuals annually and 8.11 cases/100,000 inhabitants, respectively (Yan *et al.*, 2020).

In the 2020 Atlas, the MSIF registered that about the 69% of MS patients are female, with a median female/male ratio of 2 women for every 1 man. This skew towards females seems to be even greater in the Western Pacific and South-East Asia regions, where a female/male ratio of 3-4/1 has been reported (MSIF 2020). Moreover, epidemiological data suggest that the female/male ratio changes throughout lifetime: MS affects females and males equally prior to 10 years (female/male = 0.8–1.4/1), a sharp increase in female

prevalence is registered post-puberty (female/male = 2–3/1), and eventually a moderate decline after menopause is observed (female/male = 1.4–1.9/1) (Dunn, Gunde and Lee, 2015). The increase in the incidence of MS in female population at puberty suggests that the interaction of genetic, hormonal, environmental, and socioeconomic factors could induce this sex unbalance in epidemiology of MS (Nicot, 2009).

Epidemiological, histopathological, and radiological studies demonstrated that the demyelination and neurodegeneration processes underlying the disease correlate with disease progression and disability accrual. Indeed, although some MS patients experience little disability during their lifetime, 30-50% of MS patients develop a progressive worsening of clinical symptoms after 15-30 years of disease duration (Cree *et al.*, 2016; Tintore *et al.*, 2020). Moreover, 25% of subjects in this subgroup of patients reaches a high level of disability with major implications on their quality of life and the social financial cost of the disease (Fisniku *et al.*, 2008).

Overall, epidemiological data explain the central role that MS has gained over the last 50 years in the landscape of neurological diseases and justify the huge effort made by the Academic Community to better define the disease and its underlying pathological processes.

1.2. Risk factors associated to multiple sclerosis

MS is a common, complex neurological disease whose precise aetiology is yet unknown, although epidemiological and immunological evidence described the multifactorial pathogenesis of the disease and indicated that both genetic and environmental factors are involved in its onset and natural history. In 2010, Ramagopalan et al. reviewed the previous epidemiological studies and hypothesised that environmental factors could induce an alteration in the immune system in subject genetically predisposed and trigger the pathological processes associate to the disease (***Figure 1.3***) (Ramagopalan *et al.*, 2010; Olsson, Barcellos and Alfredsson, 2016).

	Study	OR (95% CI)
Family history		
Non-twin first-degree relative affected[59]	19 615 first-degree relatives of 8205 Danish patients with MS	7·1 (5·8–8·8)
Ethnic origin		
White male compared with black male[13]	US cohort study of 4951 patients with MS and 9378 controls	1·49 (1·09–2·27)
HLA haplotype		
*HLA-DRB1*15* homozygote[16]	Canadian cohort study of 2454 patients with MS and 4639 unaffected first-degree relatives	5·42 (4·12–7·16)
*HLA-DRB1*15* heterozygote[16]	Canadian cohort study of 2454 patients with MS and 4639 unaffected first-degree relatives	2·91 (2·42–3·51)
*HLA-DRB1*15/HLA-DRB1*14* heterozygote[16]	Canadian cohort study of 2454 patients with MS and 4639 unaffected first-degree relatives	1·06 (0·56–2·03)
Immune marker genes		
Interleukin 2 receptor α (*IL2RA*)[22]	Case-control study of 4839 patients with MS and 9336 controls	1·15 (1·04–1·27)
Interleukin 7 receptor α (*IL7RA*)[22]	Case-control study of 4839 patients with MS and 9336 controls	1·13 (1·02–1·23)
C-type lectin domain family 16 A (*CLEC16A*)[22]	Case-control study of 4839 patients with MS and 9336 controls	1·15 (1·04–1·25)
CD58 (*CD58*)[22]	Case-control study of 4839 patients with MS and 9336 controls	1·30 (1·14–1·47)
Tumour necrosis factor receptor superfamily, member 1A (*TNFRSF1A*)[22]	Case-control study of 4839 patients with MS and 9336 controls	1·20 (1·10–1·31)
Interferon regulatory factor 8 (*IRF8*)[22]	Case-control study of 4839 patients with MS and 9336 controls	1·25 (1·12–1·39)
CD6 (*CD6*)[22]	Case-control study of 4839 patients with MS and 9336 controls	1·18 (1·07–1·30)
Place of birth		
Migration before *vs* after age 15 years[71]	Cohort study of 76 immigrant patients in the UK	2·07 (1·13–3·77)
Age		
Incidence at age 30 years *vs* age 55 years[4]	Cohort study of 1099 Canadian patients	4·5 (1·52–13·3)
Clinically isolated syndrome		
Abnormal MRI *vs* normal[89]	UK cohort study of 107 CIS patients	3·99 (1·65–9·65)
Presence of oligoclonal bands, independent of MRI[109]	Spanish cohort study of 415 CIS patients	1·7 (1·1–2·7)
Sex		
Female[30]	Population-based study of 27 074 Canadian patients with MS	6·62 (6·21–7·13)
EBV infection		
Infectious mononucleosis[36]	Meta-analysis of 11 case-control and 3 cohort studies totalling 1667 patients with MS and 3606 controls	2·30 (1·70–3·01)
Anti-EBNA1 antibody geometric mean titre >320 *vs* <80[141]	Nested US case-control study of 148 women with MS and 296 healthy female controls	1·66 (1·32–2·08)
Smoking		
Ever *vs* never[44]	Meta-analysis of case-control studies totalling 1155 patients with MS and 153 182 controls	1·51 (1·22–1·87)
Month of birth		
May[59]	17 874 Canadian, 11 502 British, 6276 Danish, and 6393 Swedish patients with MS compared with population controls	1·10 (1·07–1·13)
Vitamin D		
Serum 25-hydroxycholecalciferol increased in the lower quintile (<63·3 nmol/L) *vs* the upper quintile (>99·1 nmol/L)[56]	Nested US case-control study of 148 white patients with MS and 296 matched healthy white controls	1·69 (1·03–2·78)

Meta-analyses were included if available, and if not, the largest (or, in some cases, the only) study available was included. EBV=Epstein-Barr virus. EBNA1=EBV nuclear antigen 1. MS=multiple sclerosis. OR=odds ratio.

Figure 1.3. *risk factors for multiple sclerosis.* From Ramagopalan SV et al., Multiple Sclerosis: Risk Factors, Prodromes, and Potential Causal Pathways. Lancet Neurol 2010; 9: 727-739.

The evidence for the relevance of genetic factors in regulating the susceptibility to MS relies on extensive epidemiological data. The genetic bases of MS are complex and the susceptibility to the disease involve numerous loci, both within the human leukocyte antigen (HLA) region, and in more than 100 polymorphic loci outside of the HLA (Jokubaitis and Butzkueven, 2016).

The incidence of MS is 20-40 times higher in subjects with a first-degree relative affected by the disease (Nourbakhsh and Mowry, 2019). Furthermore, data on concordant twins

reported that monozygotic twins (25-30%) had a 5-6 times higher risk when compared to dizygotic twins (Weinshenker BG., 1996; Tullman, 2013). In addition, the involvement of genetic factors would partly explain the epidemiological findings reported in different ethnicity, a data extensively investigated using migration studies. Overall, the genetic background of the disease seems to be complex and to implicate several genes that would co-act in determining the MS susceptibility of the subjects (Alfredsson *et al.*, 2013; Bos *et al.*, 2016; Nourbakhsh and Mowry, 2019).

HLA types exert the strongest genetic influence on MS, although the association is not straightforward as it varies according to the geographical regions, with different HLA types both positively and negatively associated with the disease (Ramagopalan *et al.*, 2010). Alleles of the major histocompatibility complex (MHC) Class II seem to represent a risk factor for the disease, while the MHC alleles Class I would play a protective role (Bos *et al.*, 2016). HLA-DRB1*15:01 allele is the strongest risk locus found within the HLA region, contributing around 10% of the genetic risk associated with MS and increasing of 3.08 the risk of developing MS (Jokubaitis and Butzkueven, 2016). Nonetheless, numerous other common polymorphisms have been associated to a higher individual risk of MS. In fact, Genome-Wide Association (GWA) studies had identified more than 150 single-nucleotide polymorphisms (SNPs) that would be associated to MS, most of which located in noncoding introns close to genes coding for cytokines, cytokine receptors, transcription factors, adhesion molecules, and co-stimulatory molecules (Ramagopalan *et al.*, 2010; Jokubaitis and Butzkueven, 2016; Martin *et al.*, 2016).

Despite the successfully identification of loci associated with disease susceptibility, to-date, no published studies have identified risk variants significantly associated with clinical and/or paraclinical outcomes and evidence suggests that there could be numerous severity variants, each likely associated with a specific phenotypic outcome (Jokubaitis and Butzkueven, 2016)..

Although genes are clearly involved in MS development, epidemiological data equally suggest a role for the environmental factors. In 2015, Belbasis et al. provide an overview and appraisal of 44 environmental risk factors that have been associated with MS, including infections and vaccinations, comorbid diseases, surgeries, traumatic events and accidents, exposure to toxic environmental agents, and biochemical biomarkers (Belbasis *et al.*, 2015). The authors found that only (1) anti-EBNA IgG seropositivity and Epstein-

Barr virus (EBV) infectious, and (2) smoking had a strong epidemiological credibility in modulating the disease (Belbasis *et al.*, 2015). As for the first factor, more recently, Bjornevik et al. tested a cohort including more than 10 million young adults to evaluate the role of EBV in MS. In the study population, 955 subjects were diagnosed with MS. The authors found that the risk of MS increased 32-fold after infection with EBV, while it remained unchanged after infection with other viruses, including the similarly transmitted cytomegalovirus (CMV). The group also reported that serum levels of neurofilament light chain, a biomarker of neuroaxonal degeneration, increased only after EBV seroconversion (Bjornevik *et al.*, 2022). Going to smoking, evidence from randomised trials, systematic reviews, and epidemiological studies demonstrated that smokers have a higher risk of developing MS and experiencing related adverse symptoms and complications. Nonetheless, the mechanism underlying the correlation is not fully understood, with authors hypothesising that tobacco could have immunomodulatory and inflammatory effects on the CNS (Arneth, 2020).
Finally, it is still controversial the role of latitude, vitamin D, and past sunlight exposure, whose association with MS has been found inconsistent in different studies (Tullman, 2013; Belbasis *et al.*, 2015; Garg and Smith, 2015; Agnello *et al.*, 2016).

1.3. Pathogenesis of multiple sclerosis

The *primum movens* of MS is still undefined. Nonetheless, epidemiological, clinical, and experimental data have demonstrated that genetic and environmental factors interact and trigger the inflammatory and neurodegenerative processes of autoimmune aetiology that, altering the nervous conduction, are responsible for the clinical manifestations.

The nervous impulse is produced by an ionic flow, which induces a depolarization at the cellular membrane and results in an action potential or spike. This ionic flow is due to ionic channels which allow the influx and outflux of sodium and potassium, initiating a depolarisation which is propagated along the axon and permits the nervous transmission. When an impulse is conducted along a nervous fibre, sodium ions move inwards, while potassium ions move outwards producing the depolarization of the cell. In 1949, Hodgkin and Katz, studying the giant axon of a squid, demonstrated the role of sodium in the

genesis of the action potential. At resting state, the cellular membrane is more permeable to sodium than potassium. The activation of sodium channels increases the sodium permeability and produces an influx of sodium, which reverses the membrane potential and induces the depolarisation in the cell. The occurrence of the action potential produces a flux of ions in nervous fibre and consequently the impulse propagation. The depolarisation increases the membrane permeability to potassium, whose extracellular concentration rise determining a repolarisation of the cell and the onset of a refractory period during which a second depolarisation is inhibited (Huxley and Stämpfli, 1949).

Saltatory conduction (***Figure 1.2***) was discovered by Tasaki in 1939 and confirmed by Huxley and Stämpfli's studies ten years later (Huxley and Stämpfli, 1949). The authors described the phenomenon as a propagation of action potentials along myelinated axons from one node of Ranvier to the next one, with an elevation of the voltage at each successive node along the nervous fibre. The clustering of voltage-gated sodium channels at the unmyelinated nodes of Ranvier produces at each node the action potential, which is propagated and spreads passively down the myelinated nerve segment to trigger another action potential at the next node (Huxley and Stämpfli, 1949). Myelin sheaths, insulating the axon, increase the speed of impulse propagation from one node to the following one. Myelin is a dialectric material whose water content is about 40% and dry mass is characterized by a high proportion of lipid (70–85%), among which galactocerebroside (GalC) and sphingomyelin, and a low proportion of protein (15–30%), including myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), and proteolipid protein (PLP). Oligodendrocytes are responsible for myelin synthesis and maintenance in up to 40 neighbouring nerve axons in the CNS (Stadelmann *et al.*, 2019).

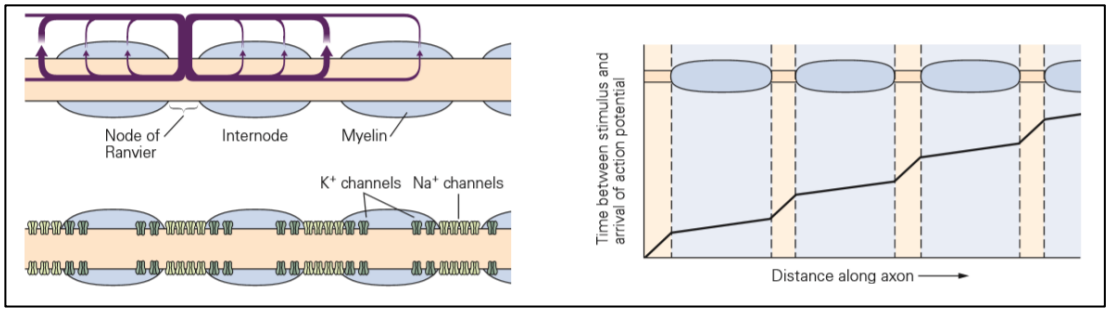


Figure 1.2. *Action potential and saltatory conduction in myelinated fibres.* From Kandel ER et al., *Principle of Neural Science* (Fifth Edition). Copyright © 2013 by The McGraw-Hill Companies, Inc.

In vivo and *in vitro* studies of neuroimmunology and the investigation of the Experimental Autoimmune Encephalomyelitis (EAE), the animal model of MS, have identified the immunological processes underlying MS onset and course. The disease seems to be due to an immunological deregulation in which not only CD4+ T cells, but also CD8+ T cells, humoral and innate immunity are involved (***Figure 1.4***) (O'Brien, Gran and Rostami, 2010).

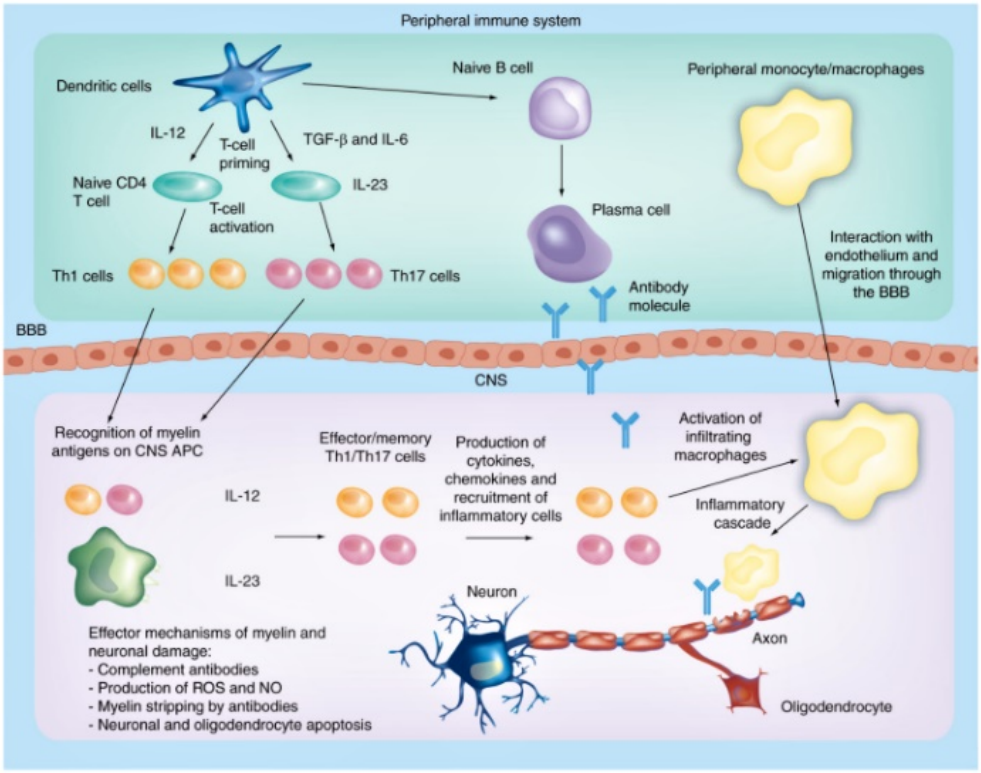


Figure 1.4. *Pathogenesis of Experimental Autoimmune Encephalomyelitis and multiple sclerosis. In the peripheral immune system, antigen-presenting cells (APCs), including dendritic cells and naïve B cells produce IL-12, TGF-β and IL-6, and activate naïve CD4+ T cells. Activated Th1 and Th17 cells cross the BBB into the CNS. Resident APCs interact with activated myelin specific Th1 and Th17 cells, which become reactivated and produce inflammatory cytokines and chemokines. These molecules initiate an inflammatory cascade and activate Infiltrating macrophages that attack the myelin sheath.* From O'Brien K et al., T Cell Immunotherapy in Experimental Autoimmmune Encephalomyelitis and Multiple Sclerosis. Immunotherapy 2010; 2: 99-115.

Evidence suggests that MS could be the result of the action of environmental factors that, in genetically susceptible subjects, would be able to activate mechanisms of cross-

reactivity and molecular mimicry, inducing the loss of immune-tolerance versus *self* myelin antigens, and provoking the massive activation of the immune system that eventually leads to demyelination and neurodegeneration (Kasper and Shoemaker, 2010; Garg and Smith, 2015; Martin *et al.*, 2016). Activated T cells penetrate into the CNS through the blood-brain barrier (BBB) and recognise the myelin sheaths as *non-self* antigen, leading to its macrophage-mediated damage. Within the brain tissue, activated leukocytes could activate microglia and macrophages, promoting an inflammatory status that causes the disruption and inflammation of the BBB, facilitating the passage of potentially pathogenic cells into the CNS. In addition to the autoreactive T cells that re-encounter antigen setting up a pro-inflammatory loop, the activate microglia expresses class II molecules and re-presents antigen to T cells leading to tissue damage and chronic inflammation (Lassmann, Brück and Lucchinetti, 2007; Liu *et al.*, 2022). Indeed, the inflammatory and demyelinating processes would determine the presentation of myelin antigens to T cells, inducing an epitope spreading that leads to the chronic, progressive pathology (Croxford, Olson and Miller, 2002).

These processes produce an inflammatory demyelination that is initially followed by remyelination (Lassmann, Brück and Lucchinetti, 2007). Gradually, the myelin repair capacity exhausted and the inflammatory activity evolves into post-inflammatory gliosis and neurodegeneration that cause the axonal damage (Frischer *et al.*, 2009; O'Brien, Gran and Rostami, 2010; Garg and Smith, 2015; Absinta, Lassmann and Trapp, 2020). The failure of lesion repairing processes leads to smouldering demyelination and axon degeneration inducing a progressive neurodegenerative state (Absinta *et al.*, 2021). Microglia seems to play a major in these phenomena, promoting injury in MS through several mechanisms (***Figure 1.5***) (Yong, 2022). The elaboration of cytokines, including TNF-α, identified as one of the major player of the excitotoxic-induced neurodegeneration (Stampanoni Bassi, Iezzi and Centonze, 2022), interferon-γ and lipopolysaccharide, and proteases (e.g., matrix metalloproteinases) are commonly associated to oligodendrocyte and neuronal injury. The release of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and other free radicals, markers of oxidative stress, leads to the damage of axons and dendrites. An unbalance in iron homeostasis may be another source of microglia-induced neurotoxicity: microglia and macrophages are responsible for the phagocytosis of deposited iron; however, the iron-induced oxidative

stress may cause these scavenger cells to degenerate and promote additional iron deposition and neuronal injury (Yong, 2022).

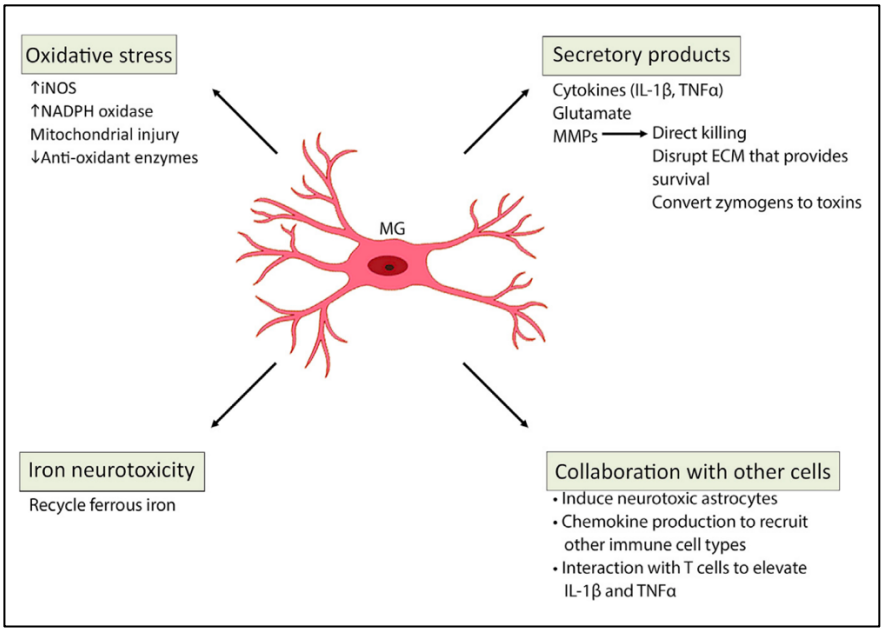


Figure 1.5. *Summary of mechanisms by which microglia promote injury in MS. Prominent factors that contribute to microglia toxicity include oxidative stress, release of pro-inflammatory products, dysregulation of iron homeostasis, and collaboration with other cell types.* From Yong VW, Microglia in Multiple Sclerosis: Protectors Turn Destroyers. Neuron 2022; 110: 3534-3548.

Already in 1868, Carchot observed that the demyelination was associated to axonal damage phenomena in the brain of patients affected by MS (Charcot, 1868). In 1998, Trapp et al., using the murine anti–non phosphorylated neurofilament (SMI-32) antibody, found that there was a spatial correlation between demyelinating lesions and axonal damage (Trapp *et al.*, 1998). However, mounting evidence has demonstrated that demyelination and neurodegeneration can occur at a distance from the T cell and B cell infiltrates, suggesting that tissue damage is driven by pro-inflammatory cytokines produced by T cells and/or B cells and capable to diffuse into the brain and induce damage either directly or indirectly through microglia activation (Absinta, Lassmann and Trapp, 2020). The existence of a MS-specific soluble demyelinating factor has been hypothesised, but the nature of this factor is so far unknown and further *in vivo* and *in vitro* studies are necessary (Absinta, Lassmann and Trapp, 2020).

Overall, the demyelination and axonal loss underlying MS and causing the clinical manifestations associated to the disease seem depend on several immunopathological phenomena (***Figure 1.6***) (Kawachi and Lassmann, 2017), including:

- oxidative burst activation of microglia/macrophages,
- mitochondrial damage and axonal energy failure,
- histotoxic hypoxia,
- Wallerian degeneration,
- age-related iron accumulation,
- meningeal inflammation in the human brain,
- activation of astrocytes.

In the early stage, the disease is dominated by active inflammatory demyelinating lesions arising with new waves of activation of the immune system and the degenerative processes are balanced by repair phenomena and synaptic plasticity (Absinta, Lassmann and Trapp, 2020). Indeed, in 1990, England and colleagues found that the voltage-gated sodium channels were present at an increased concentration along segments of previous internodal axons and suggested that this distribution was the result of the plasticity of sodium channels to recover the conduction after demyelination (England *et al.*, 1990). The authors hypothesised that remodelled sodium channels could support remyelination and re-establishment of saltatory conduction by formation of new nodes of Ranvier or could produce a continuous conduction. More recently, brain synaptic plasticity has been demonstrated to intervene to cope with the inflammatory and degenerative damage associated with the disease (Di Filippo *et al.*, 2015). Indeed, the alterations of synaptic transmission have been demonstrated to start in the early phases of the disease and, together with increased concentrations of pro-inflammatory molecules and microglial activation, it would be responsible for the excitotoxic neuronal damage and dendritic spine loss observed in MS. These findings suggest that neurodegeneration could be the result of an inflammatory synaptopathy independent from demyelination (Stampanoni Bassi, Iezzi and Centonze, 2022). Therefore, the axonal degeneration observed in the early stages of disease course would be mainly inflammation-related and would not initially cause permanent clinical disability thank to the ability of the CNS to compensate the neuroaxonal loss. However, with increasing age and disease duration, the disease gradually changes. The *progressive* accumulation of axonal loss overwhelms the mechanism of compensation determining the accrual of physical and cognitive disability

(Kawachi and Lassmann, 2017; Absinta, Lassmann and Trapp, 2020). Clinical progression would then be related to the accumulation of neuro-axonal loss in a lifelong chronic inflammatory CNS environment and the unbalance between damage, repair and brain functional reserve (Absinta, Lassmann and Trapp, 2020).

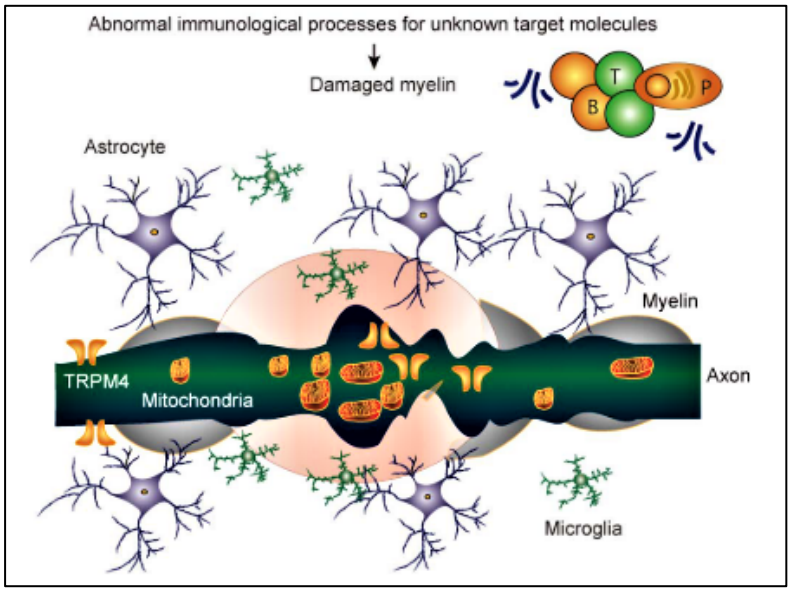


Figure 1.6. *Models of axonal degenerative pathology of multiple sclerosis. Neurodegeneration is driven by oxidative burst activation of microglia/macrophages, mitochondrial damage and axonal energy failure, histotoxic tissue hypoxia, Wallerian degeneration, age-related iron accumulation, and meningeal inflammation in the human brain.* From Kawachi I and Lassmann H, Neurodegeneration in multiple sclerosis and neuromyelitis optica. J Neurol Neurosurg Psychiatry 2017; 88(2): 137–145.

As described above, MS pathology is the result of a failure in the immunotolerance that would induce a massive and chronic activation of the immune system in the CNS. The activated immune cells are responsible for the inflammatory demyelinating processes that characterise the disease and for the alteration of the homeostasis in the CNS, which eventually leads to the neurodegeneration (Lassmann, 2018). These phenomena have been largely observed in histopathological studies.

The diagnostic hallmark of MS is the presence of large, confluent, demyelinated lesions in the white matter of the CNS. The onset of these lesions is related to perivascular inflammatory infiltrates containing T cells and B cells in close proximity to the site of active tissue damage. These *plaques* are multifocal, heterogeneous for dimensions, shape, and edges, and had their typical localisation in periventricular, juxtacortical, and

infratentorial regions of the brain and in the spinal cord. In the early stages, these areas are characterised by a complete loss of myelin and a massive destruction of oligodendrocytes, while axons are preserved to a large extent (***Figure 1.7***) (Lassmann, 2018). The demyelination and tissue damage leads to a second wave of inflammation that may be caused by adhesion molecule and chemokine expression in response to tissue injury. Indeed, in more advanced lesions, in which myelin has already been destroyed and the fragments have been taken up by macrophages, CD8+ T cells, CD4+ T cells, B cells, and monocytes colonise the lesions (Lassmann, 2018).

Historically, MS lesions were classified as acute, active, and chronic (***Figure 1.7*** and ***Figure 1.8***) (Wingerchuk, Lucchinetti and Noseworthy, 2001). The "activity" of the lesions can refer either to (1) inflammatory and/or demyelinating activity or (2) neurodegenerative activity, such as progressive axonal loss; whereas the term "chronic" is used to either describe the disease course or the histological phenotype of inactive or chronic active lesions (Kuhlmann *et al.*, 2017).

Active lesions are most frequent in MS patients with short disease duration and/or a relapsing-remitting disease course (RR-MS), although they are also detected, in lower frequency, in progressive phenotypes (P-MS) (Kuhlmann *et al.*, 2017). Active lesions are hypercellular and characterised by myelin loss and a diffuse and dense infiltration of the lesion area. CD68-positive cells are the most frequent component of the lesions, while T cells are localised both perivascularly and diffusely throughout the lesion area. Astrogliosis with increased GFAP expression is a hallmark of the active lesion (Kuhlmann *et al.*, 2017).

In 2000, Lucchinetti and colleagues identified in active lesions four different patterns of demyelination based on the distribution of myelin protein loss, the plaque geography and extension, the pattern of oligodendrocyte destruction, and the immunopathological evidence of immunoglobulin (Ig) and activated complement deposits (***Figure 1.9***) (Lucchinetti *et al.*, 2000). Pattern I and II are associated with a T cells and macrophage-dominated inflammation; prominent deposition of Ig (mainly IgG) and complement C9neo antigen at sites of active myelin destruction were found exclusively in pattern II lesions. Pattern III lesions contain inflammatory infiltrates, composed mainly of T cells, macrophages and activated microglia. Deposition of Ig and complement are absent. Demyelination in pattern III lesions was not characterised by the central veins and venules. Finally, pattern IV infiltrates are also dominated by T cells and macrophages,

without deposition of Ig and complement C9neo antigen. Demyelination is associated with oligodendrocyte death in a small rim of peri-plaque white matter, adjacent to the zone of active myelin destruction. Oligodendrocyte death is revealed by DNA fragmentation; however, the cells do not show the morphological features of apoptosis (Lucchinetti *et al.*, 2000).

Active lesions can be further separated into active demyelinating and active post-demyelinating lesions, also classified as chronic active lesions. Chronic active lesions are characterised by radial expansion (*smouldering* or slowly expanding lesions) with dense rims of microglia/macrophages and ongoing demyelination at the lesion edges or a thin rim of activated microglia with only few myelin containing macrophages (Absinta, Lassmann and Trapp, 2020). Their number increases, but their size decreases with disease duration.

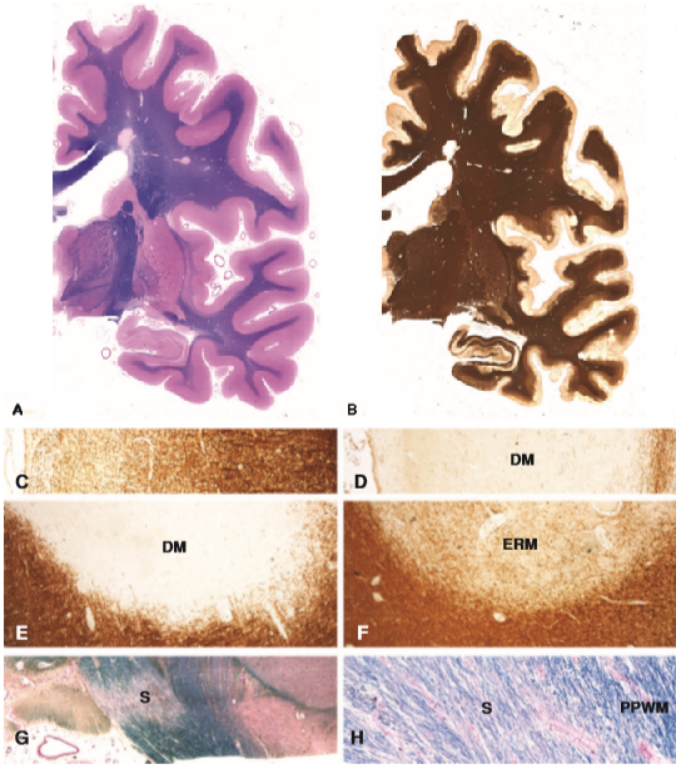


Figure 1.7. *Pathological changes in the brain of a patient with secondary progressive multiple sclerosis. Large confluent focal demyelinated lesions are present in the white matter (A). In addition, there is extensive subpial cortical demyelination, which can only be seen when sensitive immunocytochemistry for myelin proteins is used (B). In contrast to the normal pattern of myelin in the cerebral cortex, as shown in C, there is complete loss of myelin in subpial lesions (D). Demyelinated plaques in the white matter may appear as inactive demyelinated lesions (E), as early remyelinated lesions with a low density of thin myelin sheaths only visible by immunocytochemistry for myelin proteins (F) or as remyelinated shadow plaques (G, H).* From Lassman H, Multiple sclerosis pathology. Cold Spring Harb Perspect Med 2018; 8(3): 1-16.

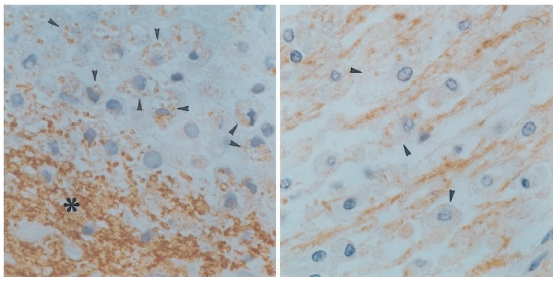


Figure 1.8. *Photomicrographs of an actively demyelinating multiple sclerosis lesion.* ***On the left****, the active edge of a multiple sclerosis lesion (asterisk), products of myelin degradation are present in numerous macrophages (arrow).* ***On the right****, macrophages containing myelin debris (arrow) are interdigitated with degenerating myelin sheaths.* From Wingerchuk DM et al., Multiple Sclerosis: Current Pathophysiological Concepts. Lab Invest 2001; 81: 263-281.

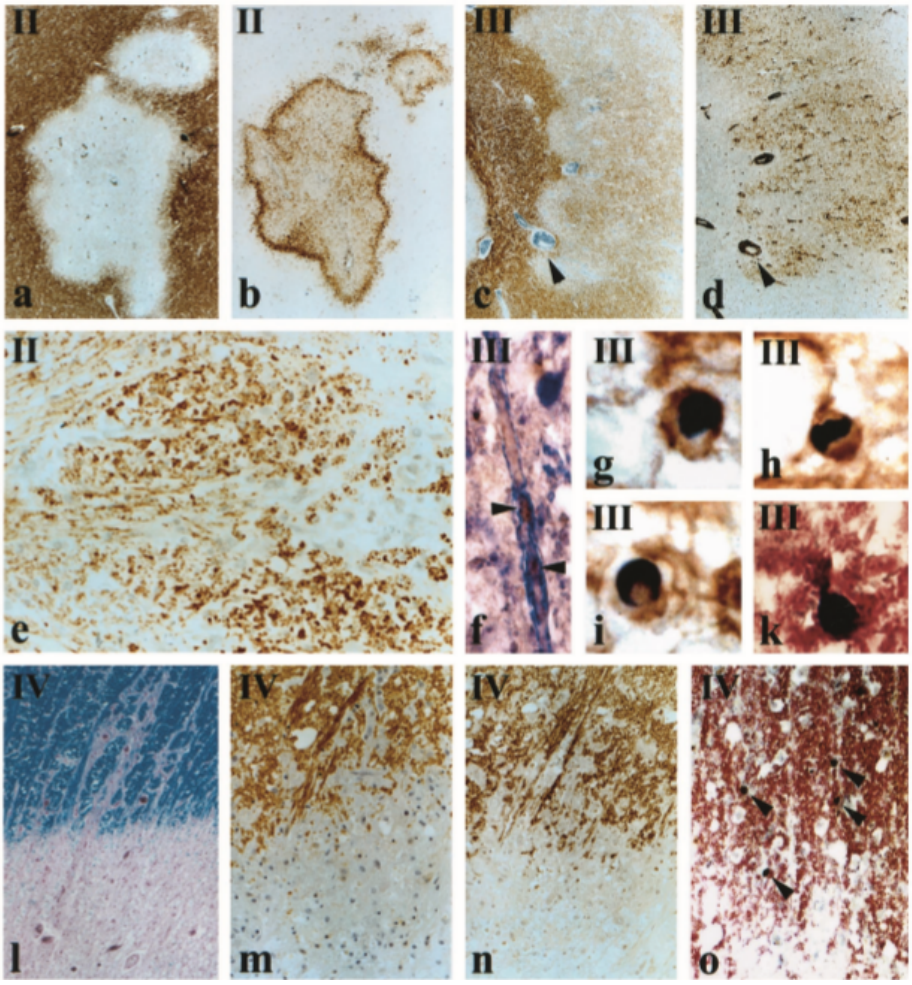


Figure 1.9. *Acute lesions in multiple sclerosis brain samples. Perivenous confluent pattern II lesion with macrophage rim at active border (a, b). Acute pattern III demyelinating lesion with ill-defined borders (c, d). The perivenous areas around inflamed vessels show lack of macrophage infiltration and demyelination (arrows). Pattern II lesion with massive C9neo deposition at the actively demyelinating border (e). C9neo Pattern III lesions (f) with actively demyelinating area. Apoptotic oligodendrocytes in pattern III lesions (g, h, I, k). Pattern IV lesion (l, m, n, o) with myelin antigens and DNA fragmentation of oligodendrocytes is seen in the peri-plaque white matter.* From Lucchinetti C et al., Heterogeneity of Multiple Sclerosis Lesions: Implications for the Pathogenesis of Demyelination. Ann Neurol 2000; 47: 707-717.

Inactive lesions are sharply demarcated, hypocellular, and almost completely depleted of mature oligodendrocytes and microglia. Only few T cells and microglia/macrophages are present within the lesion, while there is a marked axonal loss. There is no macrophage/microglia rim at the lesion border and astrocytes form a gliotic scar. Inactive lesions are the dominating plaque type in patients with disease duration of more than 15 years and/or secondary progressive MS without clinical activity (Kuhlmann *et al.*, 2017).

Shadow plaques are circumscribed regions where axons maintain uniformly thin myelin sheaths (***Figure 1.10***). These plaques represent areas of remyelination within the acute plaques or at the edge of chronic lesions. Shadow plaques are seen in conjunction with actively demyelinating patterns I and II lesions. Remyelinated shadow plaques are seen on average in similar incidence in the brains of patients with relapsing and progressive disease and their incidence varies profoundly between different cases (Kuhlmann *et al.*, 2017).

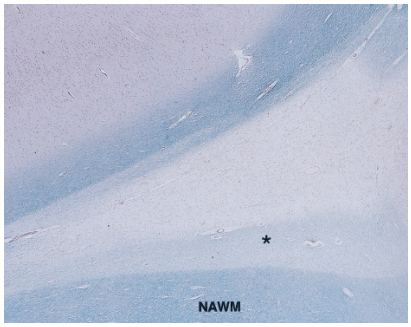


Figure 1.10. *Remyelination in a lesion associated with chronic multiple sclerosis. The area in pale blue (asterisk) represents a region of partial remyelination (a shadow plaque) along the periventricular edge of a lesion in a patient with chronic multiple sclerosis.* From Wingerchuk DM et al., Multiple Sclerosis: Current Pathophysiological Concepts. Lab Invest 2001; 81: 263-281.

MS lesions are not limited to the white matter, but can also be found in cortical and deep grey matter. Depending on the location, cortical lesions can be separated into subpial, intracortical, and leukocortical lesions (Kuhlmann *et al.*, 2017). Leukocortical lesions extend from the white matter into the cortex; intracortical lesions project radially from microvessels; and subpial lesions extend intracortically from the pia mater. Subpial,

bandlike, demyelinated plaques preferentially involve contiguous gyri (*kissing lesions*) more often in those regions of the brain engaged in attention and memory processing; these lesions develop independently from white matter lesions and may give rise to a *cortico-spinal* variant of MS, where extensive cortical demyelination is associated to few focal white matter plaques (Lucchinetti *et al.*, 2000; Kuhlmann *et al.*, 2017; Absinta, Lassmann and Trapp, 2020).

T cell and B cell infiltrates are rare within active cortical lesions, while the on-going demyelination is associated with leptomeningeal inflammation and microglia activation. In fact, post-mortem studies have revealed the presence of lymphoid-like structures in approximately 40% of progressive MS cases (Bevan *et al.*, 2018). *Meningeal immunity* includes macrophages, resident and circulating T cells, B cells, dendritic cells, and natural killer cells, all involved in meningeal immune-surveillance and exerting effector functions. These lymphoid-like structures typically develop during the transition from acute to chronic inflammation and their presence correlated with subpial cortical demyelination and neurodegeneration, and with microglia activation, suggesting that the meningeal inflammation in MS may represent an intrathecal source of inflammatory stimuli that could sustain the chronic local inflammation (Monaco *et al.*, 2020). Furthermore, meningeal inflammation is associated to an early onset, shorter and more aggressive disease course, and more rapid accumulated disability and strongly predicts the risk of extensive neocortical atrophy. The majority of neuropathological studies of cortical pathology have been conducted on progressive MS case, assuming that cortical damage may be more prominent at the later stages. However, more recently, Bevan et al. demonstrated that the mechanisms underlying cortical lesion formation could be identified also in acute MS, supporting the hypothesis that these phenomena are already present at an early stage (Bevan *et al.*, 2018).

Finally, diffuse damage and atrophy of the normal appearing white (NAWM) and grey matter (NAGM) has been described in patients with progressive disease. This diffuse white matter damage is caused by the Wallerian degeneration associated to the axonal and neuronal damage in focal lesions. Neuronal loss in cortical lesions and in NAGM contribute to the diffuse white matter degeneration (Kuhlmann *et al.*, 2017; Absinta, Lassmann and Trapp, 2020).

In conclusion, the biological basis of MS lies on the complex interaction between pathological processes of inflammation, and neurodegeneration. More recently, Kappos et al. hypothesised that the disease may be characterised by an underlying progressive disease course and a highly variable superimposed accumulation of disability resulting from relapses with incomplete recovery (Kappos *et al.*, 2020). Understanding the relation between inflammation and neurodegeneration is of key importance for future therapeutic strategies in multiple sclerosis (Frischer *et al.*, 2009).

1.4. Clinical phenotypes of multiple sclerosis

Being MS is the most common immune-mediated disease of the CNS and a leading cause of disability in young adults (Klineova and Lublin, 2018), an accurate descriptions of MS phenotypes is of paramount importance in clinical setting, as well as for research purposes. In 1996, the US National Multiple Sclerosis Society (NMSS) Advisory Committee on Clinical Trials in Multiple Sclerosis proposed the first classification of MS phenotypes (Lublin and Reingold, 1996). In 1996 classification, the clinical heterogeneity of the disease leaded to the identification of four disease phenotypes (***Figure 1.11***), whose diagnosis was generally determined retrospectively:

- Relapsing-Remitting MS (RR-MS) is the presenting phenotypes in 80% of patients. The disease course is characterised by waves of inflammation that cause the onset of clinical *relapses*. These acute episodes could recover (*remission*) completely or incompletely, determining the development of a relapse-related disability. Relapses are defined as the occurrence of at least one new neurological symptom or the recurrence or worsening of pre-existing symptoms of neurological dysfunction, whose duration last at least 24 hours, in absence of fever, infection or other diseases. A period of 30 days should separate the onset of two events to be defined as two different episodes. The intercritic periods is characterised by the absence of disability progression. At early stages, the relapse rate is usually less than one per year, and subsequently declines.
- Secondary-Progressive (SP-MS) defines the progressive phase that in 30-50% of patients follows the RR-MS after variable disease duration (usually 15-30 years). Symptoms gradually worsen due to the prevalence of neurodegenerative

processes. The standardised definition of SP-MS stated that an initial RR disease course is followed by progression with or without occasional relapses, minor remissions, and plateaus. Once progression has developed, its course is continuous, although occasional plateaus and temporary minor improvements may occur.

- Primary-Progressive (PP-MS) describe the onset phenotype in 20% of patients and is characterised by a progressive course since disease onset. Patients present a gradually accrual of neurological disability without clinical relapses.
- Relapsing-Progressive (RP-MS) is characterised by a progressive course since disease onset, with superimposed relapses that can recover completely or incompletely. The intervals between relapses are characterised by a continuous progression.

The classification provided consistency in defining patient groups according to their natural history and improved communication between clinicians and patients. However, due to MS heterogeneity in clinical manifestations and disease courses (Compston and Coles, 2008), this classification was based more on MS experts' opinion than objective biological evidence and relied on the hypothesis that there was a clear distinction between inflammatory and neurodegenerative phenotypes (Sand, 2015).

Two subclinical patterns of pre-MS phase have also been defined: the Clinically Isolated Syndrome (CIS) and Radiologically Isolated Syndrome (RIS). CIS is diagnosed by a single clinically evident episode of demyelination in the brain or spinal cord without any preceding episodes (Compston and Coles, 2008). This phenotype represents the clinical onset in 80% percent of patients. Nearly 50-60% of patients with CIS will develop MS over a period of 20 years and the percentage increases to 82% in case of abnormalities at magnetic resonance imaging (MRI) (Barkhof *et al.*, 1997; Fisniku *et al.*, 2008). The presence of oligoclonal bands (OCBs) has also been found as an independent predictor of conversion to MS (Tintore *et al.*, 2015; Martinelli *et al.*, 2017). A review of a large database of patients with CIS found that 21% presented with optic neuritis, 46% with long tract symptoms and signs, 10% with a brainstem syndrome, and 23% with multifocal abnormalities (Miller *et al.*, 2005).

RIS is defined by an incidental MRI suggestive of MS in an asymptomatic patient (Okuda *et al.*, 2009). Patients with RIS have a higher risk of developing CIS and MS, but it is still

unclear if this condition represent pre-symptomatic or benign MS, or another disease process not immediately apparent since there is by definition no clinical evidence of demyelinating disease, although brain atrophy, axonal loss, and cognitive dysfunction can be detected in patients with RIS. Around two-thirds of patients show radiological progression and one-third develop neurological symptoms within 5 years (Yamout and Al Khawajah, 2017).

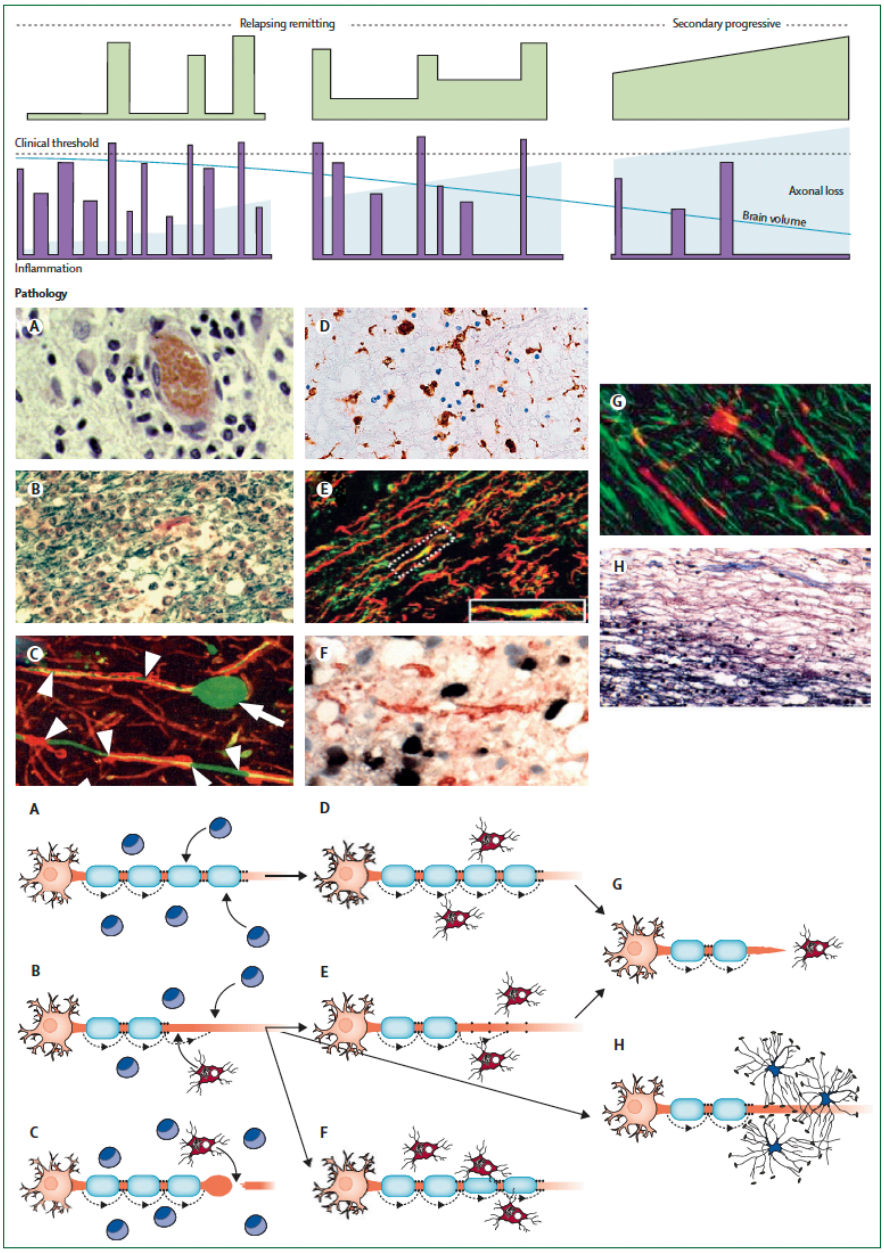


Figure 1.11. *The course and pathogenesis of multiple sclerosis. This scheme illustrates how the pathological processes of inflammation, demyelination, and axon degeneration explain the clinical course of multiple sclerosis.* From Compston A and Coles A, Multiple Sclerosis. Lancet 2008; 372: 1502–17.

In 2012, considering the modest correlation between the 1996 clinical classification and the MRI and biological findings, new descriptors of MS phenotypes were introduced (Lublin *et al.*, 2014). This new classification was based on clinical features and conventional imaging findings, recognising that the degenerative processes begin at the earliest stages of the disease and an on-going inflammatory activity is observed also in progressive clinical phenotypes (Lublin *et al.*, 2014; Sand, 2015). The Committee (i.e., NMSS and European Committee for Treatment and Research in MS and the MS Phenotype Group) identified two main phenotypes: (1) clinically isolated syndrome/relapsing-remitting disease (RR-MS) (***Figure 1.12***) and (2) progressive disease (P-MS) (***Figure 1.13***). The Committee also recognised two descriptors which provide temporal information about the on-going disease processes: (1) activity (defined by relapses and/or MRI activity, including contrast enhancing lesions and new/enlarging lesions); (2) progression (measured by clinical evaluation at least annually) in P-MS only (Lublin *et al.*, 2014).

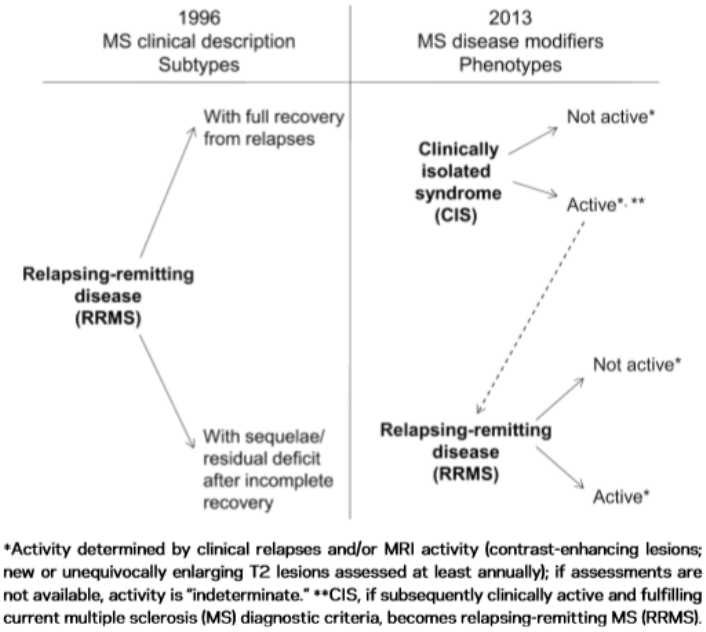


Figure 1.12. *The 1996 vs 2013 multiple sclerosis phenotype descriptions for relapsing disease.* From Lublin FD et al., Defining the Clinical Course of Multiple Sclerosis. The 2013 Revisions. Neurology 2014; 83: 278–286.

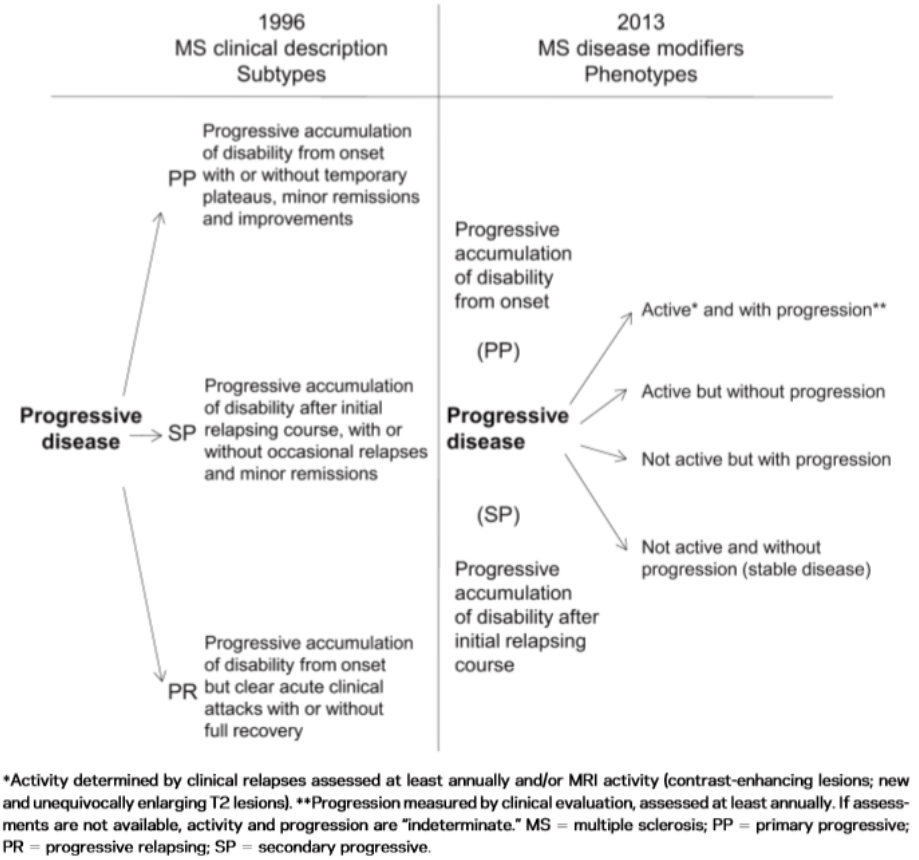


Figure 1.13. *The 1996 vs 2013 multiple sclerosis phenotype descriptions for progressive disease.* From Lublin FD et al., Defining the Clinical Course of Multiple Sclerosis. The 2013 Revisions. Neurology 2014; 83: 278–286.

More recently, histopathological findings and epidemiological studies demonstrated that patients with MS could acquire disability through (a) a step-wise accrual of impairment due to incomplete recovery from a relapse (relapse-associated worsening, RAW); and (2) progression independent of relapse activity (PIRA). This second phenomenon is thought to be the main driven in progressive MS clinical phenotype. This newer evidence, has challenged the clinical distinction between relapsing and progressive forms. Indeed, Kappos et al. observed that 80-90% of overall disability accumulation in RR-MS occurred independently of relapses confirming that MS patients acquire disability through (1) an incomplete recovery from a relapse and (2) progression independent of relapse activity (PIRA) (Kappos *et al.*, 2020). Proceeding from these data, the authors questioned the current phenotypical distinction of relapsing and progressive forms of MS (Lublin *et al.*, 2022) and suggested that MS may be a single disease continuum starting from the CIS

and ultimately evolving into the progressive phase of the disease (P-MS). The clinical manifestations of MS would then be the results of an underlying degenerative process and a highly variable superimposed inflammatory activity (Kappos *et al.*, 2020; Kuhlmann *et al.*, 2022).

1.5. Sex differences in multiple sclerosis

The concept of *sexual differentiation* in the brain has been the subject of much speculation ever since the time of Greek antiquity. However, it was only in 1854 that the German anatomist Emil Huschke described for the first time the morphological differences between male and female brain in the frontal lobe (Swaab and Hofman, 1984).

Several studies have demonstrated that the CNS is a sexual dimorphic organ and, in 2014, Ruigrok et al. described the morphological differences in volume and density observed in women and men (Ruigrok *et al.*, 2014). The embryological development of the brain is influenced by both the XX or XY genotype and the sexual hormones and these factors keep modulating the anatomo-functional characteristics of the CNS during lifetime. Ruigrok et al., studying sex differences in regional and overall brain volumes, hypothesised that biological, environmental, and gene-environment interaction mechanisms associated with sexual differentiation could shape brain development. Finally, the authors suggested that further investigations will be necessary to test whether sex differences in brain structure underlies skewed sex ratios of neurological conditions and whether brain areas affected in such conditions are caused by physiological mechanisms associated with the development of typical sex differences (Ruigrok *et al.*, 2014).

Brain sex differences arise both from the secretion of sex hormones during development, the genes on Y and X chromosomes, the female inactivation of one X chromosome, and the matrilineal mitochondrial genes. In addition, sex differences can emerge throughout life via both genetic and epigenetic mechanisms. All these factors have been shown to make important contributions to brain sexual dysmorphism and to the vast majority of sex differences involving patterns of connectivity and brain regional differences (McEwen and Milner, 2017).

In MS, the disease registered higher prevalence and incidence in the female population (The Multiple Sclerosis International Federation (MSIF), 2020). Histological evidence demonstrated that female brain has a lower percentage of white matter and that the density of oligodendrocytes in corpus callosum, fornix, and spinal cord is 20-40% lower in women when compared to men (Nicot, 2009). Nevertheless, these data could only partially explain the sex differences observed in the disease. The *clinical female phenotype* of MS is characterised by a higher risk of conversion to MS after the first demyelinating episode, a higher annual relapse rate, and overall a more severe inflammatory activity (Dunn *et al.*, 2015; Dunn, Gunde and Lee, 2015; Kalincik, 2015). On contrary, male patients have a greater risk of progression e long term disability (Confavreux, Vukusic and Adeleine, 2003), a datum in line with the epidemiological trend observed also in other neurodegenerative diseases (Voskuhl, Sawalha and Itoh, 2018). This evidence suggests that the female immune response could be more robust that their male counterpart (Nicot, 2009; Dunn *et al.*, 2015; Dunn, Gunde and Lee, 2015; Voskuhl, Sawalha and Itoh, 2018). In fact, several *in vitro* and *in vivo* studies demonstrated that the CD4+ cells are more active in women. This condition seems to be the result of (1) a massive release of immune cells from the thymus due to the loss of androgen immune suppression and (2) a greater proliferation of T cells in women (Dunn *et al.*, 2015; Dunn, Gunde and Lee, 2015). Moreover, women CD4+ T cells would have a higher tendency to produce pro-inflammatory cytokines, while the men would be more prone to differentiate to Th17 and Th2 cell (Dunn *et al.*, 2015; Dunn, Gunde and Lee, 2015). Two main factors would be involved in determining these sexual differences in the activity of the immune system: (1) the sexual chromosomes and (2) the sexual hormones (Voskuhl, Sawalha and Itoh, 2018).

The action of sexual chromosome on the immune system is related to (1) the presence or absence of Y chromosome and (2) the genetic quantitative differences related to the *parental imprinting* of the X chromosome, both factors strongly involved in the regulation of the immune system (Voskuhl, Sawalha and Itoh, 2018).

In pre-clinical studies, the Four Core Genotypes (FCG) model has been used to study sex chromosome effects not confounded by a difference in sex hormones. In FCG mice, the Sry gene, responsible for testis development, has been deleted from the Y chromosome

to produce XY mice with female phenotype and ovaries (De Vries *et al.*, 2002; Arnold and Chen, 2009). The addition of the Sry gene at an autosomal location of XX mice created a XX–Sry+ mice (De Vries *et al.*, 2002; Arnold and Chen, 2009). The models (i.e., XX–Sry+ mice, XX–Sry- mice, XY–Sry+ mice, and XY–Sry- mice) enable the comparison between XX and XY genotypes revealing the effect of sex chromosomes on a given outcome measure in a setting of identical gonadal sex, both female or male (De Vries *et al.*, 2002; Voskuhl, Sawalha and Itoh, 2018).

Creating FCG EAE mice, Smith-Bouvier et al. observed that the XX chromosome complement is disease promoting compared with the XY complement (Smith-Bouvier *et al.*, 2008). Indeed, comparisons between XX–Sry- mice versus XY–Sry- mice and those between XX–Sry+ mice versus XY–Sry+ mice revealed that the Th2 cytokine production is higher in cells derived from XY mice. Th2 cytokines, including IL-13, IL-4, and IL-10, have been previously associated with protection from disease in EAE. Therefore, the increase in Th2 cytokine production in XY mice compared with that in XX mice could underlie the decreased EAE disease severity in XY mice compared to XX mice (Smith-Bouvier *et al.*, 2008). The authors hypothesised that IL-13Rα2, located on the X chromosome, could be modulated by X inactivation. Increased expression of IL-13Rα2 could be caused by an X dosage effect in XX as the molecule acts as a decoy receptor to limit Th2 responses. Alternatively, but less likely, cytokine differences could be the cause of differences in IL-13Rα2 expressions (Smith-Bouvier *et al.*, 2008).

The authors also investigated whether differential parental imprinting of X genes could be the source of sex chromosome effects on autoimmunity. Specifically, Smith-Bouvier et al. evaluated in EAE the parental imprinting on Foxp3+, a gene involved in regulation of myelin protein specific effector T cells. The authors found that CD4+ T cells from maternal Foxp3+ mice demonstrated lower percentage methylation of the Foxp3 enhancer region as compared to those with paternal Foxp3+. Methylation causes gene silencing, so this would lead to more FoxP3 expression in males who express maternal Foxp3 genes when compared to female mice who express maternal Foxp3 genes. This result was consistent with less autoimmunity in males (Smith-Bouvier *et al.*, 2008).

Bone marrow chimeras were widely used to study the *in vivo* effects of sexual chromosomes on neurodegeneration in EAE. The irradiation of mice abolished their immune system, whose reconstitution was then induced using bone marrow from a donor

mouse. Sex chromosome effects on neurodegeneration during EAE can be determined if disease is induced in bone marrow chimeric mice that have an immune system of the same genetic sex, while having a CNS with a different genetic sex (XX or XY). These models were used to investigate the sex chromosome effects on the CNS, not confounded by differences in the immune system. Specifically, the comparison of XX and XY chimeras with male immune system developed worse EAE clinical disease severity with more neuropathology in spinal cord (axonal and myelin loss), cerebellum (Purkinje cell and myelin loss) and cerebral cortex (synaptic loss) (Voskuhl, Sawalha and Itoh, 2018). Toll-like receptor 7 (TLR7) expression in cortical neurons was higher in XY mice compared to XX mice suggesting a direct effect of sex chromosome complement on neurodegeneration in a neurological disease (Voskuhl, Sawalha and Itoh, 2018).

The role of sexual hormones is equally complex and strongly involved in the unbalance of MS epidemiology in men versus women (Dunn *et al.*, 2015; Dunn, Gunde and Lee, 2015). In 2005, Tomassini et al., investigating the relationship between serum follicle stimulating hormone (FSH), luteinising hormone (LH), 17-b oestradiol, testosterone, and dehydroepiandrosterone sulphate (DHEAs) and the radiological characteristic of MS male and female patients. The group demonstrated that women had more inflammatory, but less destructive lesions than men (Tomassini *et al.*, 2005).

It has been recognised that the androgen receptor ligands, testosterone, and its metabolite dihydroxytestosterone (DHT) have suppressive effects on the development of CNS autoimmunity and that sex differences in the levels of these hormones account for the majority of the sex differences in Th immunity (Dunn *et al.*, 2015).

The immunosuppressive role of progesterone has been largely demonstrated, suggesting that the hormone do not contribute to the more robust Th immunity in women (Dunn *et al.*, 2015). On contrary, contradictory findings have been reported on the effect of estradiol in T cell- and B cell-mediated immunity, being this hormone both immunostimulatory and immunosuppressive (Dunn *et al.*, 2015). Finally, the levels of gonadotrophin-releasing hormone (GnRH), FSH, and LH do not differ between post-pubertal male and females and thus are not likely responsible for the sex differences in Th immunity observed in adult men and women (Dunn *et al.*, 2015).

Pregnancy represents a complex hormonal setting for women and its role as major disease modifier in MS has been extensively demonstrated. Pregnancy has a beneficial effect in MS and its protective effect on the disease has been observed in EAE. Immunomodulation of the maternal immune system is known to occur naturally and has long been thought to be protective for the developing foetus (McCombe and Greer, 2013; Voskuhl and Momtazee, 2017). The rate of relapses decreases through pregnancy, mostly during the last trimester and increases 3 months after delivery. Several authors, investigating the changes occurring in MS patients during pregnancy, observed that this trend could be explained by an increase in estrogens and other sex hormones that would be responsible for shifting T-helper cells to Th2; after the delivery, this immunomodulation is reversed determining an increase in relapse rate (Varytė *et al.*, 2020). Other hormones, such as cortisol, also change during pregnancy cooperating to disease modulation (Voskuhl and Momtazee, 2017).

In contrast, evidence on long-term effect of pregnancy on permanent disability accumulation remain inconclusive with approximately equal numbers of studies arguing for either a beneficial or neutral effect (Nguyen *et al.*, 2019). The available literature includes more studies reporting no effect of pregnancy on MS disability than those reporting reduced disability or a delay in progression (Nguyen *et al.*, 2019). However, authors who reported less disability and/or a slower disease progression in parous women, have hypothesised that the repeated exposure to neuroprotective pregnancy changes could have a beneficial effect on long-term outcome (Nguyen *et al.*, 2019; Varytė *et al.*, 2020). Further studies are needed to define the long-term effect of pregnancy in the disease.

In conclusion, the available literature suggests that women are more likely to develop MS as well as other autoimmune disease, because their adaptive immune responses are more robust than in men. Female myelin-reactive Th cells proliferate more and produce higher levels of Th1 cytokines and are better able to support humoral responses than male Th cells (Dunn *et al.*, 2015). The stronger Th1 cell expansion observed in women contributes to the enhanced susceptibility to MS and evidence supports the hypothesis that the sex differences in Th1 immunity are driven by the expression of a number of genes located on sexual chromosomes. In addition, evidence on the more permissive BBB in females has been proposed as another possible factors facilitating the entry of autoreactive lymphocytes in the CNS and the subsequent inflammatory reaction. On the other hand,

there is no strong evidence to date that sex differences in Treg and microglia account for sex differences in MS initiation (Dunn *et al.*, 2015).

1.6. Diagnosis of multiple sclerosis

Diagnostic criteria for MS have deeply evolved over the past 70 years. In mid XX Century, the emerging epidemiological data on MS and the prevalence and incidence of the disease among young adults increased the interest of academic Community on the disease and emphasised the necessity to develop diagnostic criteria.

In 1965, Schumacher et al. elaborated the first diagnostic criteria, which relied mainly on the "clinical definition" of the disease. The diagnosis of "definite multiple sclerosis" was associated to the observation of the following conditions (Schumacher *et al.*, 1965):

- objective abnormalities on neurologic examination attributable to dysfunction of the CNS,
- involvemente of 2 or more separate white matter areas of the CNS (*dissemination in space*, DIS),
- presence of a specific temporal pattern (*dissemination in time*, DIT) with:
    - 2 or more episodes of worsening separated by a period of at least 30 days and lasting at least 24 hours, or
    - a slow or step-wise progression of signs and symptoms over a period of at least 6 months.

Schumacher criteria also introduced the concept of "no better explanation", a fundamental principle in MS diagnosis: the criterion of "no better explanation" stated that, in diagnosing MS, it is necessary to exclude every other neurological disease that could explain clinical symptoms (Schumacher *et al.*, 1965). The criterion was particularly relevant considering the lack of MS pathognomonic biomarkers and the importance of excluding any other neurological disease that would better explain the clinical onset of symptoms (Charil *et al.*, 2006). Finally, Schumacher et al. established that the age of the patients at the onset of the disease must fall within the range of 10 to 50 years (Schumacher *et al.*, 1965).

In 1983, Schumacher criteria were superseded by Poser criteria (Poser *et al.*, 1983). The new diagnostic criteria keep the fundamental concepts of the previous ones, but introduced the use of paraclinical and radiological evidence in diagnosing MS. Indeed, the authors suggested that paraclinical and radiological tests and cerebrospinal fluid (CSF) oligoclonal bands (OCBs) could support the fulfilment of DIS and DIT criteria in diagnosing the disease. According to these new criteria, in patients with 2 clinical attacks who did not fulfil the DIS criterion, the diagnosis of "clinically definite MS" could be supported by paraclinical evidence of another separate lesion. Paraclinical CNS lesions may be elicited by evoked potentials, Computer Tomography (CT) scan, MRI scan, and/or special urological studies. A diagnosis of "clinically probable MS" was done (a) in presence of 2 attacks and clinical evidence of one lesion; or (b) 1 attack and clinical evidence of 2 separate lesions; or (c) 1 attack, clinical evidence of 1 lesion, and paraclinical evidence of another, separate lesion. Finally, the entity of "laboratory-supported definite MS" was described by (a) 2 attacks, clinical or paraclinical evidence of 1 lesion, and CSF OCBs; or (b) 1 attack, clinical evidence of 2 separate lesions, and CSF OCBs; or (3) 1 attack, clinical evidence of 1 lesion, paraclinical evidence of another, separate lesion, and CSF OCBs (Poser *et al.*, 1983).

Over the last 40 years, the introduction of MRI in clinical practice supported the application of this new technique in diagnosing MS. Therefore, in 2001, the International Panel on the Diagnosis of MS recommended the use of updated diagnostic criteria (McDonald *et al.*, 2001). The new criteria established that radiological and laboratory data could support the diagnosis of MS demonstrating the DIS and DIT. These criteria have been largely used for both clinical and research purposes and three following revisions have been reassessed to increase their sensitivity and specificity (Polman et al. 2005; Polman et al. 2011; Thompson et al. 2018).

The last revision of McDonald criteria (***Figure 1.14*** and ***Figure 1.15***) stated that MS could be diagnosed if the patient had at least 1 clinical attack (CIS) and fulfilled the criteria of DIS and DIT (***Figure 1.16***) and if other neurological diseases are excluded (Thompson *et al.*, 2018). The International Panel on Diagnosis of MS highlighted the relevance of the "no better explanation" concept in view of the heterogeneity of disease clinical manifestations and the lack of clinical, paraclinical, or MRI pathognomonic signs.

To-date, an established diagnostic work-up to rule out MS diagnosis is not available, but the International Panel on Diagnosis of MS developed guidelines for MS differential diagnosis and defined clinical, paraclinical, and MRI *red flags* suggestive of an alternative diagnosis (Miller *et al.*, 2008).

In 2017 revised criteria, DIS can be demonstrated by at least 1 hyperintense lesions at T2-weighted (T2w) MRI scan localised in at least 2 out of 4 areas of the CNS considered typical for MS: periventricular, cortical or juxtacortical, and infratentorial brain regions, and the spinal cord. DIT can be demonstrated by the simultaneous presence of gadolinium-enhancing and non-enhancing lesions at any time or by a new T2w hyperintense or gadolinium-enhancing lesion on follow-up MRI, with reference to a baseline scan, irrespective of the timing of the baseline MRI (Thompson *et al.*, 2018). The revision enables the inclusion of symptomatic lesions in the MRI determination of DIS or DIT and recognise the role of CSF OCBs as an independent predictor of the risk of a second MS attack and marker of DIT (Thompson *et al.*, 2018).

	Number of lesions with objective clinical evidence	Additional data needed for a diagnosis of multiple sclerosis
≥2 clinical attacks	≥2	None*
≥2 clinical attacks	1 (as well as clear-cut historical evidence of a previous attack involving a lesion in a distinct anatomical location†)	None*
≥2 clinical attacks	1	Dissemination in space demonstrated by an additional clinical attack implicating a different CNS site or by MRI‡
1 clinical attack	≥2	Dissemination in time demonstrated by an additional clinical attack or by MRI§ OR demonstration of CSF-specific oligoclonal bands¶
1 clinical attack	1	Dissemination in space demonstrated by an additional clinical attack implicating a different CNS site or by MRI‡ AND Dissemination in time demonstrated by an additional clinical attack or by MRI§ OR demonstration of CSF-specific oligoclonal bands¶

If the 2017 McDonald Criteria are fulfilled and there is no better explanation for the clinical presentation, the diagnosis is multiple sclerosis. If multiple sclerosis is suspected by virtue of a clinically isolated syndrome but the 2017 McDonald Criteria are not completely met, the diagnosis is possible multiple sclerosis. If another diagnosis arises during the evaluation that better explains the clinical presentation, the diagnosis is not multiple sclerosis. An attack is defined in panel 1. *No additional tests are required to demonstrate dissemination in space and time. However, unless MRI is not possible, brain MRI should be obtained in all patients in whom the diagnosis of multiple sclerosis is being considered. In addition, spinal cord MRI or CSF examination should be considered in patients with insufficient clinical and MRI evidence supporting multiple sclerosis, with a presentation other than a typical clinically isolated syndrome, or with atypical features. If imaging or other tests (eg, CSF) are undertaken and are negative, caution needs to be taken before making a diagnosis of multiple sclerosis, and alternative diagnoses should be considered. †Clinical diagnosis based on objective clinical findings for two attacks is most secure. Reasonable historical evidence for one past attack, in the absence of documented objective neurological findings, can include historical events with symptoms and evolution characteristic for a previous inflammatory demyelinating attack; at least one attack, however, must be supported by objective findings. In the absence of residual objective evidence, caution is needed. ‡The MRI criteria for dissemination in space are described in panel 5. §The MRI criteria for dissemination in time are described in panel 5. ¶The presence of CSF-specific oligoclonal bands does not demonstrate dissemination in time per se but can substitute for the requirement for demonstration of this measure.

Table: **The 2017 McDonald criteria for diagnosis of multiple sclerosis in patients with an attack at onset**

Figure 1.14. *The 2017 McDonald criteria for diagnosis of multiple sclerosis in patients with an attack at onset.* From Thompson AJ et al., Diagnosis of Multiple Sclerosis: 2017 Revisions of the McDonald Criteria. Lancet Neurol 2018; 17: 162-173

- In a patient with a typical clinically isolated syndrome and fulfilment of clinical or MRI criteria for dissemination in space and no better explanation for the clinical presentation, **demonstration of CSF-specific OCBs in the absence of other CSF findings atypical of MS allows a diagnosis of this disease to be made**. This recommendation is an addition to the 2010 McDonald criteria.

- **Symptomatic and asymptomatic MRI lesions can be considered in the determination of dissemination in space or time**. MRI lesions in the optic nerve in a patient presenting with optic neuritis remain an exception and, owing to insufficient evidence, cannot be used in fulfilling the McDonald criteria. In the 2010 McDonald criteria, the symptomatic lesion in a patient presenting with brainstem or spinal cord syndrome could not be included as MRI evidence of dissemination in space or time.

- **Cortical and juxtacortical lesions can be used in fulfilling MRI criteria for dissemination in space.** Cortical lesions could not be used in fulfilling MRI criteria for dissemination in space in the 2010 McDonald criteria.

- **The diagnostic criteria for primary progressive MS in the 2017 McDonald criteria remain the same** as those outlined in the 2010 McDonald criteria, aside from removal of the distinction between symptomatic and asymptomatic MRI lesions and that cortical lesions can be used.

- **At the time of diagnosis, a provisional disease course should be specified (relapsing-remitting, primary progressive, or secondary progressive) and whether the course is active or not, and progressive or not based on the previous year's history.** The phenotype should be periodically re-evaluated based on accumulated information. This recommendation is an addition to the 2010 McDonald criteria.

Figure 1.15. *Main changes in 2017 revisions to the McDonald diagnostic criteria for multiple sclerosis.* From Thompson AJ et al., Diagnosis of Multiple Sclerosis: 2017 Revisions of the McDonald Criteria. Lancet Neurol 2018; 17: 162-173

Panel 5: 2017 McDonald criteria for demonstration of dissemination in space and time by MRI in a patient with a clinically isolated syndrome

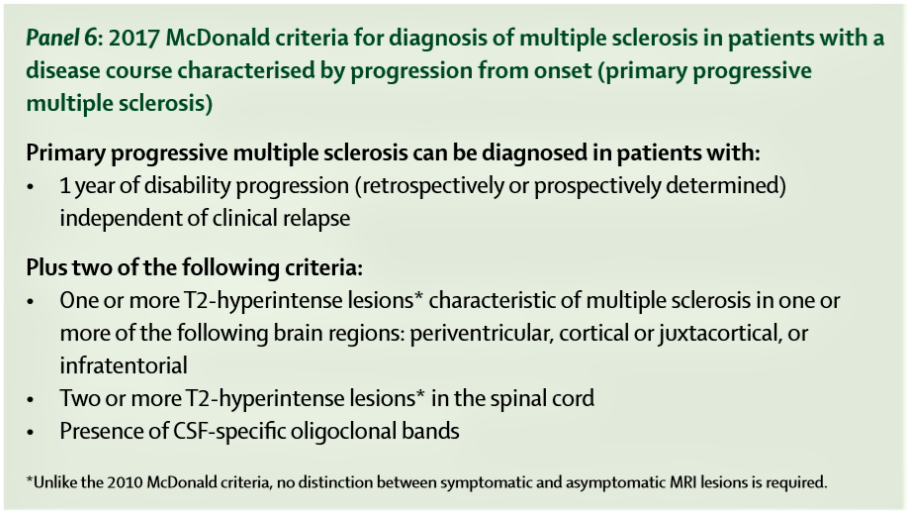
- Dissemination in space can be demonstrated by one or more T2-hyperintense lesions* that are characteristic of multiple sclerosis in two or more of four areas of the CNS: periventricular,† cortical or juxtacortical, and infratentorial brain regions, and the spinal cord
- Dissemination in time can be demonstrated by the simultaneous presence of gadolinium-enhancing and non-enhancing lesions* at any time or by a new T2-hyperintense or gadolinium-enhancing lesion on follow-up MRI, with reference to a baseline scan, irrespective of the timing of the baseline MRI

*Unlike the 2010 McDonald criteria, no distinction between symptomatic and asymptomatic MRI lesions is required. †For some patients—eg, individuals older than 50 years or those with vascular risk factors—it might be prudent for the clinician to seek a higher number of periventricular lesions.

Figure 1.16. *2017 McDonald criteria for demonstration of dissemination in space and time by MRI in a patient with a clinically isolated syndrome.* From Thompson AJ et al., Diagnosis of Multiple Sclerosis: 2017 Revisions of the McDonald Criteria. Lancet Neurol 2018; 17: 162-173

The diagnostic criteria for PP-MS remain the same in the 2017 McDonald criteria as those outlined in the 2010 criteria. However, also for the diagnosis of this phenotype, the Panel

eliminated the distinction between symptomatic and asymptomatic MRI lesions and allowed the use of cortical lesions (***Figure 1.17***) (Thompson *et al.*, 2018).



***Panel 6*: 2017 McDonald criteria for diagnosis of multiple sclerosis in patients with a disease course characterised by progression from onset (primary progressive multiple sclerosis)**

Primary progressive multiple sclerosis can be diagnosed in patients with:

- 1 year of disability progression (retrospectively or prospectively determined) independent of clinical relapse

Plus two of the following criteria:

- One or more T2-hyperintense lesions* characteristic of multiple sclerosis in one or more of the following brain regions: periventricular, cortical or juxtacortical, or infratentorial
- Two or more T2-hyperintense lesions* in the spinal cord
- Presence of CSF-specific oligoclonal bands

*Unlike the 2010 McDonald criteria, no distinction between symptomatic and asymptomatic MRI lesions is required.

Figure 1.17. *2017 McDonald criteria for diagnosis of multiple sclerosis in patients with a disease course characterised by progression from onset (primary progressive multiple sclerosis).* From Thompson AJ et al., Diagnosis of Multiple Sclerosis: 2017 Revisions of the McDonald Criteria. Lancet Neurol 2018; 17: 162-173

Finally, the Panel recognised that, although largely based on data from adult, white European and North American populations with a typical CIS, and age younger than 50 years, epidemiological evidence supported the applicability of the 2017 McDonald criteria in other populations, including African American, Asian, Latin American, and paediatric patients (Thompson *et al.*, 2018).

1.7. The use of magnetic resonance imaging and optic coherence tomography in multiple sclerosis

Accumulating data demonstrated the limited effectiveness of clinical measures in evaluating MS for both clinical and research purposes (Kuhlmann *et al.*, 2022). Indeed, the clinical definition of MS phenotype has been demonstrated to be inaccurate for evaluating the complex pathological processes underlying the disease. Moreover, the Kurtzke Expanded Disability Status Scale (EDSS), largely used in clinical practice and

research studies, is now recognised as an inadequate measure of disease activity and progression. It has become evident that the clinical evaluation should be integrated with non-clinical biomarkers (Kantarci, 2019). Among these, quantitative MRI has demonstrated to be an objective, sensitive, and specific tool for measuring outcomes and monitoring patients' evolution over time (McFarland *et al.*, 2002). MRI is an essential instrument to diagnose MS early, monitor disease progression, and evaluate response to treatments (Inglese and Petracca, 2018). The improvements in MRI techniques have enabled the *in vivo* evaluation of inflammatory and neurodegenerative mechanisms, making it possible to better investigate the interaction between these processes in the disease (Sastre-Garriga *et al.*, 2020a).

The developments of conventional MRI sequences provided sensitive and reproducible tools for assessing lesion load and brain and spinal cord volumes, offering reliable indicators of disease evolution (De Stefano, Battaglini and Smith, 2007). Brain lesion load changes are considered crucial to assess focal disease activity in MS diagnosis, monitoring, and prognosis (Wattjes *et al.*, 2021); short-term changes in brain and cervical spinal cord volumes have been established as indicators of disability and predictors of long-term clinical evolution (Sastre-Garriga *et al.*, 2020a). In 2021, the joint Panel of Magnetic Resonance Imaging in Multiple Sclerosis (MAGNIMS), Consortium of Multiple Sclerosis Centres (CMSC), and North American Imaging in Multiple Sclerosis Cooperative (NAIMS) recommended the use of the following MRI protocol for MS diagnosis and monitoring (Wattjes *et al.*, 2021):

- standardised brain protocol:
    - axial T2w sequences,
    - axial and sagittal T2-weighted 3D-fluid-attenuated inversion recovery (T2-FLAIR) sequences,
    - high-resolution T1-weighted (T1w) sequences (optional),
    - T1w sequences after gadolinium only at MS diagnosis;
- standardised spinal cord protocol:
    - sagittal T2w sequences (TSE or FSE), and/or proton density-weighted sequences (TSE or FSE), and/or short tau inversion recovery (STIR) sequences (at least two different sequences)
    - sagittal T1w sequences after gadolinium only at MS diagnosis.

Despite the improvement in MS management associated to the introduction of MRI techniques, several studies have demonstrated that conventional sequences have a modest correlation with clinical disability (Filippi, Absinta and Rocca, 2013; Inglese and Petracca, 2018). This limitation has triggered the development of advanced techniques to narrow the gap between pathology and imaging and provide specific descriptors closely associated with disability (Filippi, Absinta and Rocca, 2013; Absinta, Sati and Reich, 2016; Inglese and Petracca, 2018).

Several studies have established T1w/T2w (T1/T2 ratio) as a measure of microstructural integrity, indicating both damage and recovery processes (Boaventura *et al.*, 2022; Hannoun *et al.*, 2022; Mühlau, 2022). Due to the possibility to obtain T1/T2 ratio maps from MRI sequences that are routinely acquired in clinical practice and clinical trials, this semiquantitative measure has been increasingly used over the last years to investigate both the cerebral cortex and white matter areas, although with inconsistent results (Boaventura *et al.*, 2022; Hannoun *et al.*, 2022; Mühlau, 2022). However, because of correlations observed between T1/T2 ratio maps and clinical outcomes (Boaventura *et al.*, 2022; Hannoun *et al.*, 2022; Mühlau, 2022), it is interesting to calculate this ratio in MS.

Advanced imaging techniques have allowed to detect in vivo pathological abnormalities which were described by neuropathological studies, including cortical lesions. Cortical lesions are defined as well-demarcated, focal abnormalities entirely or partly located in the cortical grey matter (Filippi *et al.*, 2019), which differ from white matter lesions in several quantitative and qualitative aspects of cellular inflammation (Calabrese, Filippi and Gallo, 2010). The development of brain double inversion recovery (DIR), phase-sensitive inversion recovery (PSIR), and T1w Magnetization Prepared–Rapid Gradient Echo (MPRAGE) sequences have enabled the detection of these lesions, which are associated with physical and cognitive disability progression (Calabrese *et al.*, 2012).

Two relatively novel advanced imaging markers are the central vein sign (CVS) and paramagnetic rim lesions (PRLs). Pathological studies have described the presence of central vessels in MS plaques. The development of susceptibility-based imaging (SWI) has enabled the observation of the central vein in MS plaques (Sati *et al.*, 2016). Following the emerging of evidence that CVS can accurately differentiate MS from other demyelinating diseases of the CNS (Cortese *et al.*, 2018), the MRI-detectable CVS has been proposed as a biomarker of inflammatory demyelination. The presence of slow

expanding lesions with a paramagnetic rim (PRLs) is now recognise as a sign of progressive tissue injury (Dal-Bianco *et al.*, 2017). The PRLs are a subset of chronic active MS lesions. The plaques are characterised by an on-going tissue damage associated with the accumulation of microglia and/or macrophages at the lesion edge, together with failure of repair and/or remyelination and subsequent axonal loss (Absinta *et al.*, 2019). The PRLs have been identified in MS patients independently of clinical phenotype and disease-modifying therapies (DMTs), are more prevalent in patients with more severe disease, and have been proposed as a negative prognostic marker in MS (Absinta *et al.*, 2019). The development of more advanced T2w or T2-FLAIR MRI modalities have enabled the identification of these abnormalities as "lesions characterised by a hypointense rim on phase images and internal isointensity to extralesional white matter" (Lou *et al.*, 2021). These PRLs have been reported to yielded high specificity in differentiating MS from non-MS demyelinating diseases of the CNS (Maggi *et al.*, 2020). Overall, the integration of conventional and non-conventional MRI biomarkers have been established as a promising approach to investigate *in vivo* the heterogeneous pathological substrates of MS (Filippi, Absinta and Rocca, 2013; Absinta, Sati and Reich, 2016).

Optical coherence tomography (OCT) is a non-invasive technique that enables the investigation of the optic nerve. The technique is based on the principle of echo time delay of back-scattered infrared light and uses an interferometer in conjunction with a low-coherence light source (Frohman *et al.*, 2006). OCT has a good anatomical resolution that allows the differentiation of the major retinal layers and their analyses for tissue thickness and volume.

During the past decade, OCT has been largely used to investigate neurodegeneration in MS. In fact, clinical disability scales for assessing visual acuity have several limitations and do not fully capture the range of disability seen in the disease. OCT has been proposed as a useful surrogate marker considering the robustness of the association between retinal nerve fibre layer (RNFL) loss and global clinical disability scales (Petzold *et al.*, 2010). In addition, the use of OCT parameters as markers of neuroaxonal degeneration has been supported by Martinez-Lapiscina and colleagues, who reported that a reduction in RNFL thickness was associated to a higher risk of developing disability (Martinez-Lapiscina *et al.*, 2016).

OCT has also been proposed as a complementing investigation to electrophysiological techniques in assessing visual functions. Indeed, authors demonstrated an association between RNFL loss and a decrease in the amplitude of visual evoked potentials (VEP) and electroretinogram (ERG) (Petzold *et al.*, 2010). Finally, published data suggested a correlation between the structural OCT changes in RNFL thickness and measures of brain atrophy on MRI (Petzold *et al.*, 2010).

More recently, mounting literature have reported a reduction in ganglion cell-inner plexiform layer (GCIPL) thickness in eyes with a history of optic neuritis. The thinning has been attributed to retrograde transsynaptic neurodegeneration following optic neuritis damage. Furthermore, authors reported a thinning of the GCIPL in the MS patient eyes without a history of optic neuritis and without eye involvement. Indeed, a recent study by Sabaner et al. observed that RNFL and GCIPL thickness was non-significantly lower in MS eyes with previous optic neuritis when compared to those without previous episodes, suggesting that the damage in the optic nerve could be affected both by inflammatory and neurodegenerative mechanisms (Sabaner *et al.*, 2020).

In summary, the available literature suggests that OCT is valuable instrument for monitoring the disease and that this technique could provide additional information to clinical and MRI data.

In conclusion, several mechanisms, varying across and within individual patients, contribute to inflammation and neurodegeneration. It is now clear that these phenomena cannot be completely detected by clinical evaluation. The introduction of MRI and OCT in MS clinical practice and research have paved the way to shift from clinically-based to biologically-based definitions of MS, a milestone in improving the management of patient with MS.

1.8. Treatment of multiple sclerosis

Over the last 30 years, the development of a range of treatments that are able to modifying the clinical course of the disease (*disease-modifying treatments*, DMTs) had deeply changed the management of MS reducing the occurrence of relapses and radiological lesions and slowing down the accrual of brain and spine atrophy and clinical disability.

In 1993, Paty et al. demonstrated the efficacy of interferon-beta 1b (IFN-beta 1b) in decreasing disease activity and reducing disease burden (Paty and Li, 1993). This first finding triggered 25 years of intense therapeutic development that has resulted in multiple drugs that can substantially reduce the impact of MS on the lives of many patients (***Figure 1.18***) (Tintoré, Vidal-Jordana and Sastre-Garriga, 2019).

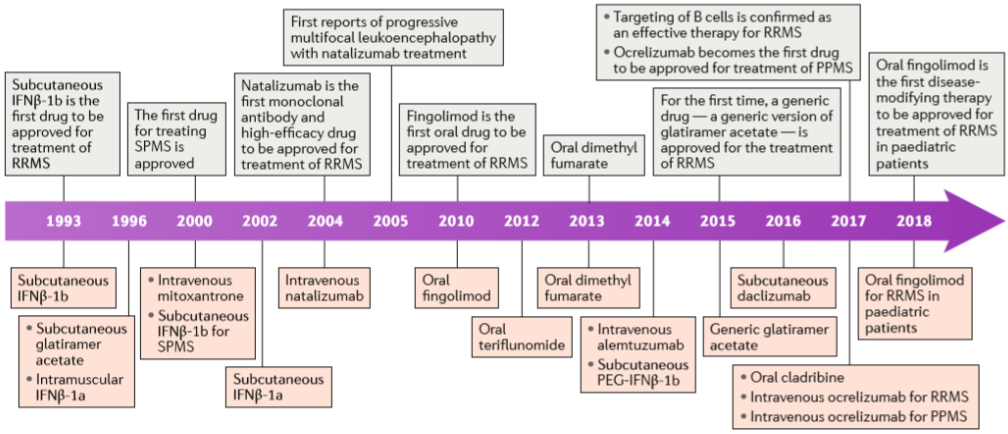


Figure 1.18. *Timeline of developments in the treatment of multiple sclerosis. Treatment are reported in the orange boxes, while important milestones in the development are shown in green.* From Tintoré M et al., Treatment of Multiple Sclerosis – Success from Bench to Bedside. Nat Rev Neurol 2019; 15(1): 53-58.

DMTs had immunomodulatory or immunosuppressive effect and reduce the activation of the immune system blocking the inflammatory processes underlying the appearance of new lesions and the development of new symptoms (***Figure 1.19***) (Garg and Smith, 2015; Martin *et al.*, 2016; Tintoré, Vidal-Jordana and Sastre-Garriga, 2019).

Injectables therapies, including IFN-beta 1b, IFN-beta 1a, and glatiramer acetate were the first type of DMTs developed for the treatment of RR-MS. The three immunomodulatory therapies had similar efficacy in reducing disease activity as measured by assessing new relapses and/or MRI lesions and have a similar safety profile (Goldschmidt and Hua, 2020). Despite the limited efficacy of these therapies, they contributed to change the attitudes towards MS revealing that the natural history of the disease could be modified with treatment (Tintoré, Vidal-Jordana and Sastre-Garriga, 2019).

The first monoclonal antibody developed for MS was natalizumab, a humanized monoclonal antibody that binds to α4 integrin, a component of very late antigen 4 (VLA4), which is present on lymphocytes. Natalizumab prevents the interaction between VLA4 and its endothelial ligand vascular cell adhesion molecule precluding lymphocytes to cross the BBB. The drug has 70% efficacy in reducing annual relapse rate (ARR) and 40% in decreasing the accrual of disability (Tintoré, Vidal-Jordana and Sastre-Garriga, 2019). However, natalizumab is associated to a higher risk of developing natalizumab-associated progressive multifocal leukoencephalopathy (PML), a serious and potentially fatal opportunistic infection of the CNS that could be developed in patients positive for John Cunningam virus (JCV) (Berger, Houff and Major, 2009).

Fingolimod was the first oral drug introduced in the treatment of MS. The therapy prevents T cells from leaving the secondary lymph organs blocking the S1P receptor-1 (S1PR1). The subsequent decrease in the number of circulating lymphocytes reduces the ARR of 50% and the risk of clinical worsening of one-third. Safety concerns in using fingolimod are associated with the reduction of heart rate at the first dose administration and the severe decrease in circulating lymphocytes. More recently, other three modulator of S1PR1, siponimod, ozanimod, and ponesimod, have been approved for treatment of MS. While fingolimod has broad receptor affinity, the other three S1PR modulators are more specific and present therefore a reduced risk of adverse events. In particular, siponimod and ozanimod are selective modulators of S1PR1 and S1PR5, while ponesimod is specific for S1PR1 (Cohan *et al.*, 2020; McGinley and Cohen, 2021). Ponesimod and ozanimod, as fingolimod, have been approved for RR-MS, while Siponimod is currently the only approved treatment for SP-MS with inflammatory activity having demonstrated its efficacy in reducing the risk of disability progression by 21% (Kappos *et al.*, 2018).

After the approval of Fingolimod, two new oral drugs have been developed, including dimethyl-fumarate and teriflunomide, both immunomodulatory drugs that have demonstrated a reduction of 30% in the occurrence of new relapses and ARR. Teriflunomide is primarily an inhibitor of dihydroorotate-dehydrogenase, a key mitochondrial enzyme involved in the *de novo* synthesis of pyrimidines in rapidly proliferating cells (Oh and O'connor, 2013). Dimethyl-fumarate has immunomodulatory

and cytoprotective functions modulating the activation of nuclear factor erythroid 2-related factor 2 (Nrf-2) and the subsequent inflammatory cascade (Tintoré, Vidal-Jordana and Sastre-Garriga, 2019). Both drugs reported limited adverse events in the short and long term (Tintoré, Vidal-Jordana and Sastre-Garriga, 2019).

More recently, Cladribine has join the list of oral treatment available for MS. The drug is administered orally in 4 short-duration courses over 2 years. After being converted to an active purine nucleoside analogue, cladribine leads to the apoptosis of lymphocytes inducing a re-programming of the cellular immune system. This treatment is considered a high efficacy therapy having showed in clinical trial a reduction in ARR of 55% and a slowing in disability progression of 30% (Tintoré, Vidal-Jordana and Sastre-Garriga, 2019).

Following the success of natalizumab, further monoclonal antibodies were developed for the treatment of RR-MS. Alemtuzumab is a humanized monoclonal antibody against CD52, a receptor that is present on lymphocytes, monocytes, and other immune and non-immune cells; the drug is administered for 5 consecutive days on year one and for 3 consecutive days on year 2. Alemtuzumab reduced the ARR by 55% and decrease the risk of confirmed disability worsening by 40%. However, the risk of developing severe events in the years following the infusions had limited the use of this drug.
CD20-binding antibodies rituximab, ocrelizumab, and ofatumumab have been demonstrated as strong agent in reducing the ARR by 45% and the disability progression by 40%. Moreover, ocrelizumab is able to slow down brain volume loss and disability progression and it is the first and only drug approved for PP-MS with inflammatory activity (Tintoré, Vidal-Jordana and Sastre-Garriga, 2019).

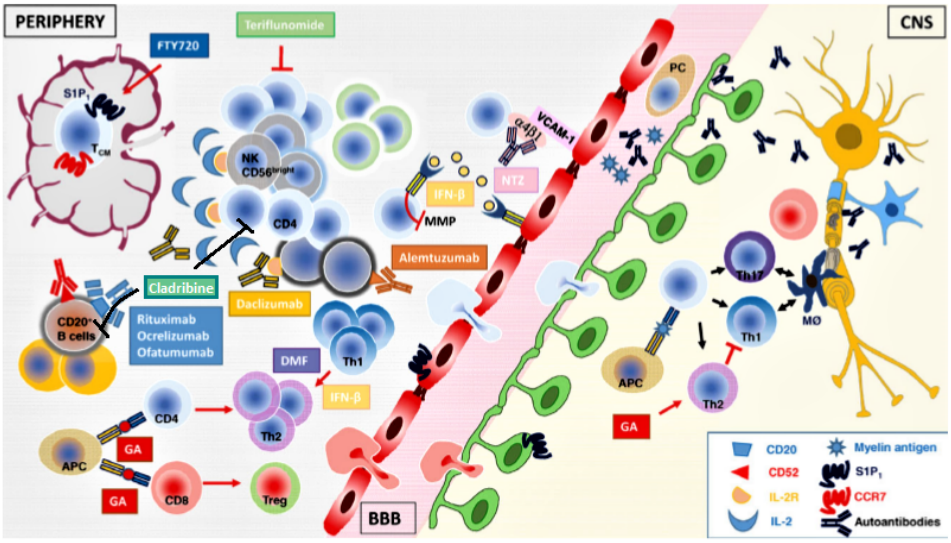


Figure 1.19. *Schematic representation of the putative cellular and molecular therapeutic targets used in the multiple sclerosis treatment.* ***On the left****, cellular and molecular targets in the periphery (lymph nodes, bone marrow, and the respective immune cells).* ***At centre****, the endothelial blood-brain barrier (red endothelial cells) at the postcapillary venule level with a perivascular space and the glia limitans including astrocytes (green).* ***On the left****, inflamed central nervous system parenchyma with a neuron (yellow) and its myelinated axon and the oligodendrocyte (blue) is shown, in addition to infiltrating immune cells (APC, Th1, Th2, Th17, and macrophages). Immune cell interactions are shown with two-headed black arrows, red arrows indicate therapeutic targets and T-shaped lines (red) indicate blocking of pathways. Cells depleted by monoclonal antibodies are indicated with the gray shade around the respective cells target. aHSCT: autologous HSCS transplantation; FTY720: fingolimod; NTZ: natalizumab; DMF: dimethyl-fumarate; GA: glatiramer acetate; BBB: blood–brain barrier; PC: plasma cell; M Ø: macrophage.* From Martin R et al., Current Multiple Sclerosis Treatments Have Improved Our Understanding of MS Autoimmune Pathogenesis. Eur J Immunol 2016; 46(9): 2078–2090

The development of many effective drugs has deeply modified the MS scenario, challenging clinical neurologist in treating patients. The availability of effective and specific drugs has changed the treatment paradigm moving to personalised precision medicine (Tintoré, Vidal-Jordana and Sastre-Garriga, 2019). The last EAN/ECTRIMS guidelines on pharmacological treatment of MS recommended the starting of a DMT in both CIS and RR-MS patients. On contrary, in P-MS a treatment is indicated only if there is evidence of disease inflammatory activity and the treatment of neurodegeneration mostly remain an unmet need in the disease (Montalban *et al.*, 2018).

Overall, pharmacological research has made several steps forward over the last 25 years. Nonetheless, MS is still a complex and heterogeneous disease and still long is the road to a complete personalised treatment. To reach this overarching goal a better understanding of the pathological mechanisms underlying the disease, the introduction of more accurate

tools to evaluate these biological processes *in vivo*, and the development of more specific treatment that can contrast both the inflammation and neurodegeneration are necessary. These goals will be the challenges of tomorrow for neurologists.

CHAPTER II

Feto-maternal microchimerism: an exchange of cells

1.1. From a trans-placenta cellular trafficking to the development of a microchimeric status

The term *microchimerism* first appeared in a peer-reviewed paper in 1977, when Liégeois et al. described the presence and proliferation of allogeneic bone marrow cells in laboratory mice (Liégeois *et al.*, 1977). Indeed, the authors observed that a small proportion of donor cells could long-term survive in the host (Liégeois *et al.*, 1977). In 1979, Herzenberg et al. identified XY fetal cells in maternal blood samples using fluorescence-activated cell sorter (FACS) (Herzenberg *et al.*, 1979). The authors detected HLA-A2-positive cells in HLA-A2-negative women blood and found that these cells, containing Y-chromatin, could enter maternal circulation during the first 15-27 weeks of pregnancy (Herzenberg *et al.*, 1979). Finally, in 1981, the group of Liégeois confirmed the presence of allogeneic fetal cells in maternal tissue during and long after pregnancy (Liégeois *et al.*, 1981).

Over the following 40 years, several studies have largely confirmed the existence of a trans-placenta, bi-directional, asymmetric trafficking of fetal and maternal cells (Lo *et al.*, 2000; Bakkour *et al.*, 2014; Boddy *et al.*, 2015). This cellular exchange has been demonstrated to start during the first weeks of pregnancy (Bianchi *et al.*, 1996) and fetal microchimeric cells (fMCs) were also found in women who reported early abortion (Nelson, 2012). Using DNA extraction and real-time Polymerase Chain Reaction (real-time PCR) amplification, Vernochet and colleagues observed that fMCs and maternal microchimeric cells (mMCs) could be detected in the placenta as early as 10 days post-coitum and their number increases as gestation progressed (Vernochet, Caucheteux and Kanellopoulos-Langevin, 2007). The author suggested that this trend could be due (1) to

an enhanced cell migration during the second half of gestation or (2) to an accumulation and/or proliferation of microchimeric cells not eliminated from the placenta. In the same study, the authors reported that maternal cells were found able to migrate to the foetus via blood circulation and colonise offspring's organs. The group speculated that, being the placenta a niche for hematopoietic stem cells, maternal cells may settle in this favourable environment (Vernochet, Caucheteux and Kanellopoulos-Langevin, 2007). Similarly, Fujiki et al. observed that the number of fMCs increases with advancing gestational age (Fujiki *et al.*, 2008) and Adams Waldorf et al. reported that the frequency of fMCs in peripheral blood mononuclear cells (PBMC) was 0% pre-pregnancy, 13% in the first trimester, 4% in the second trimester, 15% in the third trimester, and 20% after delivery, measuring a 6.2-fold increasing trend per trimester (Adams Waldorf *et al.*, 2010). The same group reported a similar trend when they analysed CD4+ and CD8+ lymphocytes, confirming that the maternal acquisition of fetal cells may be greater close to delivery (Adams Waldorf *et al.*, 2010). This temporal relationship strongly supported the hypothesis that the presence of fMCs and mMCs is mostly pregnancy-related.

Further investigation suggested that the microchimeric cells entering the host circulation during the different phases of pregnancy could potentially engraft into bone marrow and provide a renewing source of microchimeric cells for decades after delivery (O'Donoghue, 2011). Indeed, more recent studies demonstrated that fMCs and mMCs migrate to various tissues both in the mother and offspring. In 2003, Srivatsa et al. reported the presence of female nucleated cells in male newborn tissues and speculated that these female cells were most likely to be maternal in origin (Srivatsa *et al.*, 2003) (***Figure 2.1***). Similarly, several studies revealed the presence of fMCs not only in hematopoietic organs, such as spleen, liver, and blood, but also in lung, skin, thyroid gland, adrenal gland, kidney, and heart (Srivatsa *et al.*, 2003; Bayes-Genis *et al.*, 2005; Guettier *et al.*, 2005; Fujiki *et al.*, 2008; Sunami *et al.*, 2010). These results supported the hypothesis that fetal cells may migrate from the peripheral circulation into multiple organs colonising different tissues (***Figure 2.2*** and ***Figure 2.3***).

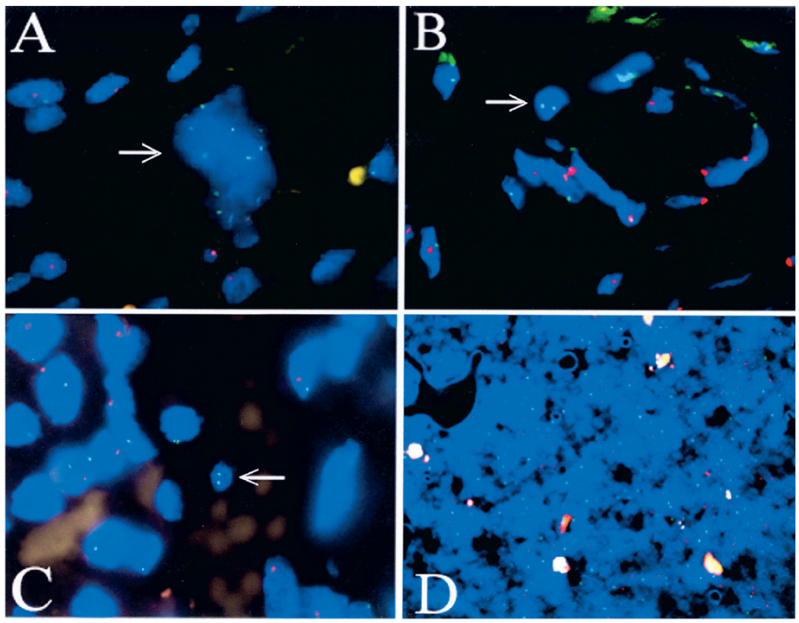
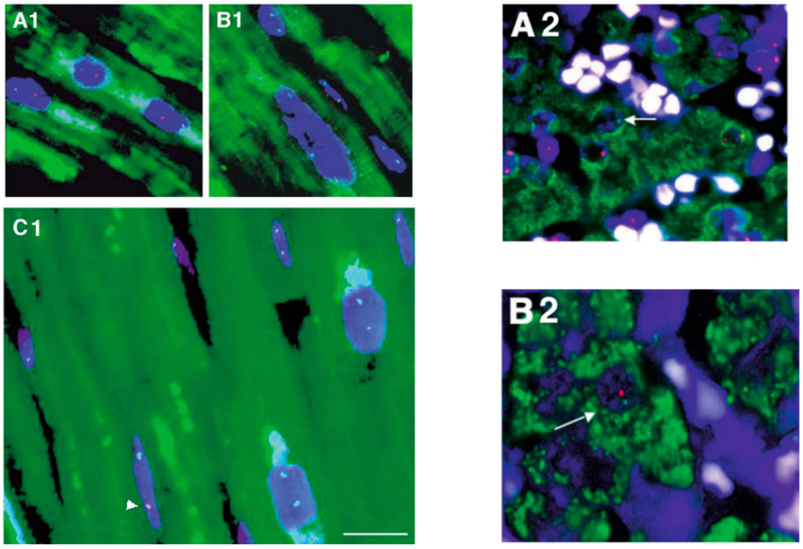
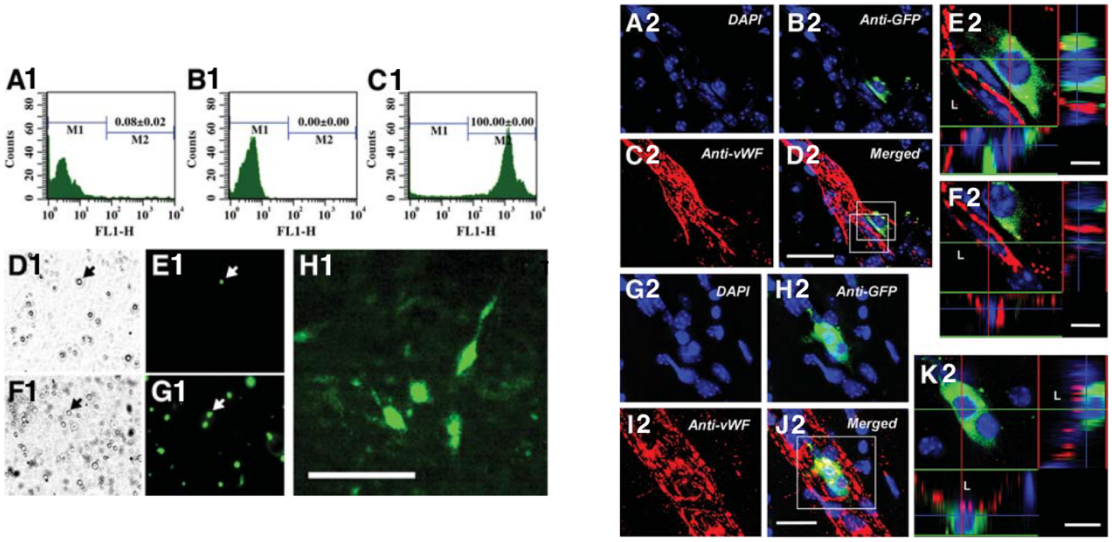


Figure 2.1. *In the figures, nuclei are stained blue: X and Y chromosomes are stained green and red, respectively. Cluster of cells containing only green intranuclear signals (XX cells) were detected in sections of thymus (A), skin (B), and liver (C) from male subject. In the section of thymus (D) the dense number of cells precluded reliable discrimination between female and male cells.* From B Srivata et al., Maternal Cell Microchimerism in Newborn Tissues. J Pediatr 2003; 142(1): 31–35.



Figures 2.2 and 2.3. ***On the left,*** *FISH technique in myocardium from male and female controls. (A) Nuclear X and Y chromosomes in male tissue. (B) Nuclear 2 X-chromosomes in female tissue. The red nuclear signal indicates the Y chromosome and the green signal the X chromosome.*. From B Srivata et al., Maternal Cell Microchimerism in Newborn Tissues. J Pediatr 2003; 142(1): 31–35). ***On the right,*** *liver from a female subject. (A2) Combined FISH with biotin-labeled X probe and digoxigenin-labeled Y probe and immunohistochemistry with anti-hepatocyte antibody identified a positive green signal from a Y chromosome in the nucleus of a hepatocyte. (B2) Combined FISH with digoxigenin-labeled X probe and biotin-labeled Y probe and immunohistochemistry with anti-hepatocyte antibody identified a positive red signal from a Y chromosome in the nucleus of a hepatocyte.* From Guettier C et al., Male Cell Microchimerism in Normal and Diseased Female Livers from Fetal Life to Adulthood. Hepatology 2005; 42(1): 35–43.

In 2005, Tan et al., using quantitative real-time PCR for enhanced green fluorescent protein (EGFP) gene, detected fetal EGFP–positive cells in the circulation and brain of wild-type murine mother. The group also found that some of these cells expressed immunocytochemical markers for neural cell types, confirming that fMCs can enter the maternal circulation in mice and cross the blood-brain barrier (BBB) (***Figure 2.4*** and ***Figure 2.5***) (Tan *et al.*, 2005). The authors reported that the number of cells identified in brain tissue by the real-time PCR analysis included both neural cells and cells engrafted into other niches, such as the perivascular environment. Moreover, larger numbers of cells were observed 4 weeks post-partum suggesting that fMCs could be progenitor or stem cells capable of proliferation. Interestingly, Tan et al. found that fMCs preferentially colonise the region of the olfactory bulb, an area that has been reported to support survival and limited proliferation, migration, and immunocytochemical differentiation of progenitor cells (Walczak *et al.*, 2004; Tan *et al.*, 2005). These findings let hypothesise that, in this facilitating niche, fMCs could be incorporated into the maternal brain, proliferate, and survive and that, when the maternal brain is injured, these cells could enter the region of the injury. Indeed, the group observed that, in the maternal brain, fetal cells usually occurred in clusters containing different morphological and immunocytochemical profiles. These findings might indicate that (1) a single fetal progenitor or stem cell entered the brain and proliferated producing cells following various differentiation pathways in subsequent generations; or (2) multiple fetal cells enter the brain at particular locations attracted by signalling molecules or due to the inhomogeneous permeability of the BBB, and proliferate following different differentiation pathways (Tan *et al.*, 2005).



Figures 2.4 and 2.5. ***On the left,*** *FACS revealed a small population of EGFP-positive cells in maternal blood from (A1) the mothers of EGFP-positive pups when normalized to (B1) control wild-type blood from virgin females and (C1) the blood of hemizygous EGFP-positive pups. M1 and M2 mark the regions selected to sort the cells into EGFP-negative and EGFP-positive cells, respectively. Phase (D1, F1) and epifluorescence (E1, G1) photomicrographs showing EGFP-positive fetal cells (arrows) in the blood of a wild-type mother of Green Mouse pups (D1, E1) and in a positive control blood sample from a hemizygous Green Mouse pup (F1, G1). Epifluorescence photomicrograph (H1) showing EGFP-positive fetal cells in the cortex of a wild-type mother of EGFP-positive pups perfused 4 weeks after giving birth.* ***On the right****, perivascular EGFP-positive fetal cells in the maternal brain. Sections were labeled with DAPI (blue) to identify nuclei (A2, G2) and by fluorescence immunocytochemistry with anti-GFP (green) (B2, H2) and anti-vWF (red) (C2, I2) antibodies. Merged images are reported for A2, B2, and C2 findings (D2) and for G2, H2, and I2 findings (J2). EGFP-positive fetal cell (green) is juxtaposed to the blood vessel, separated from the lumen (L) of the vessel only by vWF-positive maternal endothelial cells with characteristically elongated somata and nuclei (E2, F2). A putatively binucleated EGFP-positive fetal cell identified in J2 is show that the cell is closely juxtaposed to the vWF-positive endothelial wall (K2).* From Tan XW et al, Fetal Microchimerism in the Maternal Mouse Brain: A Novel Population of Fetal Progenitor or Stem Cells Able to Cross the Blood-Brain Barrier?. Stem Cells 2005; 23(10): 1443–1452.

Overall, although microchimerism and its dependency on pregnancy are currently well established, the biological processes that allow these *non-self* cells to migrate into the host, colonise the tissues, and survive are not completely defined yet.

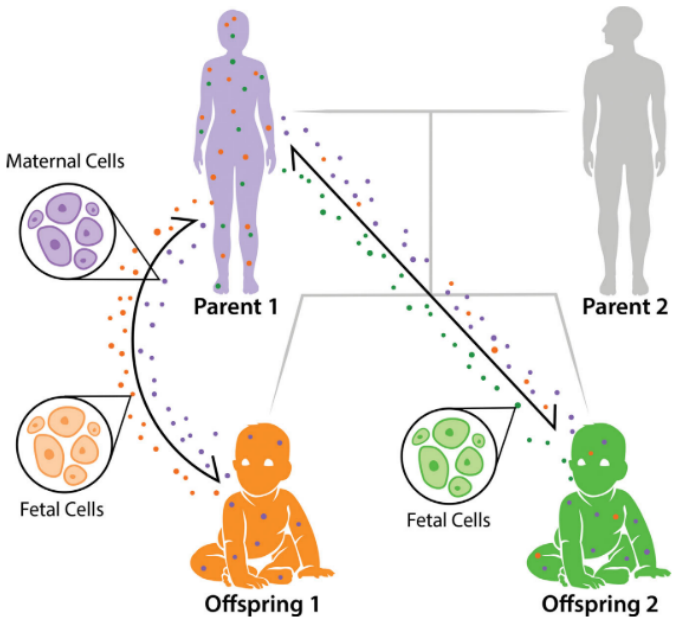
Firstly, despite being able to proliferate in different tissues, the distribution of microchimerism across organs is inhomogeneous, with lung being the organ in which fMCs present most often and in greatest abundance, followed by the spleen, liver and kidney, with lowest amounts of microchimerism found in the heart and brain (Fujiki *et al.*, 2008; Rijnink *et al.*, 2015). This pattern could be related to different explanations. It has been proposed that this distribution may be due to (1) the circulatory anatomical relationship between mother and foetus: as lung contains the first capillary bed through

which blood passes from the placenta, the organ would be the best candidate for having the highest amounts of microchimerism (Rijnink *et al.*, 2015). However, it has also been speculated that the fMCs distribution could depends on (2) the cardiac output supply or (3) an active accumulation in specific tissues due to favourable microenvironments (Rijnink *et al.*, 2015).

Another relevant issue is the biological mechanism through which the host is induced to *tolerate* these microchimeric cells. Some authors suggested that the placenta could act as a source of fetal antigens that might contribute to establish peripheral tolerance to fMCs (Adams Waldorf *et al.*, 2010). Adams Waldorf and colleagues hypothesised that maternal immature dendritic cells, interacting with fetal antigens derived from placental apoptotic debris, induce tolerogenic signals to T lymphocytes that are fetal antigen-specific (Adams Waldorf *et al.*, 2010). The presentation of fetal-derived peptides could temporarily change the maternal immunologic "self" during pregnancy to include fetal antigens (Stevens, 2016). After delivery, the loss of syncytiotrophoblast cells would block the fetal antigen presentation, interrupting maternal immunity (Kolialexi *et al.*, 2004; Adams Waldorf *et al.*, 2010). In fact, Pritchard et al. observed a two-stage pattern in murine clearance of fMCs during post-partum and reported that (1) the rapid apoptosis of fetal cells observed during the two and a half weeks immediately after delivery is followed by (2) a gradual clearance lasting 90 days and likely longer (Pritchard *et al.*, 2012). Nevertheless, as reported in Liégeois in 1981 and confirmed by following studies, a percentage of fMCs could elude the apoptotic mechanisms and survive in maternal blood and tissues for decades, determining the establishment of a microchimeric status in the mother (Liégeois *et al.*, 1981; Bianchi *et al.*, 1996; Nelson, 2012; Bakkour *et al.*, 2014). The immunological mechanism that allows these cells to survive remains unclear.

The phenomenon of microchimerism is even more complex considering the bidirectionality of fetal and maternal flow. The transfer of cells is asymmetrical, with more fMCs entering the maternal flow than vice versa. It has been largely demonstrated that fetal and maternal cells could survive in the host for decades following birth. The bidirectional flow could create a complex system of exchanges: being the mother a carrier of mMCs and previously acquired fMCs, cells from maternal grandmother and older siblings might be transferred to the feotus, creating a *multiple* or *multi-generational*

microchimerism (***Figure 2.6***) (Guettier *et al.*, 2005; Loubière *et al.*, 2006; Adams Waldorf *et al.*, 2010; Chan *et al.*, 2012; Boddy *et al.*, 2015; Müller *et al.*, 2015).



Figures 2.6. *Bidirectional exchange of fetal and maternal microchimeric cells during pregnancy. During pregnancy, fetal cells (orange and green cells) enter the maternal circulation, increasing in quantity throughout the pregnancy. Likewise, each foetus receives maternal microchimeric cells (purple cells). The younger siblings could also obtain older siblings' cells, as represented by offspring 1 cells (orange) circulating within the younger sibling's body (offspring 2).* From Boddy AM et al., Fetal Microchimerism and Maternal Health: a Review and Evolutionary Analysis of Cooperation and Conflict Beyond the Womb. BioEssays 2015; 37(10): 1106–1118.

In summary, although the presence of fMCs in the mother has been largely confirmed, not much is known about the biological meaning of these cells that originated in the foetus and become long-term residents within the maternal environment. In healthy women, cell frequencies seem to be similar to those reported for antigen-specific T cell precursors and it is so possible to speculate that a male T cell clone from a healthy woman could be stimulated and could activate an inflammatory response (Nelson, 2012). Cytotoxic lymphocytes and regulatory T cells (T_{reg} cells) specific for male minor antigens are well described in healthy females suggesting that fMCs also has antigenic functional consequences (Nelson, 2012). Being the biological significance of these cells still unknown, an increasing body of literature has linked fetal microchimerism to both pathological and healing processes in the mother.

1.2. The role of fetal microchimerism on maternal health

The biological and clinical significance of fetal microchimerism in maternal health is largely unknown, however three main hypotheses have been formulated (Johnson and Bianchi, 2004; Fugazzola, Cirello and Beck-Peccoz, 2011):

1. fetal cells are deleterious, contributing to an inflammatory response that can cause maternal tissue damage (*bad microchimerism*);
2. fetal cells are protective, with fetal progenitor cells helping to repair and maintain maternal tissues (*good microchimerism*);
3. fetal cells are simply by-standers, having no causal effect on maternal health (*neutral microchimerism*).

Moreover, it has been hypothesised that these three conditions may not be mutually exclusive and that, under specific status, fetal cells might have positive or negative effects on maternal health (Boddy *et al.*, 2015).

Microchimeric cells have been shown to exist both in healthy organs and in disease-specific tissues (Fujiki *et al.*, 2008). However, the presence of fMCs in the blood and tissues of patients with autoimmune diseases incriminated these cells to be involved in their pathogenesis inducing a *graft-versus-host disease*. Under physiological conditions, the presence of *non-self* cells induces the activation of the immune response, determining a status of inflammation. Proceeding from this principle, the first hypothesis on the biological role of fMCs focused on a possible role of the cells in triggering the occurrence of autoimmune diseases (Nelson, 1996; O'Donoghue, 2011; Boddy *et al.*, 2015).

Women are at a higher risk than men for developing autoimmune diseases, and this risk has been reported to be higher during post-partum, although, some studies reported no link between parity and autoimmune diseases (Boddy *et al.*, 2015). Considering epidemiological data and previous findings on fMCs, some authors speculated that these cells could possibly enhance susceptibility to autoimmune diseases (Nelson, 1996; O'Donoghue, 2011; Boddy *et al.*, 2015).

In 2006, Loubière et al., studying 31 healthy women probands for mMCs, found that healthy adult women harbor maternal cells among their T and B lymphocytes, monocyte/macrophages, and natural-killer (NK) cells (Loubière *et al.*, 2006). The same group estimated that mMC levels ranged from 1 to 30 gEq/100,000 proband cells and

calculated a prevalence of 100,000–2,600,000 circulating maternal T lymphocytes in healhty subjects. The data indicated that mMCs may be present at levels with the potential for immunological effects (Loubière *et al.*, 2006; Nelson, 2012). Indeed, it has been reported that the feto-maternal trafficking allows the migration of T and B lymphocytes, monocyte/macrophages, and NK cells that could activate inflammatory processes and modulate the mechanisms of antigenic tolerance (Loubière *et al.*, 2006; Nelson, 2012; Boddy *et al.*, 2015).

The association between microchimerism and systemic sclerosis has been extensively investigated and several authors have described microchimerism as a widespread phenomenon in affected women and reported a large distribution of male cells within the architecture of the tissue from the patients, but not the control subjects (Artlett, Smith and Jimenez, 1998; Johnson *et al.*, 2001). Artlett and colleagues detected fMCs in skin lesions in 58% specimens of 68 women with osteoarthritis, but in none of the controls (Artlett, Smith and Jimenez, 1998). In 2001, the group of Johnson, using fluorescence in situ hybridization (FISH), observed the presence of fMCs in lymph node, lung, adrenal gland, and skin sections of patients affected by systemic sclerosis, but in none of the controls (Johnson *et al.*, 2001). The results established that microchimerism could be observed in multiple tissue types, including those affected by the disease, such as lung and skin. Similar results have been obtained investigating the presence of mMCs in offspring affected by juvenile dermatomyositis and juvenile idiopathic inflammatory myopathies (Arlett *et al.*, 2000; Reed *et al.*, 2000).

Likewise, fMCs have been found more frequently in the blood and thyroid tissue of women with Hashimoto's thyroiditis and Graves' disease compared to healthy controls, suggesting that these cells are associated with maternal disease rather than health in the organ (Klintschar *et al.*, 2006; Lepez, Vandewoestyne and Deforce, 2012). Finally, in 2008, Fujiki et al., using a murine model, hypothesised that the migration of fMCs through the uterine vein into maternal pulmonary arteries could play an active biological or pathological role in the maternal lung and be one of the causes determining the higher risk of primary pulmonary hypertension and pulmonary thrombosis in pregnant women (Fujiki *et al.*, 2008).

On the other hand, controversy results have been found investigating other autoimmune diseases. Testing the liver of patients with primary biliary cirrhosis and controls, some authors concluded that fMCs alone does not play a significant role in the pathogenesis of

the disease, observing an equal distribution of cells in patients and control women (Johnson and Bianchi, 2004). By contrast, Fanning et al. found that fMCs were present in 8 out of 19 patients affected by primary biliary cirrhosis, but in none of the patients with either chronic hepatitis C or alcoholic liver disease and concluded that microchimerism may be involved in the pathogenesis of the disease (Fanning *et al.*, 2000). Debating findings are also reported in Sjogren's syndrome and systemic lupus erythematosus (Johnson and Bianchi, 2004).

The inconsistent findings observed studying the phenomenon of microchimerism in autoimmune diseases questioned the theory of *bad microchimerism*. Indeed, further investigations demonstrated the presence of microchimeric cells in healthy subjects and the absence of these cells in patients, discrediting the original hypothesis that chimeric T lymphocytes react to host antigens inducing chronic inflammation. As newer evidence emerged on the capacity of fMCs to differentiate in maternal tissues producing cells of mesodermal, ectodermal, endodermal, and perhaps even trophectodermal lineages (***Figure 2.7***) (Seppanen, Fisk and Khosrotehrani, 2013), an alternative hypothesis has been formulated: fMCs may give rise to stem cells, which may participate in the repair of damaged tissues according to an *allogeneic stem cell transplant* model (Guettier *et al.*, 2005). This hypothesis and a possible role of microchimerism in healing processes were also supported by the apparent capacity of these cells to transdifferentiate and assume the phenotype of the maternal tissue (Fujiki *et al.*, 2008).

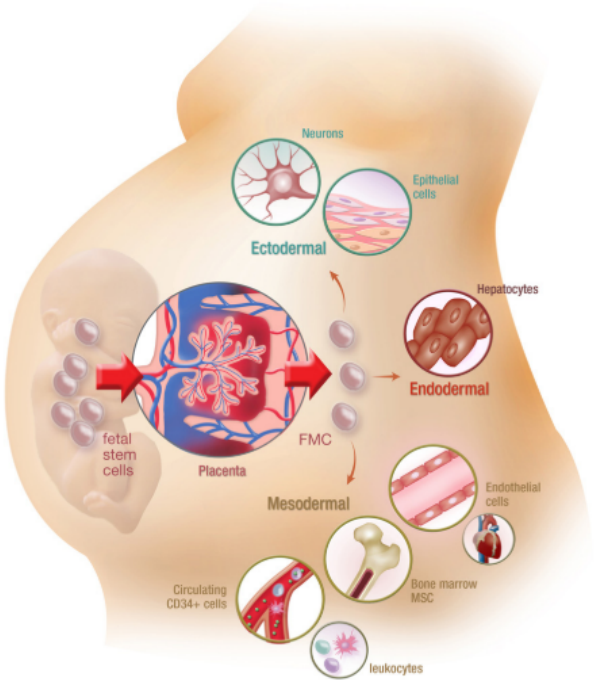
Considering these stem-like properties of the fMCs, some authors have investigated the distribution of fMCs in injured tissues from another prospective and hypothesised that these cells may provide maternal benefits, counteracting some of the negative effects of stem cell loss and damage (Boddy *et al.*, 2015).

Proceeding from this concept, some authors evaluated the presence of fMCs in peripheral blood of women with breast cancer and observed a lower frequency when compared to healthy women (Gadi and Nelson, 2007; Nelson, 2012). fMCs have also been investigated in thyroid cancer, cervical cancer, lung cancer, and melanoma with most studies proposing a beneficial role in tissue repair, repopulation and/or immune surveillance (Kallenbach, Johnson and Bianchi, 2011; Nelson, 2012). Indeed, most investigations examining solid tumours convincingly demonstrated that fetal cells are preferentially present at tumour sites and supported the hypothesis that these cells could promote

immune-surveillance and participate in tissue repair (Kallenbach, Johnson and Bianchi, 2011). Finally, in 2014, Kamper-Jørgensen et al. reported a 60% lower all-cause mortality among patients with male fMCs compared to negative women (*hazard ratio* 0,42), but a higher risk of dying from cardiovascular disease in the fMCs-positive group (*hazard ratio* 1,66) (Kamper-Jørgensen *et al.*, 2014).

Likewise, several studies suggested that fetal cells may play a role in maternal wound healing. Murine injury models have tracked fetal cells actively migrating to the site of injury in the maternal body and clustering at sites of inflammation, suggesting participation in maternal angiogenesis (Nassar *et al.*, 2012). Additionally, in humans, fetal cells were identified in healed caesarean section scars and expressed markers of cytokeratin and collagen, suggesting that fetal cells actively participate in maternal wound healing (Mahmood, Fitzgibbon and O'Donoghue., 2011). In 2010, Sunami et al. induced injuries in specific maternal organs after performing hysterectomies in female mice at different times during pregnancy and observed that fetal DNA was present only in the injured organs and that their frequency was consistent in number, irrespective of the stage when hysterectomy was performed (Sunami *et al.*, 2010). The results suggested that most of the fetal cells capable of multilineage differentiation may have entered the maternal circulation early after implantation and may concentrate in clinically affected tissues (Sunami *et al.*, 2010). Therefore, fetal cell may mediate tissue repair, have a beneficial effect on postnatal lifespan, and protect against some diseases during pregnancy.

In summary, although it remains at present unclear whether fetal cells play an active role in the recovery of damage, it is possible to speculate that the cells could modulate the biological and immunological processes of tissue repair (Kallenbach, Johnson and Bianchi, 2011; Nelson, 2012; Boddy *et al.*, 2015).



Figures 2.7. *Schematic representing feto-maternal cell trafficking and differentiation of fetal microchimeric cells (FMC) down ectodermal, endodermal, and mesodermal lineages.* From Seppanen E et al., Pregnancy-Acquired Fetal Progenitor Cells. Journal of Reproductive Immunology 2013; 97(1): 27–35.

Concluding, the available literature has not yet revealed the biological meaning of microchimerism. The results obtained over the last 40 years are controversial and it is suggestive to speculate that the role of these cells could be not univocal (Nelson, 2012). In this view, fMCs would be able, on one side, to induce immune-tolerance and activate healing processes and, on the other, to trigger autoimmune phenomena in a subset of subjects (Loubière *et al.*, 2006; Fujiki *et al.*, 2008; Nelson, 2012).

1.3. The role of fetal microchimerism in multiple sclerosis

The effect of fetal microchimerism in long-term maternal health is still controversial and, as extensively discussed above, a possible role of fMCs in the autoimmune diseases has been hypothesised by some authors (Johnson and Bianchi, 2004; Fugazzola, Cirello and Beck-Peccoz, 2011; Boddy *et al.*, 2015). Indeed, several studies suggested that, in maternal blood, progenitor cells of the fetal immune system are capable of self-renewal, proliferation, differentiation, and activation, resulting in the production of paracrine and

autocrine inflammatory cytokines and chemokines that are involved in autoimmune diseases (Miech, 2010; Pritchard *et al.*, 2012; Seppanen, Fisk and Khosrotehrani, 2013).

Multiple sclerosis (MS) is a chronic, disabling, autoimmune disorder of the central nervous system (CNS) characterised by both inflammatory and neurodegenerative processes which result in demyelination and axonal damage (Chapter 1). Immunological, histopathological, genetic, and therapeutic evidence suggests the autoimmune origin of MS, but the etiopathogenetic mechanism which triggers the autoimmune reaction is still unknown. The incidence and prevalence of the disease among the female population is almost twice the figures for male patients, with a ratio of women to men that varies throughout geographical areas (MSIF 2020). Although this phenomenon has been observed also in other autoimmune diseases (Ngo, Steyn and McCombe, 2014; Kronzer, Bridges and Davis, 2020), the causes behind the greater propensity of women to develop chronic autoimmune diseases during their reproductive years remain unclear with hormonal differences not completely explaining this phenomenon (Dunn *et al.*, 2015; Dunn, Gunde and Lee, 2015; Johnson *et al.*, 2020; Kronzer, Bridges and Davis, 2020).

The available data concerning the role of the microchimerism in MS are still limited. Pregnancy is considered to be a protective factor in MS, an effect likely due to the modulation of the immune system and to possible changes in the brain during pregnancy (McCombe and Greer, 2013). In 2005, Tan et al. observed that fMCs could cross the BBB and enter the brain, where they can express morphological and immunocytochemical characteristics of various cell types, including neuronal cells, endothelial cells, and perivascular macrophage (***Figure 2.8*** and ***Figure 2.9***) (Tan *et al.*, 2005). The authors also hypothesised that fetal cells could proliferate in the brain or migrate into the organ in response to specific signals, suggesting that when the maternal brain is injured, the migration could increase due to the damage of BBB and/or the release of signalling molecules that recruit fMCs. This hypothesis seems to be supported by the detection of fMCs both closely juxtaposed to blood vessels and within the brain parenchyma with no obvious association to blood vessels, a distribution that may result from a response to cues from the host (Tan *et al.*, 2005).

Five years later, the same group confirmed their hypothesis on the capacity of fMCs to engraft maternal brain and undergo neural differentiation (Zeng *et al.*, 2010). In fact, the

authors observed an increase in fetal detectable cells during postpartum, when the fMCs tend to disappear in the peripheral blood. Furthermore, Zeng et al. showed that these cells could also express immature neuronal markers and go through a cellular and molecular maturation process similar to those observed during adult neurogenesis (Zeng *et al.*, 2010). The group reported that fMCs may increase axonal-dendritic complexity over time and enable the maturation and time-dependent integration of these cells into the brain (Tan *et al.*, 2005; Zeng *et al.*, 2010). Finally, the authors investigated the frequency and distribution of fetal cells in a murine Parkinson's disease (PD) model. They found lower total frequency in PD mothers, suggesting that the disease environment did not support their long-term maintenance or survival. They also observed that fetal cells had a different distribution in the hippocampus of lesioned and non-lesioned mothers, hypothesising that the lesioned tissue could reduce the survival of fMCs and that pathological microenvironment might play a crucial role impacting the outcome of any cell-based regenerative strategy (Zeng *et al.*, 2010).

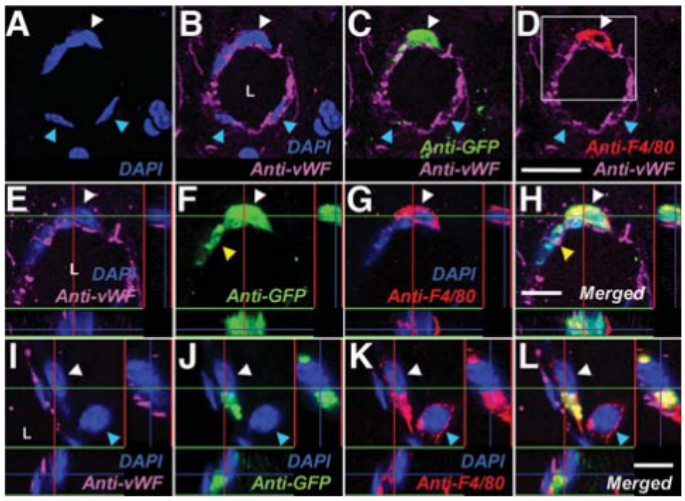


Figure 2.8. *Orthogonal slices from perivascular Green Mouse fetal cells in maternal brain. The white arrowheads indicate large perivascular EGFP-positive fetal cells double-labelling for the macrophage marker F4/80 (red) antibodies, but not labelling for the endothelial marker vWF (purple) antibodies. In images F and H, yellow arrowheads indicate what appears to be evidence of an EGFP-positive process from a fetal perivascular macrophage wrapping around adjacent endothelial cells. The blue arrowheads indicate maternal cells labelling for vWF antibodies (A–D) and F4/80 atnibodies (I–L). The F4/80-positive fetal cells exhibit a similar size and location to the maternal perivascular macrophage.* From Tan XW et al, Fetal Microchimerism in the Maternal Mouse Brain: A Novel Population of Fetal Progenitor or Stem Cells Able to Cross the Blood-Brain Barrier?. Stem Cells 2005; 23(10): 1443–1452.

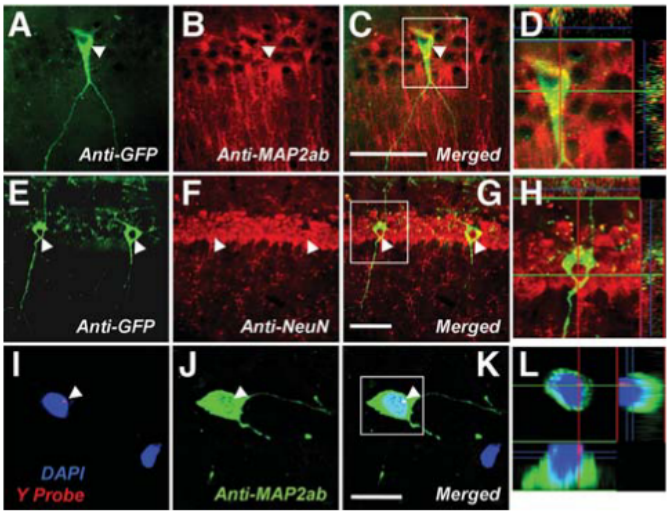


Figure 2.9. *Fetal cells can express neuronal immunocytochemical markers in the maternal brain. Orthogonal slices through the cells in the regions identified by the white boxes in (C, G, K) are shown in (D, H, L), respectively. Fetal cells were identified by fluorescence immunocytochemistry with an anti–green fluorescent protein (anti-GFP) antibody (A, E) or by Y chromosome–specific FISH (I). Sections were immunostained (red in B–D, F–H; green in J–L) for neural cell type markers for neuronal cells (MAP2ab in B–D, J–L and NeuN in F–H). White arrowheads indicate fetal cells double-labelled either by anti-GFP immunocytochemistry (A, E) or Y chromosome FISH (I) and neural cell type markers MAP2ab (B, J) and NeuN (F).* From Tan XW et al, Fetal Microchimerism in the Maternal Mouse Brain: A Novel Population of Fetal Progenitor or Stem Cells Able to Cross the Blood-Brain Barrier?. Stem Cells 2005; 23(10): 1443–1452.

In 2012, Chan et al., evaluating the presence of fMCs in human female brain, reported that male fMCs could be detected in up to 63% of subjects and are distributed in multiple brain regions, suggesting that microchimerism could potentially persistent across the human lifespan (Chan *et al.*, 2012). The group also investigated male fMCs in patients affected by Alzheimer's disease (AD) and healthy controls. AD is neurodegenerative disease characterized by elevated levels of amyloid plaques, cerebrovascular amyloidosis, and neurofibrillary tangle. Sex differences in the AD have been reported, indicating that women are protected relative to men at the prodromal phases, but later exhibit steeper cognitive decline and higher rates of brain atrophy (Ferretti *et al.*, 2018). Also, the disease prevalence is higher in parous than nulliparous women and pregnancy-related risk factors have been proposed as possible causes of this unbalance (Ferretti *et al.*, 2018). Chan et al. reported findings similar to those observed in murine PD: AD was significantly associated with lower fMCs prevalence and the risk of having AD in fMCs-positive subjects was 60% lower than in subjects who tested negative for fMCs (***Figure 2.10*** and ***Table 2.1***).

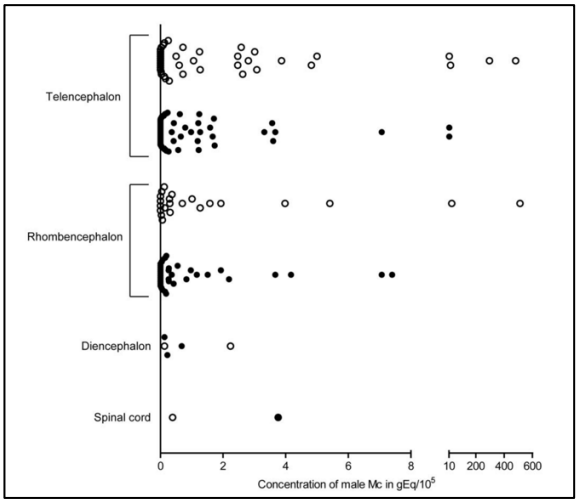


Figure 2.10. *Concentration of male fMCs in female human brain regions of females without any neurologic disease (open circles) or with AD (filled circles). Telencephalon consists of neocortical regions (frontal, parietal, temporal, and occipital lobes), limbic regions (hippocampus, amygdala, and cingulate gyrus), and regions of the basal ganglia (putamen, caudate, and globus pallidus). Diencephalon consists of thalamus. Rhombencephalon consists of medulla, pons, and cerebellum.* From Chan WFN et al., Male Microchimerism in the Human Female Brain. PLoS ONE 2012; 7(9): e45592.

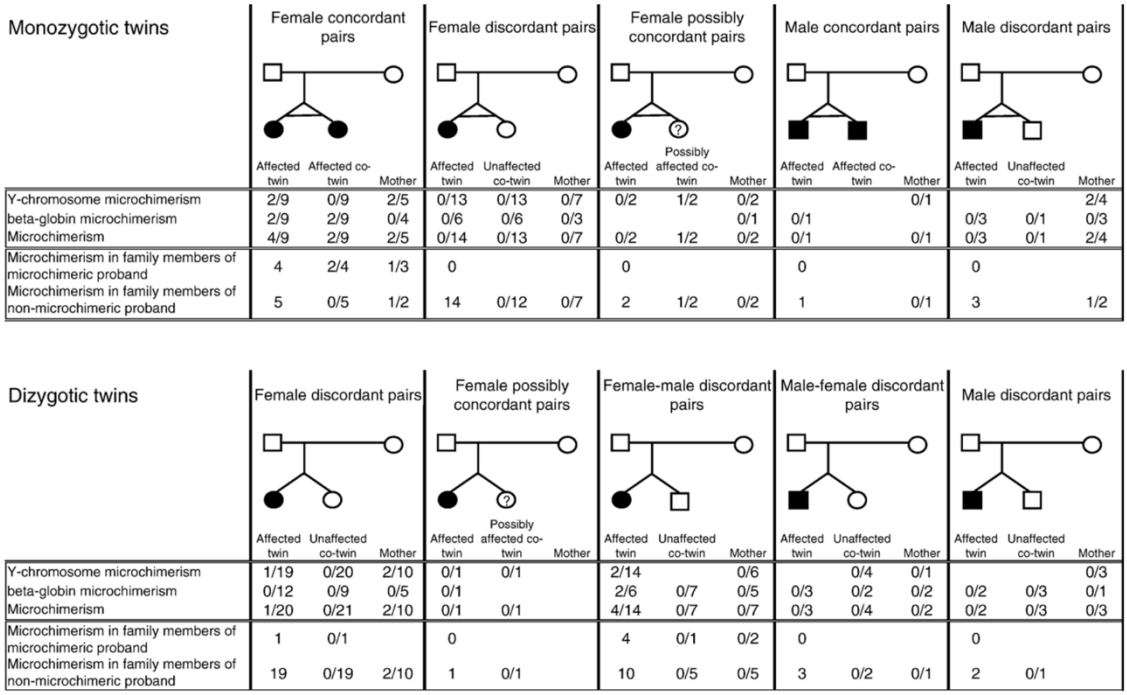
	Proportion of samples tested positive for male Mc (%)		
Brain region	**Total**	**No neurologic disease**	**Alzheimer's disease**
I. Telencephalon			
i) *Neocortical*			
Frontal	0/3 (0)	–	0/3 (0)
Parietal	7/17 (41)	3/4 (75)	4/13 (31)
Temporal	12/26 (46)	6/13 (46)	6/13 (46)
Occipital	1/5 (20)	–	1/5 (20)
ii) *Limbic*			
Hippocampus	7/20 (35)	3/7 (43)	4/13 (31)
Amygdala	1/2 (50)	–	1/2 (50)
Cingulate gyrus	11/26 (42)	7/14 (50)	4/12 (33)
iii) *Basal ganglia*			
Putamen	0/4 (0)	–	0/4 (0)
Caudate	1/9 (11)	0/1 (0)	1/8 (13)
Globus pallidus	1/5 (20)	1/1 (100)	0/4 (0)
II. Diencephalon			
Thalamus	2/5 (40)	1/2 (50)	1/3 (33)
III. Rhombencephalon			
Medulla	7/8 (88)	1/1 (100)	6/7 (86)
Pons	11/35 (31)	7/17 (41)	4/18 (22)
Cerebellum	2/16 (13)	1/4 (25)	1/12 (8)
IV. Spinal cord	1/2 (50)	0/1 (0)	1/1 (100)
All regions*	64/183 (35)	30/65 (46)	34/118 (29)

*P = 0.03 comparing the overall prevalence of male Mc between the two groups. Individual brain regions were not compared due to limited sample sizes.
doi:10.1371/journal.pone.0045592.t002

Table 2.1. *Prevalence of male microchimeric cells within individual brain regions in women without neurologic disease or with Alzheimer's disease.* From Chan WFN et al., Male Microchimerism in the Human Female Brain. PLoS ONE 2012; 7(9): e45592.

The biological role of fMCs in the brain remain still unclear. Up-to-date, the available literature suggests that fMCs could enter into the brain and interact with the microenvironment (Tan *et al.*, 2005; Zeng *et al.*, 2010; Chan *et al.*, 2012). However, the outcome of this interaction is still undefined. In other non-neurological autoimmune diseases, authors have suggested that fMCs could induce the activation of a chronic inflammation (Miech, 2010). MS is an autoimmune disease likely resulting from the interaction of genetic and environmental factors and the subsequent triggering of dysregulation of the immune system, loss of *self-tolerance* toward myelin and other CNS antigens, the massive activation of the immune system and the onset a chronic inflammatory status (Garg and Smith, 2015). Proceeding from these knowledges, some authors have hypothesised a possible role of fMCs in the pathogenesis of the disease.

In 2006, Willer et al. studied a total of 190 samples from twins with MS, their unaffected co-twins, mothers, husbands and twins' husbands. The authors found a higher rate of microchimerism in female twins with MS compared to unaffected co-twins (Willer et al, 2006). Comparing the rate of microchimerism in female twins with a diagnosis of MS to those of unaffected, Willer et al. observed that 15.9% of affected females had fMCs, a percentage significantly higher than those registered in unaffected female co-twins (0.00%; p-value = 0.006) (***Figure 2.11***). Similarly, the authors found that the frequency of microchimerism was significantly higher in females from concordant monozygotic pairs than in discordant female monozygotic twins (33.3% vs 0.00%, p-value = 0.0023) (***Figure 2.11***). Interestingly, the group tested the husbands of microchimeric-positive subjects and found that, in nearly all cases, the husband carried the allele that was present in the microchimeric cells. The authors concluded that the higher rate of microchimerism observed in female twins with MS compared to unaffected co-twins supported the hypotheses that microchimerism is related to having MS.



Monozygotic twins	Female concordant pairs			Female discordant pairs			Female possibly concordant pairs			Male concordant pairs			Male discordant pairs		
	Affected twin	Affected co-twin	Mother	Affected twin	Unaffected co-twin	Mother	Affected twin	Possibly affected co-twin	Mother	Affected twin	Affected co-twin	Mother	Affected twin	Unaffected co-twin	Mother
Y-chromosome microchimerism	2/9	0/9	2/5	0/13	0/13	0/7	0/2	1/2	0/2			0/1			2/4
beta-globin microchimerism	2/9	2/9	0/4	0/6	0/6	0/3			0/1	0/1			0/3	0/1	0/3
Microchimerism	4/9	2/9	2/5	0/14	0/13	0/7	0/2	1/2	0/2	0/1		0/1	0/3	0/1	2/4
Microchimerism in family members of microchimeric proband	4	2/4	1/3	0			0			0			0		
Microchimerism in family members of non-microchimeric proband	5	0/5	1/2	14	0/12	0/7	2	1/2	0/2	1		0/1	3		1/2

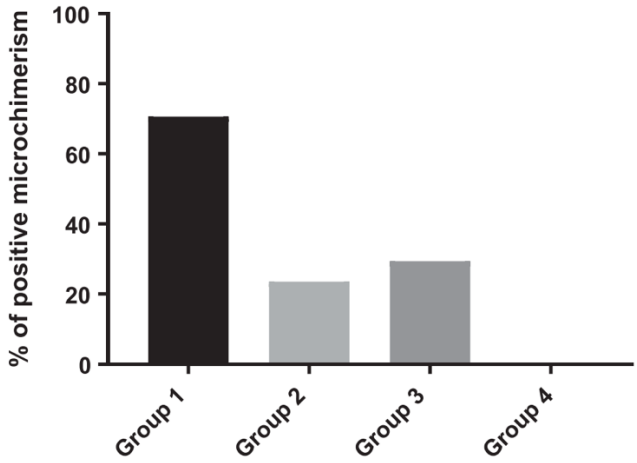
Dizygotic twins	Female discordant pairs			Female possibly concordant pairs			Female-male discordant pairs			Male-female discordant pairs			Male discordant pairs		
	Affected twin	Unaffected co-twin	Mother	Affected twin	Possibly affected co-twin	Mother	Affected twin	Unaffected co-twin	Mother	Affected twin	Unaffected co-twin	Mother	Affected twin	Unaffected co-twin	Mother
Y-chromosome microchimerism	1/19	0/20	2/10	0/1	0/1		2/14		0/6		0/4	0/1			0/3
beta-globin microchimerism	0/12	0/9	0/5	0/1			2/6	0/7	0/5	0/3	0/2	0/2	0/2	0/3	0/1
Microchimerism	1/20	0/21	2/10	0/1	0/1		4/14	0/7	0/7	0/3	0/4	0/2	0/2	0/3	0/3
Microchimerism in family members of microchimeric proband	1	0/1		0			4	0/1	0/2	0			0		
Microchimerism in family members of non-microchimeric proband	19	0/19	2/10	1	0/1		10	0/5	0/5	3	0/2	0/1	2	0/1	

Figure 2.11. *Rates of Y-chromosome, beta-globin, and total microchimerism in Canadian twins with MS and their mothers. The authors observed higher frequency of microchimeric cells in affected female twins than in unaffected co-twins and in concordant monozygotic pairs than in discordant monozygotic pairs.* From Willer CJ et al., Association between Microchimerism and Multiple Sclerosis in Canadian Twins. Journal of Neuroimmunology 2006; 179(1–2): 145–151.

A second study reported similar results suggesting that a low-level microchimerism is associated with MS (Bloch *et al.*, 2011). The authors tested a population including (1) 27 women affected by MS and with a history of male pregnancy, (2) 24 MS female patients without known male pregnancy, and (3) 22 unaffected siblings of MS patients with a history of male pregnancy. Overall, the group found that among the 51 women with MS, 21 (41.2%) had fMCs, including 10/27 (37.0%) MS patients with a history of male pregnancy and 11/24 (45.8%) MS patients without known male pregnancy. Among 22 MS-negative siblings with a history of male pregnancy, 4 (18.2%) had male fMCs. Bloch et al. suggested that their pilot study supported the hypothesis that a low-level microchimerism is associated with MS.

A more recent study by Jafarinia et al. investigated the presence of male genome in (a) MS patients who gave birth to a male son, (b) MS nulliparous patients, (c) healthy controls (MS-negative mother or sister of MS-positive subjects) who gave birth to a male son, and (d) healthy controls without a history of pregnancy (Jafarinia *et al.*, 2020). In their publication, the group reported that the percentage of male genome-positive women was

statistically higher in MS-positive women who had given birth to a son (12/17 subjects, 70.6%) in comparison with the other three groups (p-value <0.008) (***Graph 2.1***). They also estimated that in MS-positive women without a known pregnancy and in healthy controls who had given birth to a son, the percentage of microchimerism-positive subjects was 23.5% (4/17 subjects) and 29.4% (5/17 subjects), respectively. None of the healthy controls without a history of pregnancy had fMCs. Overall, of 34 women with MS, 16 (47%) subjects had fMCs. The authors suggested the existence of an association between microchimerism and the autoimmune disease and speculated that these *non-self* cells could trigger an inflammatory process activating the immune host cells or could down-regulate host immune-regulation mechanisms, releasing the control on autoreactive host cells.



Graph 2.1. *Percentage of male genome-positive women. Patients were classified as: (a) MS-positive women have given birth to a son (Group 1), (b) MS-positive women reported had never a known pregnancy (Group 2), (c) MS- negative mother or sister of MS-positive subjects given birth to a son (mother or sister of group 1 subjects) (Group 3), and (d) MS-negative women without a history of pregnancy (Group 4).* From Jafarinia M et al., Male Microchimerism in Peripheral Blood from Women with Multiple Sclerosis in Isfahan Province. International Journal of Immunogenetics 2020; 47(2): 175–179.

Finally, Snethen et al. analysed fresh frozen tissue from 6 male progressive MS (P-MS) patients and 6 male controls to identify mMCs using FISH (Snethen *et al.*, 2020). The group observed that frequencies of mMCs were 0.032 (range 0.013–0.083) in control samples, 0.029 (range 0–0.12) in normal appearing MS samples and 0.062 (range 0–0.139) in active MS lesion samples, although none of the comparison was statistically significant (***Figure 2.12***). They also reported that immunofluorescent labelling for

phenotypic markers, including βIII tubulin for neuronal cells and CD45 for immune cells, showed that the largest portion of female cells identified in control and lesion tissue were of neuronal lineage or of immune origin. The authors concluded that a low levels of maternal microchimerism are very common. However, the findings were unable to demonstrated whether there is a clear and understandable role for fMCs in MS and they envisioned that further studies are needed to tease out how, why and when microchimeric cells migrate to the human brain, and what role, if any, they play in MS disease susceptibility and development.

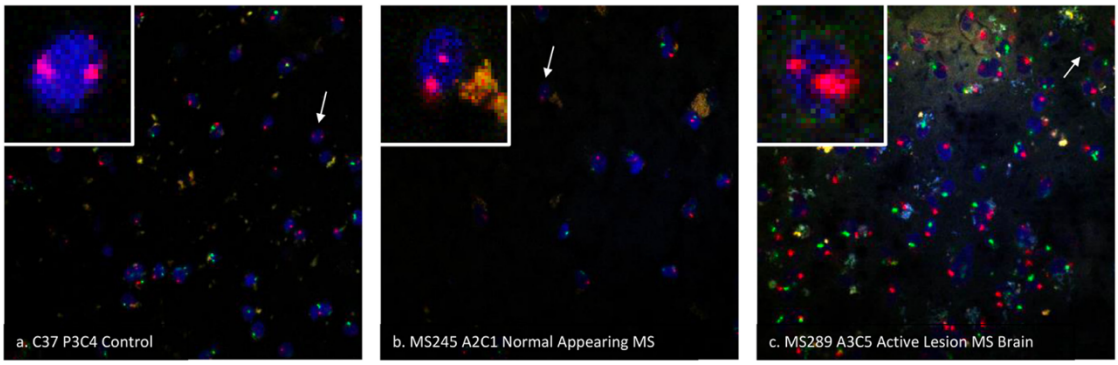


Figure 2.12. *Maternal microchimeric cells in human brain sections. Sections have undergone FISH for X (spectrum orange) and Y (Spectrum green) chromosomes. Nuclei are stained with DAPI in blue. Arrows show nuclei with two X chromosomes (enlarged in the panels at top left). Images refer to a control sample (a), the normal appearing white matter region of MS affected brain (b), and active lesion within MS affected brain (c).* From Snethen H et al., Maternal Micro-Chimeric Cells in the Multiple Sclerosis Brain. Multiple Sclerosis and Related Disorders 2020; 40: 101925.

To-date, these are the only four studies conducted to evaluate the relationship between microchimerism and MS. The data suggested a relationship between MS and fMCs, though a positive effect of pregnancy on disease course has been largely reported (McCombe and Greer, 2013). The first three studies reported a higher prevalence of fMCs in MS patients when compared to healthy controls and Snethen et al. speculated that a role of microchimeric cells in neuroprotection or regeneration in neurological diseases such as MS could not be ruled out. Concluding, the available literature has not yet revealed the biological effects of fMCs on maternal health and further studies are needed to evaluate which are the biologic and clinical implications on this phenomenon and whether these cells have a *good*, *bad* o *neutral effect*.

CHAPTER III

A retrospective study to evaluate the relationship between sex of offspring and disease features in a large cohort of patients derived from a population-based study

3.1. Background and aims

In 1977, Liégeois et al. proposed the term "microchimerism" to describe long-term donor cell survival in small proportion in the host (Liégeois *et al.*, 1977). Two years later, in 1979, Herzenberg et al. demonstrated the presence of fetal cells in the maternal circulation (Herzenberg *et al.*, 1979) and, in 1981, Liégeois et al. confirmed the presence of allogeneic fetal cells in maternal tissue during and long after pregnancy (Liégeois *et al.*, 1981). Over the following 40 years, several authors have confirmed the existence of fetal cells in maternal blood and confirmed their pregnancy-related origin, demonstrating that pregnancy may establish a long-term, low-grade chimeric state in women (Bianchi *et al.*, 1996; O'Donoghue, 2011; Nelson, 2012).

Although the presence of living fetal microchimeric cells (fMCs) that transfer from the fetal blood into the maternal circulation during the pregnancy, colonise maternal tissues, and persist for decades has been largely demonstrated, not much is known about their biological meaning (Miech, 2010; Nelson, 2012). In healthy women, the observed fMCs frequencies are similar to those registered for antigen-specific T cell precursors and it is so possible that a male activated T cell clone from a healthy woman could trigger the inflammatory response (Nelson, 2012). Cytotoxic lymphocytes and regulatory T cells (T_{reg} cells) specific for male minor antigens are well described in healthy females suggesting that fMCs also has antigenic functional consequences (Nelson, 2012). Being the biological significance of these cells still unknown, an increasing body of literature

has investigate the role of microchimerism in the autoimmune diseases (Nelson, 2012; Seppanen, Fisk and Khosrotehrani, 2013; Boddy *et al.*, 2015) and some authors suggested that progenitor fetal cells could activate and induce the production of paracrine and autocrine inflammatory cytokines and chemokines that are involved in autoimmune diseases (Miech, 2010; Pritchard *et al.*, 2012; Seppanen, Fisk and Khosrotehrani, 2013).

The robust data reported in the previous chapter on the association between pregnancy and microchimerism suggested that women with at least a previous pregnancy should be carriers of fMCs. Furthermore, it is possible to speculate also that subjects with only female daughters have exclusively XX fMCs, while subjects with at least a male pregnancy should have XY fMCs. In addition, starting the microchimeric trans-placenta flow as early as 10 days post-coitum (Vernochet, Caucheteux and Kanellopoulos-Langevin, 2007), also women with previous unsuccessful pregnancies are likely to carry fMCs. On contrary, being these cells pregnancy-derived, patients without a history of previous pregnancy should be negative for these cells. Proceeding from these assumptions, it is possible to speculate that obstetric history and sex of offspring could be considered an indirect marker of fMCs.

The autoimmune origin of multiple sclerosis (MS) has been well established, nonetheless the etiopathogenetic mechanism that trigger the inflammatory and neurodegenerative processes is still unknown and both genetic and environmental factors are likely involved in these pathological phenomena. Pregnancy is considered a protective factor in MS, an effect likely due to the modulation of the immune system and to the brain changes occurring during gestation (McCombe and Greer, 2013). However, the relationship between MS and microchimerism remains unclear and the available data are still limited.

Proceeding from the available literature on microchimerism and MS, I hypothesised that fMCs could have a role in the multifactorial background underlying the disease. The hypothesis that fetal cells induce a *graft-versus-host-like* immune response in mothers was investigated and led to a concentration of reports on the involvement of microchimerism in other autoimmune diseases. However, evidence has also emerged on a possible role of these cells in the tissue repair processes. Three previous *in vivo* studies reported a higher rate of microchimerism in affected women when compared with

unaffected women (Willer *et al.*, 2006; Bloch *et al.*, 2011; Jafarinia *et al.*, 2020). However, none of them focused on the effect of fMCs on maternal disease features. More recently, Snethen et al. tried to take a first step in this direction investigating the presence and distribution of maternal microchimeric cells (mMCs) in adult human brain of MS patients. The authors demonstrated that the largest portion of microchimeric cells were of neuronal or immune lineage and that the frequency of the cells tended to be higher in active lesions when compared with normal appearing MS samples and control samples (Snethen *et al.*, 2020). These previous findings and the published data on human AD brain and murine PD brain reported in the previous chapters let me hypothesise that fMCs could either (a) play a triggering role in MS, modulating the immune response and being involved in the inflammatory processes underlying the disease, or (b) have a beneficial role and participate in the repair mechanisms activated to contrast the neurodegeneration. Moving from this first hypothesis, I also postulated that fMCs could have a different effect according to their sexual genotype. In fact, the available literature on the role of sexual chromosomes in modulating the immune system suggests that male and female genotype could influence differently the molecules and cells involved in the immune response (Smith-Bouvier *et al.*, 2008). Proceeding from this statement, I hypothesised that XY fMCs and XX fMCs could have a different effect on MS.

Being the available data on MS and microchimerism limited, my first project focused on the analysis of epidemiological data from a large population of MS patients to generate a hypothesis. The aim of my first project was then to evaluate a large population of MS patients and to compare their disease features to estimate whether there was any clinical, radiological, and/or paraclinical difference according to their pregnancy status and the sex of their offspring, two indirect markers of microchimeric cells. Starting from the results obtained during this first project, I would have generated a hypothesis and designed a prospective study on a smaller population of patients.

3.2. Population, methods and materials

I reviewed data from a previous population-based study (Ragonese *et al.*, 2022) to extract information about MS female patients in the provinces of Palermo and Trapani. In the

population-based study, we collected data using the medical records of all the MS Centres in the provinces of Palermo and Trapani, including:

- the Centre for the Diagnosis and Treatment of MS and Demyelinating Disorders of the University Hospital "Paolo Giaccone", Palermo,
- the MS Centre of "Villa Sofia" Hospital, Palermo,
- the MS Centre of "Istituto Giglio" Hospital, Cefalù (PA).

Medical records and datasets were used to review the MS diagnosis according to the revised McDonald diagnostic Criteria (Thompson *et al.*, 2018) for all the identified patients. Data collected from MS Centre databases were cross-referenced to identified duplicated patients. We reviewed MS data on onset and diagnosis to select all the patients with a confirmed diagnosis of MS on the established day of prevalence (30th June 2018). Finally, we contacted the Neurology Units of "Buccheri La Ferla" Hospital (Palermo) and "San Antonio Abate" Hospital (Trapani), and the Pediatric Neuropsychiatric Hospital "Di Cristina" (Palermo) to collect further information on identified patients and check that no additional patients were missing. Overall, we identified 2,557 patients, including 1,729 women and 828 men.

Subsequently, I reviewed data from the identified patients and selected all the female patients attending the Centre for the Diagnosis and Treatment of MS and Demyelinating Disorders of the University Hospital "Paolo Giaccone", Palermo. Overall, I identified 1,458 female patients. Unfortunately, the running of my first project corresponds to the spreading of Coronavirus disease (COVID19) Pandemic, due to which, it was not feasible to directly test patients for microchimeric cells. Therefore, I proceeded from the robust available data on the association between pregnancy and microchimerism and classified patients according to their obstetric history. Using medical records and telephone interviews, I obtained information on pregnancy history for a subgroup of 354 patients, who were classified as follow:

- 87 nulliparous subjects supposedly without microchimeric cells (NLp),
- 188 subjects with history of at least a male pregnancy and supposedly carrying XY microchimeric cells (XYp),
- 79 subjects with history of only female pregnancy and supposedly carrying XX microchimeric cells, but not XY microchimeric cells (XXp).

As some patients have had a pregnancy in-between onset and last clinical follow-up, I also collected data on patients' pregnancy status at disease onset and diagnosis. I obtained data on pregnancy status at disease onset for 366 patients, distributed as follow:

- 203 patients were NLp,
- 116 subjects had at least a male pregnancy (XYp),
- 47 subjects had only female pregnancy (XXp).

None of the patients had changed her status between onset and diagnosis.

Finally, I used the medical records of our MS Centre to retrospectively collect clinical, radiological, and paraclinical data at onset, diagnosis, and last clinical follow-up for this large cohort of patients. The following data were collected:

- presence and type of comorbidity;
- age and disease onset;
- type of clinical presentation at onset;
- functional system(s) involved at first clinical episode;
- severity of clinical symptoms at first clinical episode;
- recovery after first clinical episode;
- treatment with steroid at first clinical episode;
- magnetic resonance imaging (MRI) features at diagnosis, including number of MRI brain T1-weighted (T1w) and T2-weighted (T2w) lesions and number of T1w gadolinium enhancing (Gd+) lesions and number of MRI spine T1w, T2w, and Gd+ lesions;
- data on visual evoked potentials (VEP), brainstem acoustic evoked potentials (BAEP), sensory evoked potentials (SEP), and motor evoked potentials (MEP) at diagnosis;
- presence and number of oligoclonal bands (OCBs) in the cerebrospinal fluid (CSF) and diagnosis;
- Link index in the CSF at diagnosis;
- disease duration at the first access to a MS Centre,
- Expanded Disability Status Scale (EDSS) score and ambulation score at EDSS at year 3, 5, and 10 of disease duration;
- age and disease duration at last clinical follow-up;

- EDSS score and functional system scores at last clinical follow-up;
- total number of relapses and annual relapse rate (ARR);
- total number of previous disease-modifying treatments (DMTs);
- MRI features at last radiological follow-up, including number of MRI brain T1w, T2w, and Gd+ lesions and number of MRI spine T1w, T2w, and Gd+ lesions.

The study was conducted in accordance with the International Conference on Harmonisation guidelines for Good Clinical Practice and the Declaration of Helsinki. All patients gave informed consent upon data collection and analysis.

3.3. Statistical analysis

Demographic, radiological, clinical, and paraclinical characteristics of patients were summarised through counts and percentages for categorical variables. Quantitative variables were synthesised through mean ± standard deviation (SD) and median and interquartile range (IQR) when the variable distribution was asymmetric. The analysis of data at last clinical follow-up was performed considering the pregnancy status at the last clinical assessment, while data at disease onset and diagnosis were analysed according to the pregnancy status of patients before disease onset.

ANOVA test were performed for comparisons of continuous variables and Chi-square test for trend were used to compare categorical variables between nulliparous women, women with at least one male son, and women with exclusively female daughters ($p < 0.05$). Bonferroni correction was applied to reduce the chances of obtaining type I errors. Multiple regression models with adjustments for age and disease duration were performed to evaluate the relationship between the sex of offspring and disease features, testing the possible effects of confounding or modification factors. Additional adjustments for age at disease onset, number of previous DMTs, and number of previous pregnancies were performed where applicable.

Pearson correlation coefficient and Spearman's rank correlation were used to evaluate the linear and non-linear correlations between disease features and microchimeric status. Correlation matrixes will be also applied to evaluate the relationships between multiple variables.

3.4. Results

3.4.1. Participant characteristics

Overall, I obtained data on obstetric history for 354/1,458 (24.28%) patients: 87/354 (24.58%) patients were nulliparous (NLp), while 267/354 (75.42%) had a previous pregnancy, among whom 188/354 (53.11%) had at least a male pregnancy (XYp), while 79/354 (22.32%) had only female daughters (XXp). For the analysis of data at onset and diagnosis, I included 366/1,458 (25.10%) patients for whom I obtained data on pregnancy history at first clinical episode. At onset and diagnosis, 203/366 (55.46%) patients were nulliparous (NLp), 116/366 (31.69%) had at least a male pregnancy (XYp), while 47/366 (12.84%) had only female daughters (XXp).

The NLp had a mean age of 40.4 ± 11.1 years and were younger than XYp (48.0 ± 10.1 years; coeff = 7.563, p-value <0.001, 95%CI = 4.857 to 10.268) and XXp (48.2 ± 11.3 years; coeff = 7.740, p-value <0.001, 95%CI = 4.498 to 10.983). I did not find any difference comparing the prevalence of comorbidity and number of comorbidities between the three groups (***Table 3.1***).

Characteristics	NLp (n=87)	XYp (n=188)	XXp (n=79)	p-value[§]
Demographic characteristics				
Age (years), mean ± sd	40.4 ± 11.1	48.0 ± 10.1	48.2 ± 11.3	p <0.001
Disease duration (years), mean ± sd	14.9 ± 9.8	16.0 ± 10.2	16.6 ± 11.0	p = 0.579
Comorbidity, prevalence (%)	8/87 (9.20%)	10/188 (5.32%)	5/79 (6.33%)	p = 0.478
Comorbidity (number), mean ± sd	1.63 ± 0.74	1.70 ± 0.67	1.00 ± 0.00	p = 0.136

[§]Comparison between the three groups using ANOVA test

Table 3.1. *Demographic characteristics of the enrolled population.*

3.4.2. Differences in clinical, paraclinical, and radiological features at onset and diagnosis between the three groups

I collected clinical, radiological and paraclinical data at onset and diagnosis (***Table 3.2*** and ***Table 3.3***). At disease onset, NLp were younger than XYp (24.0 ± 6.8 years vs 36.6 ± 7.7 years; coeff = 12.670, p-value <0.001, 95%CI = 10.943 to 14.397) and XXp (24.0

± 6.8 years vs 36.2 ± 8.9 years; coeff = 12.186, p-value <0.001, 95%CI = 9.783 to 14.589). I found that pyramidal onset was more frequent in XXp patients when compared to NLp (40/191 [20.94%] vs 16/43 [37.21%]; coeff = 0.172, p-value = 0.045, 95%CI = 0.004 to 0.340). The former group also reported a more frequent use of steroids (1000 mg/die of methylprednisolone for 5 days) at first relapse when compared to NLp (55/59 [93.22%] vs 10/10 [100.00%], coeff = -0.232, p-value = 0.022, 95%CI = 0.034 to 0.430). However, this difference disappeared when the comparison was adjusted for the severity of the first relapse (mild vs moderate vs severe) (coeff = 0.396, p-value = 0.083, 95%CI = -0.054 to 0.848). The remaining trends of distribution for clinical presentations at onset (***Table 3.2***) (all p-value >0.050) were similar in the three groups (all p >0.050).

Comparing the paraclinical and MRI data at diagnosis, I did not identify any difference in abnormalities of VEP, BAEP, SEP, and MEP, frequency and number of OCBs in the CSF, Link Index, and number of T1w, T2w, and Gd+ lesions (all p-value >0.050) (***Table 3.3***).

At the first visit at MS Centre, the disease duration was shorter in both XYp (34.7 ± 56.2 months) and XXp (49.0 ± 60.6 months) when compared to NLp (59.0 ± 87.9 months; *NLp vs XYp*: coeff = -75.149, p-value <0.001, 95%CI = -92.889 to -57.409; *NLp vs XXp*: coeff = -62.931, p-value <0.001, 95%CI = -86.322 to -39.540). The difference was confirmed also after adjusting for severity of the first MS attack (*NLp vs XYp*: coeff = -115.439, p-value <0.001, 95%CI = -152.037 to -78.841; *NLp vs XXp*: coeff = -96.608, p-value <0.001, 95%CI = -148.438 to -44.778). I did not find any other difference comparing clinical data at the first visit at MS Centre (all p-value >0.050).

Disease features at onset	NLp (n=203)	XYp (n=116)	XXp (n=47)	NLp vs XYp^	NLp vs XXp^	XYp vs XXp^
Age at onset (years), mean ± sd	24.0 ± 6.8	36.6 ± 7.7	36.2 ± 8.9	**p < 0.001**	**p < 0.001**	p = 0.711
Functional system involved at onset, prevalence (%)						
Supratentorial	35/40 (87.50%)	15/23 (65.22%)	6/9 (66.67%)	p = 0.882	p = 0.868	p = 0.758
Brainstem	67/71 (94.37%)	28/33 (84.85%)	15/17 (88.24%)	p = 0.920	p = 0.872	p = 0.797
Visual	52/57 (91.23%)	23/31 (74.19%)	6/9 (66.67%)	p = 0.682	p = 0.523	p = 0.697
Spinal	76/80 (95.00%)	51/55 (92.73%)	21/22 (95.45%)	p = 0.690	p = 0.929	p = 0.648
Progression at onset, prevalence (%)	8/189 (4.23%)	14/108 (12.96%)	3/44 (6.82%)	p = 0.605	p = 0.112	p = 0.216
Functional system involved at first episode, prevalence (%)						
Visual	47/191 (24.61%)	22/105 (20.95%)	5/43 (11.63%)	p = 0.443	p = 0.552	p = 0.200
Brainstem	59/191 (30.89%)	32/105 (30.48%)	11/43 (25.58%)	p = 0.222	p = 0.086	p = 0.434
Pyramidal	40/191 (20.94%)	34/105 (32.38%)	16/43 (37.21%)	p = 0.085	**p = 0.045**	p = 0.504
Cerebellum	16/191 (8.38%)	4/105 (3.81%)	1/43 (2.33%)	p = 0.062	p = 0.074	p = 0.760
Sensory	83/191 (43.46%)	45/105 (42.86%)	21/43 (48.84%)	p = 0.477	p = 0.183	p = 0.419
Sphincter	10/191 (5.24%)	9/105 (8.57%)	2/43 (4.65%)	p = 0.575	p = 0.855	p = 0.504
Cortical	3/191 (1.57%)	3/105 (2.86%)	3/43 (6.98%)	p = 0.895	p = 0.139	p = 0.146
Recovery after first relapse, prevalence (%)						
No recovery	8/157 (5.10%)	5/88 (5.68%)	1/33 (3.03%)	p = 0.861	p = 0.329	p = 0.373
Incomplete	27/157 (17.20%)	30/88 (34.09%)	10/33 (30.30%)			
Complete	122/157 (77.7%)	53/88 (60.23%)	22/33 (66.67%)			
Severity of first relapse, prevalence (%)						
Mild	19/56 (33.93%)	8/28 (28.57%)	3/11 (27.27%)	p = 0.858	p = 0.618	p = 0.473
Moderate	34/56 (60.71%)	19/28 (67.86%)	6/11 (54.55%)			
Severe	3/56 (5.36%)	1/28 (3.57%)	2/11 (18.18%)			
Treatment with steroid at first relapse, prevalence (%)	55/59 (93.22%)	31/35 (88.57%)	10/10 (100.00%)	p = 0.143	**p = 0.022**	p = 0.152

^Comparison between the groups using a linear regression model with adjustment for age at onset

Table 3.2. *Comparison of multiple sclerosis features at onset between the three groups.*

Disease features at diagnosis	NLp (n=203)	XYp (n=116)	XXp (n=47)	NLp vs XYp^	NLp vs XXp^	XYp vs XXp^
Age at diagnosis (years), mean ± sd	28.0 ± 8.2	38.4 ± 8.1	39.1 ± 8.2	p = 0.856	p = 0.385	p = 0.447
MRI T1w brain lesions, prevalence (%)	38/54 (70.37%)	27/39 (69.23%)	4/6 (66.67%)	p = 0.459	p = 0.415	p = 0.666
MRI T1w Gd+ brain lesions, prevalence (%)	39/73 (54.42%)	18/44 (40.91%)	4/7 (57.14%)	p = 0.768	p = 0.244	p = 0.289
MRI T2w brain lesions (number), prevalence (%)						
0 lesions	1/76 (1.32%)	1/51 (1.96%)	0/15 (0.00%)	p = 0.918	p = 0.393	p = 0.440
1-2 lesions	9/76 (11.84%)	3/51 (5.88%)	2/15 (13.33%)			
3-8 lesions	18/76 (23.68%)	11/51 (21.57%)	5/15 (33.33%)			
≥9 lesions	48/76 (63.16%)	36/51 (70.59%)	8/15 (53.33%)			
MRI T1w spine lesions, prevalence (%)	3/53 (5.66%)	5/45 (11.11%)	2/18 (11.11%)	p = 0.655	p = 0.700	p = 0.987
MRI T1w Gd+ spine lesions, prevalence (%)	9/67 (13.43%)	11/49 (22.45%)	1/18 (5.56%)	p = 0.291	p = 0.421	p = 0.087
MRI T2w spine lesions (number), prevalence (%)						
0 lesions	31/90 (34.44%)	20/58 (34.48%)	8/26 (30.77%)	p = 0.286	p = 0.318	p = 0.901
1lesion	36/90 (40.00%)	25/58 (43.10%)	13/26 (50.00%)			
≥2 lesions	23/90 (25.56%)	13/58 (22.41%)	5/26 (19.23%)			
Link index, mean ± sd	1.10 ± 1.18	1.09 ± 0.48	0.68 ± 0.14	p = 0.806	p = 0.205	p = 0.110
Positive OCBs, prevalence (%)	111/143 (77.6%)	66/83 (79.52%)	28/33 (84.85%)	p = 0.198	p = 0.228	p = 0.850
Number of OCBs, mean ± sd	4.74 ± 2.57	3.85 ± 2.19	5.14 ± 3.80	p = 0.154	p = 0.867	p = 0.250
Abnormal VEP, prevalence (%)	23/52 (44.23%)	21/37 (56.76%)	8/14 (57.14%)	p = 0.130	p = 0.234	p = 0.985
Abnormal BAEP, prevalence (%)	8/40 (20.00%)	4/37 (10.81%)	2/9 (22.22%)	p = 0.111	p = 0.654	p = 0.459
Abnormal SEP, prevalence (%)	10/32 (31.25%)	11/32 (34.38%)	3/10 (30.00%)	p = 0.611	p = 0.394	p = 0.585
Abnormal MEP, prevalence (%)	2/16 (12.50%)	6/19 (31.58%)	3/9 (33.33%)	p = 0.394	p = 0.519	p = 0.949

^Comparison between the groups using a linear regression model with adjustment for age at onset

Table 3.3. *Comparison of multiple sclerosis features at diagnosis between the three groups.*

3.4.3. Differences in clinical, paraclinical, and radiological features at last clinical follow-up between the three groups

Evaluating clinical, radiological and paraclinical data at last clinical follow-up (***Table 3.4***), I found that NLp were younger than XYp (40.4 ± 11.1 vs 48.1 ± 10.1 years; coeff = 7.563, p-value <0.001, 95%CI = 4.858 to 10.268) and XXp (40.4 ± 11.1 vs 48.2 ± 11.3 years; coeff = 7.740, p-value <0.001, 95%CI = 4.498 to 10.983). NLp group had also a lower disease duration when compared with XYp (14.9 ± 9.8 vs 16.0 ± 10.2 years; coeff = -3.151, p-value = 0.009, 95%CI = -5.502 to -0.802). I found a similar difference between NLp with XXp, but it did not reach the statistical significance (14.9 ± 9.8 vs 16.6 ± 11.0 years; coeff = -2.666, p-value = 0.060, 95%CI = -5.448 to 0.115).

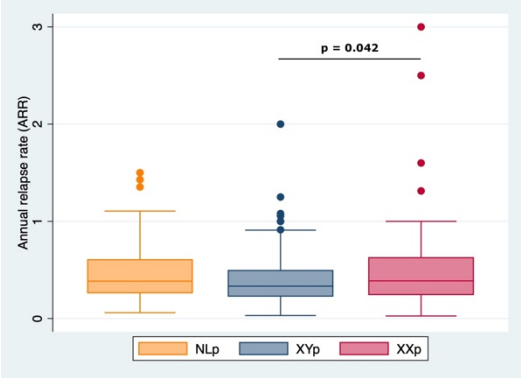
At follow-up, I found some differences between XYp and XXp (***Table 3.4***):

- XXp had higher ARR than XYp (0.42 ± 0.31 vs 0.52 ± 0.50; coeff = 0.100, p-value = 0.042, 95%CI = 0.004 to 0.197) (***Graph 3.1***).
- XXp registered higher EDSS scores both at 3 years (2.59 ± 2.00 vs 3.45 ± 2.20; coeff = 0.879, p-value = 0.023, 95%CI = 0.121 to 1.637) and 5 years of disease duration (2.57 ± 2.02 vs 3.25 ± 2.42; coeff = 0.826, p-value = 0.039, 95%CI = 0.041 to 1.611) (***Graph 3.2*** and ***Graph 3.3***). The differences were confirmed after adjusting for age at onset and number of previous DMTs (*year 3*: coeff = 0.924, p-value = 0.017, 95%CI = 0.167 to 1.681; *year 5*: coeff = 0.831, p-value = 0.041, 95%CI = 0.036 to 1.625).
- the mean ambulation score at EDSS at 3 years of disease duration was higher in XXp compared to XYp (0.82 ± 1.85 vs 1.75 ± 2.49; coeff = 0.862, p-value = 0.034, 95%CI = 0.067 to 1.657). The difference was confirmed after adjusting for age at onset and number of previous DMTs (coeff = 0.877, p-value = 0.029, 95%CI = 0.091 to 1.663).

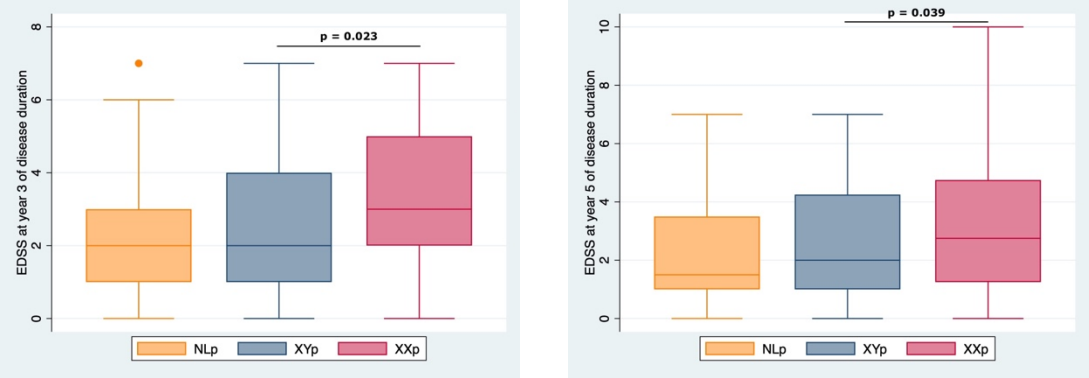
Disease features at last clinical visit	NLp (n=87)	XYp (n=188)	XXp (n=79)	NLp vs XYp^	NLp vs XXp^	XYp vs XXp^
Age (years), mean ± sd	40.4 ± 11.1	48.1 ± 10.1	48.2 ± 11.3	**p <0.001**	**p <0.001**	p = 0.901
Disease duration (years), mean ± sd	14.9 ± 9.8	16.0 ± 10.2	16.6 ± 11.0	**p = 0.009**	p = 0.060	p = 0.683
Age at conversion to progressive phenotype (years), mean ± sd	42.3 ± 8.5	43.4 ± 9.2	44.5 ± 11.4	p = 0.072	p = 0.247	p = 0.599
Disease duration at conversion to progressive phenotype (years), mean ± sd	163.8 ± 141.4	117.9 ± 213.9	227.3 ± 384.2	p = 0.505	p = 0.484	p = 0.130
EDSS at 3 years of disease duration, median (range)	2.0 (0.0 – 7.0)	2.0 (0.0 – 7.0)	3.0 (0.0 – 7.0)	p = 0.767	p = 0.084	**p = 0.023**
Ambulation score at 3 years of disease duration, median (range)	0.0 (0.0 – 6.0)	0.0 (0.0 – 7.0)	0.0 (0.0 – 7.0)	p = 0.331	p = 0.304	**p = 0.034**
EDSS at 5 years of disease duration, median (range)	1.5 (0.0 – 7.0)	2.0 (0.0 – 7.0)	2.75 (0.0 – 10.0)	p = 0.319	p = 0.307	**p = 0.039**
Ambulation score at 5 years of disease duration, median (range)	0.0 (0.0 – 8.0)	0.0 (0.0 – 6.0)	0.0 (0.0 – 7.0)	p = 0.419	p = 0.506	p = 0.130
EDSS at 10 years of disease duration, median (range)	2.0 (0.0 – 7.5)	2.5 (0.0 – 7.25)	3.75 (0.0 – 7.0)	p = 0.507	p = 0.484	p = 0.782
Ambulation score at 10 years of disease duration, median (range)	0.0 (0.0 – 6.0)	0.0 (0.0 – 7.0)	0.5 (0.0 – 7.0)	p = 0.962	p = 0.692	p = 0.622
ARR (number), mean ± sd	0.48 ± 0.31	0.41 ± 0.31	0.51 ± 0.49	p = 0.991	p = 0.090	**p = 0.042**
MRI T1w brain lesions, prevalence (%)	71/85 (83.53%)	33/42 (78.57%)	12/15 (80.00%)	p = 0.684	p = 0.670	p = 0.914
MRI T1w Gd+ brain lesions, prevalence (%)	12/121 (9.92%)	8/64 (12.50%)	5/22 (22.73%)	p = 0.558	p = 0.897	p = 0.692
MRI T2w brain lesions (number), prevalence (%) **0 lesions** **1-2 lesions** **3-8 lesions** **≥9 lesions**	 0/28 (0.00%) 0/28 (0.00%) 5/28 (17.86%) 23/28 (82.14%)	 1/66 (1.52%) 1/66 (1.52%) 13/66 (19.70%) 51/66 (77.27%)	 0/21 (0.00%) 0/21 (0.00%) 4/21 (19.05%) 17/21 (80.95%)	p = 0.337	p = 0.627	p = 0.166
DMTs (number), mean ± sd	2.41 ± 1.74	2.46 ± 1.84	2.11 ± 1.71	p = 0.871	p = 0.241	p = 0.117

^Comparison between the groups using a linear regression model with adjustment for age and disease duration

Table 3.4. *Comparison of multiple sclerosis features at last clinical follow-up between the three groups.*



Graphs 3.1. *Differences in annual relapse rate in the three groups.*



Graphs 3.2 and 3.3. *Differences in EDSS score at year 3 (**left**) and year 5 (**right**) of disease duration in the three groups.*

3.5. Discussion

The aim of this hypothesis-generating, retrospective study was to indirectly evaluate the role of fMCs in MS investigating the impact of the sex of offspring on the clinical, paraclinical, and radiological features of the disease. Overall, I found that, at disease onset, NLp were younger than XYp and XXp and that the same group reported a longer disease duration when attending the first visit at MS Centre. In addition, data showed that NLp had less frequently a pyramidal onset when compared to XXp. Comparing XYp and XXp patients, I observed that XXp had higher ARR, a higher disability after 3 and 5 years of disease duration, and more severe ambulation scores at EDSS at 3 years of disease duration.

The effect of fetal microchimerism in long-term maternal health is still controversial and its role in MS is still uncertain. Pregnancy is considered a protective factor in MS, an effect likely due to the modulation of the immune system and to the changes that occur in the brain during pregnancy (McCombe and Greer, 2013). In my analysis, I observed that NLp had a younger age at disease onset. A recent study by Nguyen et al. to investigate the association of pregnancy with time to clinically isolated syndrome (CIS) onset reported that women with previous pregnancies and childbirths had a later disease onset compared with women who had never been pregnant and those who had never given birth (Nguyen *et al.*, 2020). The authors observed a median delay of 3.3 years in women with previous pregnancies and 3.4 years in women with previous childbirths, while the number of previous pregnancies and childbirth was not associated with a later onset (Nguyen *et*

al., 2020). The group did not propose any causative factors for explaining this association. However, several pregnancy-associated changes, including circulating hormones and epigenetic changes, have been previously suggested as protective variables in pregnancy and it is possible to speculate that the same biological mechanisms could suppress or postpone the first demyelinating episode (McCombe and Greer, 2013; Graves, 2020). Moreover, considering that I detected this difference both comparing NLp vs XYp and NLp vs XXp, it is likely that several pregnancy-related factors could cooperate in delaying the disease onset. I hypothesised that microchimerism could be one of these modulating factors. However, due to the retrospective nature of the study, I could not directly investigate the presence of XX and XY fMCs at onset and, therefore, it was not possible to determine whether the findings were related to fMCs and/or other pregnancy-related factors. Nonetheless, being microchimerism an established phenomenon in pregnancy, it is possible to speculate that, in a multifactorial background and likely interacting with other pregnancy-related factors, fMCs could participate in the modulation of the immune system and delay the disease onset. This finding would support the postulation that fMCs could have a beneficial role more than triggering MS onset.

It is interesting to note that XXp patients had more frequently pyramidal symptoms at onset when compared to NLp and, accordingly to more severe manifestations, had been treated more frequently with steroids at the first relapse. Older age at symptom onset and the presence of pyramidal signs have been associated with aggressive phenotypes of MS and a worst prognosis (Malpas *et al.*, 2020). However, in our population XXp patients had not developed higher disability at follow-up or experienced a quicker worsening of the disease. This outcome might be related to several factors that could occur during the disease course and it is not possible, considering the retrospective collection of data, to identify a univocal reason for the observed phenomenon.

Study results showed that patients with a previous pregnancy had a shorter disease duration when attending the first visit at MS Centre. The data let hypothesise that this difference could be related to a *selection bias*. In fact, it is likely that patients with a previous pregnancy are more medicalised that nulliparous patients and tend to access more frequently to healthcare facilities. This phenomenon could be also associated to the older age of parous patients at onset.

Finally, the study detected some differences in comparing patients with previous male pregnancies and patients with only previous female pregnancies. Indeed, I found that XXp had higher inflammatory activity and had experienced a more rapid disability accrual. Interestingly, these differences were confirmed after adjusting for several confounding factors, including, age, disease duration, age at onset, and number of previous DMTs. These data suggest that the disease could be more aggressive in patients with female pregnancies. Considering that I did not identify any significant difference comparing the two group at baseline and diagnosis and that both XYp and XXp had at least a previous pregnancy, I speculated that these unbalances could be related to the sex of offspring. To best of my knowledge, the changes occurring during pregnancy, such as hormonal changes and epigenetic modification, are the same in both male and female gravidity. On contrary, fMCs are genetically different in male and female pregnancies and therefore these cells could be a good candidate for the role. Indeed, previous studies have demonstrated the role of sexual chromosome in modulating the immune system and authors agreed on the disease-promoting effect of XX chromosome compared to XY complement (Smith-Bouvier *et al.*, 2008). Evidence suggests that the female immune response would be more robust that the male one (Nicot, 2009; Voskuhl, Sawalha and Itoh, 2018), while XY genotype could be associated to neurodegeneration and a subsequent more severe long-term disability (Smith-Bouvier *et al.*, 2008; Voskuhl, Sawalha and Itoh, 2018).

The main limitation of this study was the retrospective collection of data. The onset of COVID19-Pandemic made it necessary to choose this design, due to which (1) it was not possible to collect blood samples for the direct research of fMCs at onset and last clinical visit; and (2) data were collected from clinical databases. Medical records are reliable for clinical and demographic data that are directly collected and entered by the physician. On contrary, radiological data are generally limited and not always accurate in clinical databases as they are translated from MRI reports by a clinical neurologist (Eder *et al.*, 2005). To overcome this limitation, I design a second study to analyse the MRI scans of the patients enrolled in this first project and evaluate the presence of radiological differences in the three groups.

To-date, only four studies were conducted to investigate the relationship between microchimerism and MS (Willer *et al.*, 2006; Bloch *et al.*, 2011; Jafarinia *et al.*, 2020; Snethen *et al.*, 2020). The first three studies reported a higher prevalence of fMCs in MS patients when compared to healthy controls (Willer *et al.*, 2006; Bloch *et al.*, 2011; Jafarinia *et al.*, 2020) and Snethen et al. speculated that a role of microchimeric cells in neuroprotection or regeneration in neurological diseases, such as MS, could not be ruled out. This first project had the aim (1) to investigate the association between the sex of offspring, considered as an indirect marker of XX and XY fMCs, and disease features and (2) to formulate a hypothesis on the effect of fMCs in MS. The available literature has not revealed the biological effects of fMCs on maternal health yet, but my preliminary findings suggested that the sex of foetus could modulate the inflammatory disease activity. Being this variable the only known distinctive factors between the groups in my classification, I hypothesised that (1) independently from the sexual chromosome bearing, fMCs could participate in the modulation of the immune system during pregnancy, and (2) that the sexual chromosome in the fMCs could produce a different modulation determining the differences observed in mothers with and without previous male pregnancies.

CHAPTER IV

A retrospective study to evaluate the impact of the sex of offspring on the radiological features in women with multiple sclerosis

4.1. Background and aims

In the previous Chapters, I have extensively reported that the available data concerning the role of microchimerism in multiple sclerosis (MS) are still limited. Pregnancy is considered to be a protective factor in MS (McCombe and Greer, 2013), but the effects of fetal microchimerism have not been defined yet (Willer *et al.*, 2006; Bloch *et al.*, 2011; Jafarinia *et al.*, 2020; Snethen *et al.*, 2020).

Over the last decade, several authors challenged the limited effectiveness of clinical measures in evaluating MS for both clinical and research purposes (Kuhlmann *et al.*, 2022). Indeed, mounting literature showed the inaccuracy of clinical definition of MS phenotypes for evaluating the complex pathological processes underlying the disease. Similarly, the Kurtzke Expanded Disability Status Scale (EDSS), largely used in clinical practice and research studies, has been long questioned as an inadequate instrument for monitoring disease activity and progression. Proceeding from these issues, it has become evident that the clinical evaluation should be integrated with non-clinical biomarkers (Kantarci, 2019). Among these, magnetic resonance imaging (MRI) has demonstrated to be an objective, sensitive and specific tool that measures outcomes and monitors patients' evolution over time (McFarland *et al.*, 2002). In fact, MRI is an essential instrument to diagnose MS, monitor disease progression, and evaluate the response to treatments (Inglese and Petracca, 2018). Furthermore, high improvements in MRI techniques have enabled the *in vivo* evaluation of inflammatory and neurodegenerative mechanisms,

making it possible to better investigate the interaction between these processes in the disease (Sastre-Garriga *et al.*, 2020a).

The developments of conventional MRI sequences provided sensitive and reproducible tools for assessing lesion load and brain and spinal cord volumes, offering reliable markers of disease evolution (De Stefano, Battaglini and Smith, 2007). Monitoring lesion load and its changes over time has become a crucial aspect in evaluating the focal, inflammatory disease activity at diagnosis and throughout disease course (Wattjes *et al.*, 2021). Similarly, short-term changes in brain and spinal cord volumes have been established as valuable predictors of long-term clinical evolution (Sastre-Garriga *et al.*, 2020a). Finally, the translation of MRI measures into clinical practice has narrowed the gap between biological and clinical features and provided effective descriptors both in managing MS patients and investigating MS pathology (Filippi, Absinta and Rocca, 2013; Absinta, Sati and Reich, 2016; Inglese and Petracca, 2018).

The results reported in the previous chapter suggested that the sex of offspring may influence the inflammatory activity of the disease and its evolution over time. Nonetheless, I had not found any effect on radiological outcomes collected from medical records. The available literature suggests that medical records are sufficiently reliable for clinical and demographic data which are directly collected and entered by the physician. Conversely, radiological data are generally limited and not always accurate in clinical database. These data are frequently translated from MRI report by a clinical neurologist and, particularly for those patients with high lesion load, is frequently difficult to convert clinical reports in standardised data (Eder *et al.*, 2005). Furthermore, clinical MRI report do not provide any measure for brain volumes, a fundamental biomarkers in monitoring the disease progression (De Stefano, Battaglini and Smith, 2007). Considering the potential of MRI in evaluating the biological processes underlying MS and to overcome these limitations, I design a second retrospective, observational study to analyse the MRI scans of the patients enrolled in my first project and evaluate the presence of radiological differences in the three groups.

4.2. Population, methods and materials

I identify from the previous study 54 patients attending the Centre for the Diagnosis and Treatment of Multiple Sclerosis and Demyelinating Disorders of the University Hospital "Paolo Giaccone" of Palermo, Italy and fulfilling the diagnosis of MS according to the revised McDonald criteria (Polman *et al.*, 2011; Thompson *et al.*, 2018). Patients were categorised according to their obstetric history in 3 groups:

- nulliparous subjects supposedly without fMCs (NLp),
- subjects with only female daughters, who were supposed to carry only XX fMCs (XXp),
- subjects with at least a male son, who were supposedly carriers of XY fMCs (XYp).

At recruitment time, 26 patients were nulliparous, 8 had only female daughters, and 20 had at least a male son. As some patients have had a pregnancy after disease onset, I also collected data on patients' obstetric history at disease onset and diagnosis: I found that, at the first episode, 36 patients were nulliparous, 7 had only female daughters, and 11 had at least a male son. None of the patients had changed her status between onset and diagnosis. I used medical records and interviews to collect additional data on the obstetric history of patients, including:

- age at menarche and regularity of period;
- age and type of menopause;
- number and duration of pregnancies;
- type of delivery;
- number and type of abortions and duration of pregnancy before abortion;
- duration and type of breastfeeding;
- type and duration of contraception.

Patients were included if at least one 1.5T magnetic resonance (MR) scan was available. MRI scan should include three-dimensional (3D) T2-weighted (T2w) fluid attenuated inversion recovery (FLAIR) with fat saturation (Fat Sat) and 3D T1-weighted (T1w) fast spoiled gradient echo (FSPGR) pulse sequences or 3D Turbo Field Echo (TFE) pulse sequences. MRI images were acquired with two MR scanners:

- Signa HDxt – GE Medical System, Milwaukee, WI, USA and Achieva,

- Philips Medical Systems, Cleveland, OH, USA.

Both systems were equipped with an 8-channel phased-array head coil. On GE scanner, 3D T2w-FLAIR Fat Sat and 3D T1w FSPGR pulse sequences were acquired, while Philips scanner was set to acquire 3D T2w-FLAIR Fat Sat and 3D TFE pulse sequences (technical parameters reported in ***Table 4.1***).

MRI data for lesion number and volume was obtained. Lesion load volume was segmented from 3D T2w FLAIR sequences by the lesion growth algorithm (Schmidt *et al.*, 2012) as implemented in the Lesion Segmentation Tool (LST) (www.statistical-modelling.de/lst.html) for Statistical Parametric Mapping (SPM) software. Whole-brain and regional grey matter and white matter volumes were estimated on 3D T1w pulse sequences using FreeSurfer software suite (https://surfer.nmr.mgh.harvard.edu/). I applied the Aseg, Aparc, Wmparc Free Surfer Atlases to obtain information on cortical areas, subcortical areas, and white matter volumes (***Appendix 1***), respectively. Age and disease duration on MRI scan day was obtained.

MR pulse sequences	Slice thickness (mm)	TR (ms)	TE (ms)	IT (ms)	Matrix	NEX	FOV (cm)
3D T1w FSPGR (GE)	1	12.4	5.2	450	256X256	1	25.6x25.6
3D T2w FLAIR Fat Sat (GE)	1.2	6000	126	1860	224x224	1	26.0x26.0
3D T1w TFE (Philips)	1	7.4	3.4	--	256x256	1	25.6x25.6
3D T2w FLAIR Fat Sat (Philips)	1.12	4800	281	1650	228x225	1	26.0x26.0

Table 4.1. *MRI acquisition protocol technical details.*

Medical records and questionnaires were used to collect the following information:

- demographic information, including age, working status, and level of Education;
- prevalence of comorbidity, including every type of comorbidity and specifically other autoimmune (AI) comorbidity;
- family history for MS or other AI diseases;
- clinical data at onset, including:
    - age at onset,
    - type of clinical presentation at onset;
    - functional system(s) involved at first clinical episode;

    - recovery after first clinical episode;
- clinical, radiological, and paraclinical data at diagnosis, including:
    - time-gap between onset and diagnosis,
    - number of MRI brain T1w, T2w, and gadolinium enhancing (Gd+) lesions and number of MRI spine T1w, T2w, and Gd+ lesions at diagnosis;
    - visual evoked potentials (VEP), brainstem acoustic evoked potentials (BAEP), sensory evoked potentials (SEP), and motor evoked potentials (MEP) at diagnosis;
    - presence and number of oligoclonal bands (OCBs) in the cerebrospinal fluid (CSF) and diagnosis;
- clinical features at last clinical follow-up, including:
    - age and disease duration at last clinical follow-up;
    - EDSS score and functional system scores at last clinical follow-up;
    - number of relapses withing 3 years from onset;
    - total number of relapses and annual relapse rate (ARR);
    - clinical phenotype at last clinical follow-up;
    - time-gap between onset and conversion to progressive MS,
    - total number of previous disease-modifying treatments (DMTs) and number of previous first and second line DMTs;
- clinical features during and after pregnancy, including:
    - time-gap between pregnancy and disease onset;
    - number of relapses during pregnancy;
    - number of relapses during 6 months and 3 years within delivery (post-partum);
    - EDSS score at last pregnancy;

The study was conducted in accordance with the International Conference on Harmonisation guidelines for Good Clinical Practice and the Declaration of Helsinki. All patients gave informed consent upon data collection and analysis.

4.3. Statistical analysis

Statistical analysis was performed using STATA software version 17.0 (StataCorp LLC 2021). Demographic, neuroradiological, clinical, and paraclinical characteristics of patients were summarised through counts and percentages for categorical variables. Quantitative variables were reported as mean ± standard deviation (SD) and median and interquartile range (IQR) when the variable distribution was asymmetric.

The analysis of neuroradiological data was performed considering the pregnancy status at the MRI (NLp vs XXp vs XYp at recruitment). I built a linear regression model with adjustments for age, disease duration, and MRI scan to evaluate the relationship between the sex of offspring and disease features at recruitment. Additional adjustments for age at disease onset, number of previous DMTs, and number of previous pregnancies were performed where applicable. Bonferroni correction was applied to reduce the chances of obtaining type I errors.

Data at disease onset and diagnosis were analysed according to the pregnancy status of patients before disease onset (NLp vs XXp vs XYp at onset). A linear regression model with adjustments for age at onset were applied to evaluate the association between the sex of offspring and disease features at onset and diagnosis. Bonferroni correction was applied to reduce the chances of obtaining type I errors.

An exploratory statistical analysis was performed re-classifying patients as follow: (a) nulliparous patients, (b) patients with only female daughters, (c) patients with only male sons, and (d) patients with male and female offspring. I built a linear regression model with adjustments for age, disease duration, and MRI scan to evaluate the relationship between the sex of offspring and disease features. Additional adjustments for number of previous DMTs and number of previous pregnancies were performed where applicable.

4.4. Results

4.4.1. Participant characteristics

Fifty-four consecutive patients were recruited in the study. NLp patients had a mean age of 34.7 ± 8.8 years and were younger than XXp (43.8 ± 12.6 years; coeff = 9.096, p-value = 0.014, 95%CI = 1.897 to 16.295) and XYp (40.4 ± 7.2 years; coeff = 5.696, p-value

0.036, 95%CI = 0.400 to 10.992). I did not find any difference comparing the prevalence of comorbidity and family history for MS or other autoimmune diseases between the three groups (***Table 4.2***).

When the obstetric history of patients with at least a child was reviewed, I found that the mean number of pregnancies was non-significantly lower in XXp compared to XYp (1.88 ± 1.46 vs 2.75 ± 1.29; p-value >0.05), while the time-gap between pregnancy and onset was similar in the two groups (48.7 ± 61.0 months vs 51.6 ± 48.2; p-value >0.05) (***Table 4.2***).

Demographic characteristics	NLp (n=26)	XXp (n=8)	XYp (n=20)	NLp vs XXp^	NLp vs XYp^	XXp vs XYp^
Age, mean ± sd§	34.7 ± 8.8	43.8 ± 12.6	40.4 ± 7.2	**p = 0.014***	**p = 0.036***	p = 0.374
Comorbidity, prevalence (%)	11/26 (42.31%)	7/8 (87.50%)	13/20 (65.00%)	p = 0.063	p = 0.241	p = 0.208
Others AI comorbidity, prevalence (%)	0/26 (0.00%)	2/8 (25.00%)	3/20 (15.00%)	p = 0.124	p = 0.222	p = 0.738
Family history for MS, prevalence (%)	2/25 (8.00%)	1/8 (12.50%)	3/20 (15.00%)	p = 0.734	p = 0.475	p = 0.870
Family history for non-MS AI diseases, prevalence (%)	3/25 (12.00%)	2/8 (25.00%)	3/20 (15.00%)	p = 0.386	p = 0.786	p = 0.550
Pregnancy (number), mean ± sd	-	1.88 ± 1.46	2.75 ± 1.29	-	-	p = 0.130
Total months on pregnancy, mean ± sd	-	15.00 ± 7.35	19.25 ± 7.35	-	-	p = 0.062

^ Comparison between the groups using a linear regression model with adjustment for age
§ Comparison between the groups not adjusted for age

Table 4.2. *Demographic characteristics of the enrolled population.*

4.4.2. Differences in clinical features at onset, diagnosis, and last clinical follow-up between the three groups

I collected clinical, radiological, and paraclinical data at onset, diagnosis, and last clinical follow-up (***Table 4.3*** and ***Table 4.4***). At onset, NLp patients were younger than XYp (23.8 ± 7.5 years vs 34.9 ± 6.8 years; coeff = 11.076, p-value <0.001, 95%CI = 5.816 to 16.335) (***Table 4.3***). The group was also younger than XXp, although the difference was not significant (23.8 ± 7.5 years vs 28.7 ± 9.2 years; p-value >0.050). Comparing NLp and XYp, I also observed that NLp had less frequently pyramidal involvement at onset (6/36 [16.67%] vs 5/11 [45.45%]; coeff = 0.288, p-value = 0.047, 95%CI = 0.004 to 0.572) and more frequently sensory involvement (20/36 [55.56%] vs 2/11 [18.18%]; coeff = -0.374, p-value = 0.029, 95%CI = -0.709 to -0.039) (***Table 4.3***). I did not find any additional difference between the group either at onset or diagnosis (all p-value >0.050).

At last clinical follow-up, NLp were again younger (34.7 ± 8.8 years) than XXp (43.8 ± 12.6 years; coeff = 9.096, p-value = 0.014, 95%CI = 1.897 to 16.295) and XYp (40.4 ± 7.2 years; coeff = 5.696, p-value = 0.036, 95%CI = 0.400 to 10.992). Data also showed that the percentage of patients with an EDSS score ≥3.0 was higher in NLp when compared with XYp (1/26 [38.46%] vs 6/19 [31.58%]; coeff = -0.277, p-value = 0.031, 95%CI = -0.527 to -0.026) (***Table 4.4***). This difference was confirmed when the analysis was adjusted for age at MS onset and number of previous DMTs (coeff = -0.267, p-value = 0.04195%CI = -0.523 to -0.012).

MS characteristics	NLp (n=36)	XXp (n=7)	XYp (n=11)	NLp vs XXp	NLp vs XYp	XXp vs XYp
			MS onset^			
Age, mean ± sd	23.8 ± 7.5	28.7 ± 9.2	34.9 ± 6.8	p = 0.126	**p <0.001****	p = 0.120
Polysymptomatic onset, prevalence (%)	10/36 (27.78%)	2/7 (28.57%)	2/11 (18.18%)	p = 0.966	p = 0.538	p = 0.630
Pyramidal symptoms, prevalence (%)	6/36 (16.67%)	1/7 (14.29%)	5/11 (45.45%)	p = 0.889	**p = 0.047***	p = 0.192
Visual symptoms, prevalence (%)	11/36 (30.56%)	3/7 (42.86%)	5/11 (45.45%)	p = 0.543	p = 0.378	p = 0.920
Sensory symptoms, prevalence (%)	20/36 (55.56%)	2/7 (28.57%)	2/11 (18.18%)	p = 0.183	**p = 0.029***	p = 0.630
Cerebellar symptoms, prevalence (%)	3/36 (8.33%)	0/7 (0.00%)	1/11 (9.09%)	p = 0.455	p = 0.935	p = 0.442
Brainstem symptoms, prevalence (%)	9/36 (25.00%)	3/7 (42.86%)	2/11 (18.18%)	p = 0.336	p = 0.658	p = 0.281
Sphincter symptoms, prevalence (%)	1/36 (2.78%)	1/7 (14.29%)	1/11 (9.09%)	p = 0.235	p = 0.433	p = 0.751
Progression at onset, prevalence (%)	1/36 (2.78%)	0/7 (0.00%)	1/11 (9.09%)	p = 0.728	p = 0.345	p = 0.442
Relapses within the first 3 years from onset, mean ± sd	3.28 ± 2.31	4.29 ± 1.70	2.64 ± 2.11	p = 0.275	p = 0.404	p = 0.102
Time-gap from delivery to onset (months), mean ± sd	-	48.71 ± 61.00	51.55 ± 48.20	-	-	p = 0.725
			MS diagnosis°			
Time-gap onset-diagnosis (months), mean ± sd	33.5 ± 86.5	83.7 ± 101.1	27.2 ± 41.9	p = 0.143	p = 0.824	p = 0.115
EDSS, median (range)	1.5 (0.0 – 6.0)	3.5 (1.5 – 4.0)	2.8 (0.0 – 6.0)	p = 0.537	p = 0.447	p = 0.175
Positive OCBs, prevalence (%)	21/27 (77.78%)	5/6 (83.33%)	5/9 (55.56%)	p = 0.834	p = 0.274	p = 0.125

^ Comparison between the groups using a linear regression model with adjustment for age at onset
° Comparison between the groups using a linear regression model with adjustment for age and disease duration at diagnosis

Table 4.3. *Comparison of multiple sclerosis features at onset and diagnosis between the three groups.*

MS characteristics	NLp (n=26)	XXp (n=8)	XYp (n=20)	NLp vs XYp^	NLp vs XXp^	XYp vs XXp^
Age, mean ± sd	34.7 ± 8.8	43.8 ± 12.6	40.4 ± 7.2	**p = 0.014**	**p = 0.036**	p = 0.374
Disease duration (years), mean ± sd	9.4 ± 9.5	16.2 ± 12.4	10.4 ± 7.6	p = 0.080	p = 0.730	p = 0.143
Progression, prevalence (%)	1/26 (3.85%)	0/8 (0.00%)	2/20 (10.00%)	p = 0.350	p = 0.671	p = 0.354
EDSS at follow-up, median (range)	1.5 (0.0 – 6.5)	4.3 (0.0 – 7.0)	2.3 (1.0 – 6.5)	p = 0.829	p = 0.245	p = 0.252
Patients with EDSS ≥3.0, prevalence (%)	10/26 (38.46%)	5/8 (62.50%)	6/19 (31.58%)	p = 0.669	**p = 0.031**	p = 0.201
Time from onset to EDSS 3.0 (months), mean ± sd	123.3 ± 151.8	159.8 ± 123.9	48.0 ± 40.4	p = 0.598	p = 0.255	p = 0.065
Annual relapse rate, mean ± sd	0.80 ± 0.45	0.60 ± 0.47	0.76 ± 0.47	p = 0.931	p = 0.949	p = 0.942
DMTs (number), mean ± sd	2.22 ± 1.20	2.00 ± 1.31	1.85 ± 1.04	p = 0.566	p = 0.473	p = 0.961
First-line DMTs (number), mean ± sd	1.52 ± 0.79	1.38 ± 0.52	1.35 ± 0.88	p = 0.479	p = 0.558	p = 0.775
Second-line DMTs (number), mean ± sd	0.70 ± 0.82	0.63 ± 0.92	0.50 ± 0.61	p = 0.905	p = 0.646	p = 0.825
DMTs before EDSS 3.0 (number), mean ± sd	1.77 ± 0.75	1.57 ± 1.13	2.14 ± 0.86	p = 0.706	p = 0.219	p = 0.241
DMTs before EDSS 6.0 (number), mean ± sd	2.20 ± 1.23	2.25 ± 1.26	2.00 ± 0.89	p = 0.463	p = 0.865	p = 0.888
EDSS at last pregnancy, median (range)	-	0.0 (0.0 – 1.0)	0.0 (0.0 – 2.0)	-	-	p = 0.692
Relapses within 6 months post-partum (number), mean ± sd	-	0.43 ± 0.53	0.00 ± 0.00	-	-	p = 0.801
Relapses within 3 years post-partum (number), mean ± sd	-	1.57 ± 1.27	1.09 ± 0.94	-	-	p = 0.502

^Comparison between the groups using a linear regression model with adjustment for age and disease duration

Table 4.4. *Comparison of multiple sclerosis features at last clinical follow-up between the three groups.*

4.4.3. Differences in brain MRI measures between the three groups

Main findings for whole-brain and regional grey and white matter volumes are reported in ***Table 4.5*** and ***Table 4.6***. All regional volumes are detailed in ***Appendix 2***, ***Appendix 3***, and ***Appendix 4***.

Data on white matter lesion number and volume, and on whole-brain grey matter, white matter, and subcortical volumes are reported in ***Table 4.5***. I observed that NLp had the highest number of lesions (*NLp*: 27.68 ± 26.86; *XXp*: 18.25 ± 9.25; *XYp*: 24.34 ± 30.49), while XYp registered the highest white matter lesion volume (*NLp*: 14.17 ± 22.50; *XXp*: 11.73 ± 9.99; *XYp*: 20.12 ± 37.10). However, the comparisons did not reach the statistical significance (all p-values >0.05). Similarly, I found that XXp had lower grey matter volume, white matter volume, subcortical structures volume, and cortical grey matter volume, although non-significantly (all p-values >0.05).

MRI whole-brain volumes	NLp (n=26)	XXp (n=8)	XYp (n=20)	NLp vs XXp^	NLp vs XYp^	XXp vs XYp^
White matter lesion (number), mean ± sd	27.68 ± 26.86	18.25 ± 9.25	24.34 ± 30.49	p = 0.509	p = 0.581	p = 0.814
White matter lesion (volume, cm^3), mean ± sd	14.17 ± 22.50	11.73 ± 9.99	20.12 ± 37.10	p = 0.484	p = 0.984	p = 0.739
Gray matter (volume, cm^3), mean ± sd	564.47 ± 58.38	533.15 ± 31.51	562.72 ± 46.58	p = 0.597	p = 0.633	p = 0.248
White matter (volume, cm^3), mean ± sd	380.57 ± 49.34	365.77 ± 48.06	390.70 ± 57.15	p = 0.684	p = 0.567	p = 0.361
Subcortical structures (volume, cm^3), mean ± sd	48.03 ± 5.95	46.96 ± 5.55	49.33 ± 5.81	p = 0.778	p = 0.329	p = 0.588
Cortical grey matter (volume, cm^3), mean ± sd	415.40 ± 46.06	389.72 ± 29.31	414.81 ± 38.82	p = 0.587	p = 0.552	p = 0.224

^ Comparison between the groups using a linear regression model with adjustment for age and disease duration

Table 4.5. *Comparison of radiological data for whole-brain volumes between the three groups.*

Analysing the subcortical structures, the 4th ventricle was larger in NLp group when compared with XYp group (2.02 ± 0.59 vs 1.70 ± 0.41; coeff = -0.382, p-value = 0.022, 95%CI = -0.708 to -0.057) and the right globus pallidus was smaller in NLp group (1.54 ± 0.23 vs 1.68 ± 0.22; coeff = 0.149, p-value = 0.049, 95%CI = 0.001 to 0.297) (***Graph 4.1***). These results were confirmed when additional adjustments for age at onset and number of previous DMTs were applied (*4th ventricle*: coeff = -0.404, p-value = 0.023, 95%CI = -0.749 to -0.0591; *right globus pallidus*: coeff = 0.166, p-value = 0.039, 95%CI = 0.009 to 0.322). Compering NLp and XYp using the multi-covariate regression model, we also found that NLp had lower brainstem volume (3.31 ± 0.44 vs 3.43 ± 0.44; coeff = 1.411, p-value = 0.050, 95%CI = -0.001 to 2.824), right cerebellum volume (11.41 ± 1.59 vs 12.10 ± 1.75; coeff = 1.104, p-value = 0.024, 95%CI = 0.152 to 2.055), and right ventral diencephalon (18.23 ± 2.20 vs 19.04 ± 2.23; coeff = 0.279, p-value = 0.032, 95%CI = 0.025 to 0.533), while they had higher optic nerve volume (0.20 ± 0.04 vs 0.19 ± 0.04; coeff = -0.031, p-value = 0.024, 95%CI = -0.058 to -0.004).

In the cortical areas, NLp had lower thickness in the left paracentral cortex when compared with XXp (2.34 ± 0.16 vs 2.39 ± 0.17; coeff = 0.131, p-value = 0.043, 95%CI = 0.004 to 0.258) and XYp (2.34 ± 0.16 vs 2.46 ± 0.17; coeff = 0.142, p-value = 0.004, 95%CI = 0.047 to 0.238) (***Graph 4.2***). The results were confirmed after adjusting for age at onset and number of previous DMTs (*NLp vs XXp*: coeff = 0.138, p-value = 0.037, 95%CI = 0.009 to 0.268; *NLp vs XYp*: coeff = 0.153, p-value = 0.004, 95%CI = 0.052 to 0.253). NLp group also had lower thickness in comparison to XXp in left precuneus (2.27 ± 0.11 vs 2.34 ± 0.16; *adjusted for age, disease duration, and MRI scanner*: coeff = 0.118, p-value = 0.046, 95%CI = 0.002 to 0.234; *adjusted for age, disease duration, MRI scanner, age at disease onset, and number of previous DMTs*: coeff = 0.124, p-value =

0.041, 95%CI = 0.005 to 0.244) and right lateral occipital cortex (2.14 ± 0.11 vs 2.25 ± 0.08; *adjusted for age, disease duration, and MRI scanner*: coeff = 0.138, p-value = 0.006, 95%CI = 0.041 to 0.234; *adjusted for age, disease duration, MRI scanner, age at disease onset, and number of previous DMTs*: coeff = 0.131, p-value = 0.010, 95%CI = 0.033 to 0.229). NLp also had lower thickness in comparison to XYp in left pericalcarine (1.64 ± 0.14 vs 1.72 ± 0.12; coeff = 0.094, p-value = 0.041, 95%CI = 0.004 to 0.184) and right paracentral areas (2.34 ± 0.17 vs 2.42 ± 0.14; coeff = 0.127, p-value = 0.015, 95%CI = 0.026 to 0.228) (***Graph 4.2***). However, only the latter comparison was confirmed after adding adjustment for age at onset and number of previous DMTs (coeff = 0.140, p-value = 0.011, 95%CI = 0.033 to 0.246). Compering NLp and XYp using the multi-covariate regression model including age, disease duration, MRI scanner, age at disease onset, and number of previous DMTs, we also found that NLp had lower right postcentral thickness (coeff = 0.080, p-value = 0.035, 95%CI = 0.006 to 0.154), right precentral thickness (coeff = 0.107, p-value = 0.044, 95%CI = 0.003 to 0.212), and left superior-frontal thickness (coeff = 0.116, p-value = 0.037, 95%CI = 0.007 to 0.224).

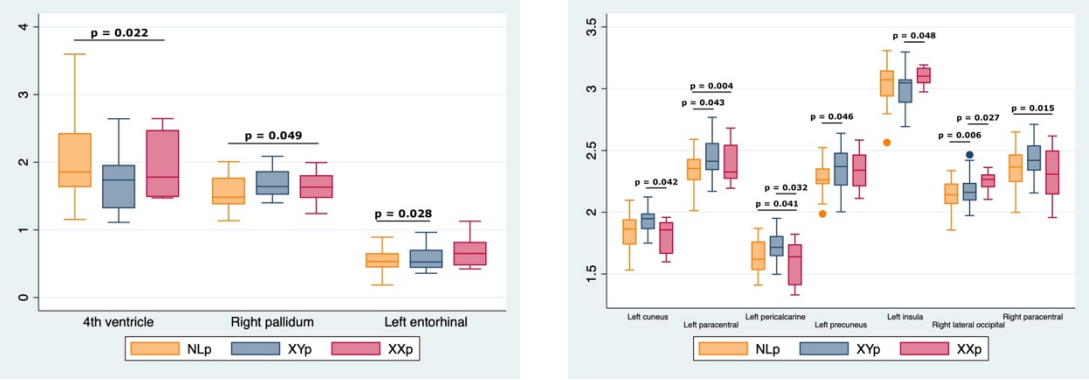
Comparing the cortical thickness in patients with at least a child, we found that XYp had higher thickness in left cuneus (1.80 ± 0.14 vs 1.93 ± 0.10; coeff = 0.115, p-value = 0.042, 95%CI = 0.005 to 0.225), left pericalcarine cortex (1.59 ± 0.19 vs 1.72 ± 0.12; coeff = 0.148, p-value = 0.032, 95%CI = 0.014 to 0.282), and left insula (3.10 ± 0.08 vs 3.00 ± 0.16; coeff = -0.127, p-value = 0.048, 95%CI = -0.253 to -0.001) and lower thickness in right lateral occipital cortex (2.25 ± 0.08 vs 2.18 ± 0.13; coeff = -0.110, p-value = 0.027, 95%CI = -0.207 to -0.014) (***Graph 4.2***). The comparisons were confirmed after adjusting for age at onset and number of previous DMTs only in left pericalcarine cortex (coeff = 0.124, p-value = 0.045, 95%CI = 0.003 to 0.244), left insula (coeff = -0.124, p-value = 0.045, 95%CI = -0.246 to -0.003), and right lateral occipital cortex (coeff = -0.109, p-value = 0.030, 95%CI = -0.207 to -0.011), but not in left cuneus.

Finally, the analysis of white matter volumes demonstrated that the left entorhinal volume was lower in NLp (0.55 ± 0.17) when compared with XXp (0.68 ± 0.25; *adjusted for age, disease duration, and MRI scanner*: coeff = 0.188, p-value = 0.028, 95%CI = 0.021 to 0.354; *adjusted for age, disease duration, MRI scanner, age at disease onset, and number of previous DMTs*: coeff = 0.194, p-value = 0.026, 95%CI = 0.024 to 0.365) (***Graph 4.1***).

Regional volumes	NLp (n=26)	XXp (n=8)	XYp (n=20)	NLp vs XYp^	NLp vs XXp^	XYp vs XXp^
Subcortical volumes (cm³, mean ± sd)						
4th ventricle	2.02 ± 0.59	1.95 ± 0.50	1.70 ± 0.41	p = 0.233	p = 0.022	p = 0.545
Right pallidum	1.54 ± 0.23	1.63 ± 0.24	1.68 ± 0.22	p = 0.146	**p = 0.049**	p = 0.877
Cortical volumes (mm, mean ± sd)						
Left cuneus	1.84 ± 0.15	1.80 ± 0.14	1.93 ± 0.10	p = 0.684	p = 0.057	**p = 0.042**
Left paracentral	2.34 ± 0.16	2.39 ± 0.17	2.46 ± 0.17	**p = 0.043**	**p = 0.004**	p = 0.741
Left pericalcarine	1.64 ± 0.14	1.59 ± 0.19	1.72 ± 0.12	p = 0.571	**p = 0.041**	**p = 0.032**
Left precuneus	2.27 ± 0.11	2.34 ± 0.16	2.34 ± 0.17	**p = 0.046**	p = 0.079	p = 0.599
Left insula	3.05 ± 0.17	3.10 ± 0.08	3.00 ± 0.16	p = 0.656	p = 0.095	p = 0.048
Right lateral occipital	2.14 ± 0.11	2.25 ± 0.08	2.18 ± 0.13	**p = 0.006**	p = 0.409	**p = 0.027**
Right paracentral	2.34 ± 0.17	2.31 ± 0.23	2.42 ± 0.14	p = 0.407	**p = 0.015**	p = 0.245
White matter volumes (mm, mean ± sd)						
Left entorhinal	0.55 ± 0.17	0.68 ± 0.25	0.59 ± 0.20	**p = 0.028**	p = 0.318	p = 0.158

^Comparison between the groups using a linear regression model with adjustment for age, disease duration, and MRI scanner

Table 4.6. *Comparison of radiological data for regional volumes between the three groups.*



Graphs 4.1 and 4.2. *Differences in subcortical volumes and white matter volumes* ***(left)*** *and in cortical thickness* ***(right)*** *in the three groups. P-values are reported for linear regression models adjusted for age, disease duration, and MRI scanner.*

4.4.4. Exploratory analysis comparing nulliparous patients, patients with only female daughters, patients with only male sons, and patients with male and female offspring

I found additional differences when we re-classified patients as follow: (a) nulliparous patients, (b) patients with only female daughters, (c) patients with only male sons, and (d) patients with male and female offspring.

Comparing nulliparous patients and patients with only daughters, I found that nulliparous patients reported lower disability in pyramidal functional system at diagnosis (1.0 [0.0 – 3.0] vs 3.0 [1.0 – 3.0]; coeff = -1.274, p-value = 0.022, 95%CI = -2.356 to -0.192). the same group was also younger at follow-up (34.4 ± 7.9 vs 50.0 ± 6.0; coeff = -6.740, p-value = 0.079, 95%CI = -14.299 to 0.818). I also detected that, nulliparous patients had lower volumes in left paracentral cortex (2.34 ± 0.15 vs 2.39 ± 0.17; coeff = -0.137, p-value = 0.035, 95%CI = -0.263 to -0.010), left precuneus cortex (2.27 ± 0.11 vs 2.34 ± 0.16; coeff = -0.122, p-value = 0.042, 95%CI = -0.239 to -0.005), right lateral-occipital cortex (2.14 ± 0.11 vs 2.25 ± 0.08; coeff = -0.137, p-value = 0.007, 95%CI = -0.235 to -0.039), and left entorhinal white matter (0.55 ± 0.17 vs 0.68 ± 0.25; coeff = -0.176, p-value = 0.034, 95%CI = -0.339 to -0.014).

I found that nulliparous patients were younger at disease onset when compared to patients with only male sons (24.6 ± 8.3 vs 29.5 ± 10.1; coeff = -13.667, p-value = 0.001, 95%CI = -21.678 to -5.655) and the former group had also lower right transverse temporal thickness (2.39 ± 0.21 vs 2.43 ± 0.15; coeff = -0.163, p-value = 0.034, 95%CI = -0.312 to -0.013).

Comparing patients with only daughters and patients with only male sons, I observed that the former groups reported higher thickness in left insula cortex (2.57 ± 0.15 vs 2.48 ± 0.13; coeff = -0.200, p-value = 0.013, 95%CI = -0.356 to -0.045) and left pars opercularis cortex when compared to XYp (3.10 ± 0.08 vs 2.97 ± 0.17; coeff = -0.168, p-value = 0.037, 95%CI = -0.326 to -0.010).

Findings also showed some difference comparing patients with previous pregnancies. I found that, when compared to patients with only daughters, patients with male and female offspring had lower volumes in optic chiasm (0.22 ± 0.04 vs 0.18 ± 0.04; coeff = -0.039, p-value = 0.043, 95%CI = -0.076 to -0.001), right lateral-occipital cortex (2.25 ± 0.08 vs 2.17 ± 0.16; coeff = -0.109, p-value = 0.044, 95%CI = -0.216 to -0.003), and left entorhinal white matter (0.68 ± 0.25 vs 0.51 ± 0.16; coeff = -0.186, p-value = 0.040, 95%CI = -0.362 to -0.009). On contrary, patients with male and female offspring had higher thickness in left cuneus (1.80 ± 0.15 vs 1.94 ± 0.11; coeff = 0.129, p-value = 0.039, 95%CI = 0.007 to 0.251) and left pericalcarine cortex (1.59 ± 0.19 vs 1.71 ± 0.11; coeff

= 0.133, p-value = 0.047, 95%CI = 0.002 to 0.263). Comparing patients with only male sons and patients with male and female offspring, I found that visual onset was more frequent in the latter group (0/4 [0.00%] vs 4/6 [66.67%]; coeff = 0.667, p-value = 0.032, 95%CI = 0.061 to 1.272).

Finally, I found that patients with only male sons had higher prevalence of progressive phenotype at onset than nulliparous patients (1/4 [25.00%] vs 1/36 [2.78%]; coeff = -0.222, p-value = 0.029, 95%CI = -0.421 to -0.024), patients with only female daughters (1/4 [25.00%] vs 0/7 [0.00%]; coeff = -0.250, p-value = 0.038, 95%CI = -0.486 to -0.014), and patients with male and female offspring (1/4 [25.00%] vs 0/6 [0.00%]; coeff = -0.250, p-value = 0.044, 95%CI = -0.493 to -0.007). The same group also had higher prevalence in family history for MS when compared to nulliparous patients (1/4 [25.00%] vs 4/35 [11.43%]; coeff = -0.295, p-value = 0.022, 95%CI = -0.545 to -0.045) and patients with male and female offspring (1/4 [25.00%] vs 0/6 [0.00%]; coeff = -0.375, p-value = 0.010, 95%CI = -0.656 to -0.094).

4.5. Discussion

The aim of this retrospective study was to investigate the impact of the sex of offspring on radiological features of MS female patients. I collected data on obstetric history of patients and categorised them in three groups: (1) NLp were supposed not to have fMCs, (2) XXp were supposed to carry only XX fMCs (XXp), and (3) XYp were supposed to be carriers of XY fMCs. The data showed that NLp had lower brain volumes in several cortical areas, as well as in some subcortical and white matter volumes. More specifically, comparing NLp and XXp, I found that the former group had larger 4^{th} ventricle and smaller right pallidum and left enthorinal volumes. NLp also reported lower thickness in left paracentral cortex, left precuneus cortex, and right lateral occipital cortex when compared with XXp. A similar trend was observed comparing NLp and XYp: NLp group had lower thickness in left paracentral cortex, left pericalcarine cortex, and right paracentral cortex. Interestingly, at the comparison between XYp and XXp, I observed that the thickness was higher in XYp in the left cuneus cortex, left pericalcarine cortex, and left insula, while XXp had a higher thickness in the right lateral occipital cortex.

Overall, the findings suggested that having a previous pregnancy was associated to some radiological differences in MS. Indeed, the results showed that patients with at least a son, and supposedly carriers of fMCs, had developed lower atrophy in several cortical areas, subcortical volumes, and white matter regions. The neurodegeneration of both grey matter and white matter seems to start in the early phases of MS and to progress throughout the disease course. This phenomenon is considered to be associated not only to the demyelination and the secondary axonal loss, but also to other pathological processes, including the oxidative burst activation of microglia and macrophages, mitochondrial dysfunction, and neuronal energy failure. The occurrence of an hypoxic state triggers the Wallerian degeneration, iron accumulation, meningeal inflammation, and astrocytes activation, starting a cascade that result in axonal loss (Frischer *et al.*, 2009; Kawachi and Lassmann, 2017).

The data reported in this study showed that women with at least a previous pregnancy had higher regional brain volumes and thickness. A similar trend was also observed analysing the clinical data. At onset XYp had experienced more frequently pyramidal involvement and less frequently sensory involvement than NLp. This condition is considered a negative prognostic factor for developing of long-term disability (Malpas *et al.*, 2020). Nonetheless, at the last clinical follow-up, NLp reported higher percentage of patients with an EDSS score ≥3.0 than XYp, showing that the latter group had not progressed more or more quickly.

Several pregnancy-associated changes, including circulating hormones and epigenetic changes, have been previously suggested as protective variables in pregnancy and it is possible to speculate that the same biological mechanisms could influence the radiological outcomes (McCombe and Greer, 2013; Graves, 2020). As my results also highlighted some differences in comparing NLp vs XXp and NLp vs XYp, I again hypothesised that, in a multifactorial background and likely interacting with other pregnancy-relate factors, microchimerism could be one of the modulating factors intervening in MS. Indeed, the sexual chromosomes in fMCs are a distinctive factor between male and female pregnancies, while other pregnancy-associated changes are thought to be similar in male and female offspring. Therefore, the results of this study were in line with the protective effect of pregnancy previously described (McCombe and Greer, 2013), but taking a step further, they also supported the speculation that the

differences observed comparing XXp and XYp to NLp could be the result of XX and XY fMCs.

In 2005, Tan et al. reported that at least some of the fMCs that spontaneously migrate in the maternal circulation during pregnancy are capable of entering the brain (Tan *et al.*, 2005). The same group suggested that, in case of injury, inflammatory signalling pathways can activate fMCs migration to sites of damage (Tan *et al.*, 2005), where they undergo cellular and molecular maturation processes similar to those observed in adult neurogenesis (Zeng *et al.*, 2010). The same group confirmed their hypothesis analysing mice mothers with experimentally induced Parkinson's disease (PD) and observing an increase in fMCs in the lesioned areas of the brain (Zeng *et al.*, 2010). The authors concluded that feto-maternal microchimerism in the brain represents a model of neural differentiation similar to adult neurogenesis and that fMCs can activate processes of cell maturation and time-dependent integration into the brain, inducing a "regeneration" that could act a role in neurodegenerative diseases (Zeng *et al.*, 2010). Moreover, in 2012, Chan et al. evaluated the role of male fMCs in women brain affected by Alzheimer's disease (AD). The findings suggested that AD patients had a lower prevalence of male fMCs in the brain and, particularly, in the regions most affected by the disease and that AD was significantly associated with lower fMCs prevalence (Chan *et al.*, 2012).
Considering data previously reported (Tan *et al.*, 2005; Zeng *et al.*, 2010; Chan *et al.*, 2012), the findings observed in this study let speculate that fMCs cells in MS patients could be one of the factors involved in the protective role of pregnancy. Indeed, the data suggested that these cells could act a neuroprotective role, homing in grey matter and white matter areas, activating processes of neurogenesis and circuit integration, and modulating the neurodegeneration associated to MS.

It is worth to note that most of the differences were observed in cortical areas. In MS, the intrathecal inflammation associated to the clinical and pathological progression is characterised by the accumulation of B cells in tertiary lympoid-like structures within the inflamed meninges (Monaco *et al.*, 2020). Indeed, during the last 10 years, it has it become increasingly clear that MS cortical damage is strongly associated to the activation of meningeal compartment and microglia and that the mechanisms underlying grey matter pathology substantially differs from those observed in white matter (Magliozzi, Reynolds

and Calabrese, 2018). The meningeal and CSF inflammatory profile of MS patients with cortical damage is characterised by pro-inflammatory cytokines (IFNγ, TNF, IL2, and IL22) and molecules related to sustained B-cell activity and lymphoid-neogenesis (CXCL13, CXCL10, LTα, IL6, IL10), suggesting that, in this compartment, the immune system could sustain chronic compartmentalised inflammation and directly mediate and/or exacerbate cortical pathology and the associated disease progression (Magliozzi *et al.*, 2018). The cortical damage would then strongly correlate with the activation of the microglia, which would be able to induce an intrathecal inflammatory profile through differente mechanisms: (1) releasing cytokines and proteases, (2) producing nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and other free radicals, (3) unbalancing iron homeostasis, and (4) activating T cells (Yong, 2022).

In 1983, Masseyeff et al. had speculated that fMCs preferentially homed to maternal lymphoid organs where they possessed suppressor characteristics (Masseyeff *et al.*, 1983). Furthermore, in 2008, Khosrotehrani et al. demonstrated that fetal lymphoid progenitors entering the maternal circulation during pregnancy have the capacity to colonise the thymus and develop into T cells and B cells (Khosrotehrani *et al.*, 2008). The authors also speculated that these fetal T cells that develop in maternal thymus may undergo positive and negative selection to eliminate anti-maternal allogenic responses and, therefore, could be involved in immune modulation processes. These findings let hypothesise that fMCs with stem-like properties could provide maternal benefits, replenishing maternal stem cell niches and contributing to their survival (Boddy *et al.*, 2015). Several studies suggested that fMCs may play a role in maternal wound healing, clustering at sites of inflammation and participating in maternal angiogenesis (Nguyen Huu *et al.*, 2007; Nassar *et al.*, 2012). In the brain, fMCs have been demonstrated to be able to differentiate into neuronal cells, integrate into maternal circuitry, and express appropriate immunocytochemical markers (Tan *et al.*, 2005; Zeng *et al.*, 2010). More recently, Snethen et al. observed that the frequencies of maternal microchimeric cells (mMCs) were higher in MS active lesions than in control samples and normal appearing MS samples and suggested that these cells, mainly of neuronal lineage or of immune origin, might play a protective or regenerative role in neurological diseases such as MS (Snethen *et al.*, 2020).

In line with these previous findings, I hypothesise that the fMCs could colonise the lympoid-like structures and, from this niche, could migrate to areas of cortical chronic

inflammation participating in the repair processes that lead to a preservation of cortical gray matter. In support to my hypothesis, I found some differences not only comparing patients with and without previous pregnancy, but also analysing patients with male and female previous pregnancy, supposedly carrying XY fMCs and XX fMCs, respectively. As for the epidemiological study described in Chapter III, these findings again supported the existence of a pregnancy-associated factors that should be different in male and female pregnancy, as XX and XY fMCs, and could explain the observed discrepancy between the groups with different sex of offspring. The available data suggest that X and Y sexual chromosomes could differently modulate the immune system: evidence suggests that the female immune response would be more robust that the male one (Nicot, 2009; Voskuhl, Sawalha and Itoh, 2018), while XY genotype could be associated to neurodegeneration and a subsequent more severe disability (Smith-Bouvier *et al.*, 2008; Voskuhl, Sawalha and Itoh, 2018). These findings, together with the different results found in comparing NLp vs XXp and NLP vs XYp and with the results obtained in the exploratory analysis, let hypothesise that XY and XX fMCs could act differently also in the chronic inflammation and neurodegenerative processes, explaining the differences observed in our population between patients with only daughters and patients with at least a male son. As most of pregnancy-associated changes are thought to be independent from the sex of offspring, fMCs are again a good candidate in this sex-specific modulation of the immune system.

The retrospective selection of patients and the subsequently lack of blood samples at MRI acquisition for the direct research of fMCs constituted the main limitations of my study. To overcome this potential bias, data were collected from official medical records and information about pregnancies and abortions were obtained directly from the patients. Therefore, the available literature on acquisition of microchimeric cells during pregnancy and the high accuracy in data collection supported my classification of patients. A second limitation is linked to the unbalanced numerosity between the three groups, which could have reduced the statistical power in some analysis and covered additional differences. Moving from these two hypothesis-generating studies and their results, I designed a prospective, longitudinal study on a larger population of patients, which is still in progress.

CHAPTER V

A prospective study to evaluate the relationship between microchimeric cells in maternal blood and clinical, radiological, and paraclinical disease features

5.1. Background and aims

As extensively reported in the previous Chapters, the available data concerning the role of the microchimerism in multiple sclerosis (MS) are still limited. Three previous studies reported higher rate of fetal microchimeric cells (fMCs) in affected women when compared with unaffected women (Willer *et al.*, 2006; Bloch *et al.*, 2011; Jafarinia *et al.*, 2020). However, none of the studies investigated the clinical, radiological, ophthalmological, and paraclinical features of disease comparing patients with and without fMCs. A fourth tissue-based study conducted by Snethen et al. revealed that maternal microchimeric cells (mMCs) in male sons accumulate in MS active lesions more than in normal appearing MS samples and control samples (Snethen *et al.*, 2020).

The findings observed in the previous two studies suggested that pregnancy status and the sex of offspring may influence clinical disease features and brain atrophy, supporting the hypothesis that a pregnancy-related factor varying in male and female pregnancies could be involved in the inflammatory and degenerative processes underlying MS. I hypothesised that fMCs could be identified as this factor, but the retrospective design of the previous studies limited the quantity and quality of available data and made impossible to test biological samples for a direct search of fMCs in the investigated population of MS patients.

Accumulating data demonstrated the limited effectiveness of clinical measures in evaluating the complex pathological processes underlying the disease and it is now clear that the use of other instruments is of paramount importance to better understand these phenomena. This evidence suggested that clinical assessments would be inadequate for evaluating the role of fMCs in the pathological mechanisms underlying MS.

Magnetic Resonance imaging (MRI) is a well-establish method to monitor disease activity in patient and it has been largely applied in MS. Lesion load changes are considered crucial to assess focal disease activity in MS diagnosis, monitoring, and prognosis (Wattjes *et al.*, 2021). Short-term changes in brain and spinal cord volumes have been established as good predictors of long-term clinical evolution (Sastre-Garriga *et al.*, 2020b). The translation of MRI measures into clinical practice has narrowed the gap between biological and clinical features and provided valuable descriptors in investigating MS (Filippi, Absinta and Rocca, 2013; Absinta, Sati and Reich, 2016; Inglese and Petracca, 2018).

Similarly, optical coherence tomography (OCT) has been proposed as a complementing investigation of optic nerve pathology and visual function. The non-invasive technique has been extensively applied in MS (Frohman *et al.*, 2006) and it has been proposed as a useful surrogate marker of neurodegeneration and inflammation (Petzold *et al.*, 2010) with mounting literature reporting a correlation between retinal nerve fibre layer (RNFL) and ganglion cell-inner plexiform layer (GCIPL) thickness, other biomarkers, and clinical visual assessment in MS patients (Sabaner *et al.*, 2020).

Starting from the available literature data and my preliminary results, I hypothesised that (1) independently from the sexual chromosome bearing, fMCs could participate in the modulation of the inflammation and degeneration observed in MS pathology, and (2) that the sexual chromosome in the fMCs could produce a different modulation of these processes explaining the discrepancy observed in mothers with and without previous male pregnancies. In order to test my hypothesis, I designed a prospective study with the following aims:

1. to confirm *in-vivo* the presence of microchimeric cells in MS patients;
2. to evaluate whether XY microchimeric cells could influence clinical outcomes;

3. to evaluate whether XY microchimeric cells could influence radiological, ophthalmological, and paraclinical features of MS more strongly associated to the biological mechanisms underlying the disease.

5.2. Population, methods and materials

To address my aims, I planned to enrol an overall population of 60 female MS patients, including 20 nulliparous subjects without microchimeric cells (NLp), 20 subjects with history of pregnancy and with XY microchimeric cells (XYp), and 20 subjects with history of pregnancy and only female offspring without XY microchimeric cells (XXp). Unfortunately, due to Coronavirus disease (COVID19) Pandemic, the recruitment phase was delayed and it is still on-going. The inclusion criteria for study were the following:

- age between 18-65 years;
- confirmed diagnosis of MS according to the revised McDonald Criteria (Thompson *et al.*, 2018);
- availability of obstetric history data.

To-date, a total of 43 patients were enrolled. The population includes 18 nulliparous subjects supposedly without microchimeric cells (NLp), 19 subjects with history of pregnancy and supposedly with XY microchimeric cells (XYp), and 6 subjects with history of pregnancy and only female offspring supposedly with XX microchimeric cells and without XY microchimeric cells (XXp). All patients were classified according to their obstetric history and underwent a blood test analysis to determine the microchimeric group. Genomic DNA was extracted from blood samples and real-time qPCR was used to amplify the Y chromosome-specific sequences, such as AMELY gene or the recently proposed TSPY gene. The analysis is still in progress, so patients have been preliminary classified according to their obstetric history for the purposes of this thesis.

For each subject demographic data and data concerning the disease features at onset and diagnosis were collected at recruitment. These data included:

- date of birth and age;
- family history for autoimmune diseases;

- comorbidities;
- age at disease onset;
- symptom(s) and Expanded Disability Status Scale (EDSS) at onset;
- clinical course at onset according to revised Lublin criteria (Lublin *et al.*, 2014);
- number of relapses within the first 3 years from disease onset;
- annual relapse rate (ARR);
- time (months) from onset to EDSS 4.0 and to EDSS 6.0;
- disease modifying therapies (DMTs) and duration of each treatment;
- symptomatic treatments;
- magnetic resonance imaging (MRI) features at diagnosis, including number of MRI brain T1-weighted (T1w) and T2-weighted (T2w) lesions and number of T1w gadolinium enhancing (Gd+) lesions and number of MRI spine T1w, T2w, and Gd+ lesions;
- presence of abnormal visual evoked potentials (VEPs), brainstem acoustic evoked potentials (BAEPs), sensory evoked potentials (SEPs), and motor evoked potentials (MEPs) at diagnosis;
- positivity for oligoclonal bands (OCBs) on cerebrospinal fluid (CSF) at diagnosis.

Then, each patient underwent a clinical visit to record the following data:

- age and disease duration;
- presence of comorbidities;
- clinical course according to revised Lublin criteria (Lublin *et al.*, 2014);
- disability established through the EDSS (neurostatus);
- Multiple Sclerosis Functional Composite (MSFC) score, including the Timed 25-Foot Walk Test (T25FW), the 9-Hole Peg Test (9HPT), and the Paced Auditory Serial Addition Test (PASAT);
- Brief International Cognitive Assessment for MS (BICAMS) score, including the Rao adaptation of the Symbol Digit Modalities Test (SDMT, oral version), the California Verbal Learning Test (CVLT), and the revised Brief Visuospatial Memory Test (BVMT).

The EDSS, MSFC, and BICAMS are well-established batteries for assessing longitudinally the physical status (EDSS, T25FW, and 9HPT) of patients and their cognition (PASAT, SDMT, CVLT, and BVMT).

- The EDSS is a well-known and commonly used scale to define the global status of patients.
- The T25FW is a quantitative measure of lower extremity function. The patient is directed to one end of a clearly marked 25-foot course and is instructed to walk as quickly as possible, but safely. The task is immediately administered again by having the patient walk back the same distance. Patients may use assistive devices when doing this task. The test is largely used to score the lower limbs functioning (Meyer-Moock *et al.*, 2014).
- In the 9HPT both the dominant and non-dominant hands are tested twice (two consecutive trials of the dominant hand, followed immediately by two consecutive trials of the non-dominant hand). The patient is required to pick up the pegs one at a time, using one hand only, and put them into the holes as quickly as possible until all the holes are filled. Then, without pausing, the patient has to remove the pegs one at a time and return them to the container as quickly as possible. The test is largely used to score the upper limbs functioning (Meyer-Moock *et al.*, 2014).
- The PASAT is a measure of cognitive function that specifically assesses auditory information processing speed and flexibility, as well as calculation ability. Single digits are presented every 3 seconds and the patient must add each new digit to the one immediately prior to it (Meyer-Moock *et al.*, 2014).
- The SDMT provides processing speed information. The test consists of a number of single digits, each paired with a particular abstract symbol. The patient is presented with rows of the 9 symbols that are arranged pseudo-randomly, and patients must say the numbers that go with each symbol in turn (Corfield and Langdon, 2018).
- The CVLT-II comprises 5 learning trials and is an examination of immediate verbal recall. Patients are read a list of words at a slightly slower rate than 1item per second, and asked to recall as many items as possible in any order, across five trials. The test is a 16-item word list, with 4 items belonging to each of 4 categories, arranged randomly (Corfield and Langdon, 2018).

- The BVMT-R is an assessment of immediate visual recall. The test requires the patient to observe 2 per 3 stimulus arrays of abstract geometric figures. There are 3 learning trials, each 10 seconds in length. The array is hidden from view, and the patient is required to draw the geometric figures in the correct position from memory after each 10 second exposure (Corfield and Langdon, 2018).

Z-scores were calculated for both physical and cognitive tests, including T25FW, 9HPT, PASAT, SDMT, CVLT, and BVMTR. Adjustments for age and years of Education were applied in scoring the cognitive tests.

Signa HDxt – GE Medical System, Milwaukee, WI, USA and Achieva was used to acquire brain MRI scans, including three-dimensional (3D) T2w fluid attenuated inversion recovery (FLAIR) with fat saturation (Fat Sat) sequences, 3D T1w fast spoiled gradient echo (FSPGR) pulse sequences. Scanner was equipped with an 8-channel phased-array head coil. MRI group analysis will be run at the end of the recruitment and therefore they are not currently available. MRI data for lesion number and volume will be obtained. Lesion load volume will be segmented from 3D T2w FLAIR sequences by the lesion growth algorithm (Schmidt *et al.*, 2012) as implemented in the Lesion Segmentation Tool (LST) (www.statistical- modelling.de/lst.html) for Statistical Parametric Mapping (SPM) software. Whole-brain and regional grey matter and white matter volumes will be estimated on 3D T1w pulse sequences using FreeSurfer software suite (https://surfer.nmr.mgh.harvard.edu/). Several Free Surfer Atlases will be used to obtain information on cortical areas, subcortical areas, and white matter volumes.

Each patient underwent OCT scan using Toccon DRI OCT Triton machine to obtain data on RNFL and GCIPL of the optic nerve. Appropriate quality assurance was undertaken to ensure comparability and scans were excluded if international consensus quality control criteria were violated (Tewarie *et al.*, 2012).

5.3. Statistical analysis

Demographic, radiological, clinical, and paraclinical characteristics of patients were summarised through counts and percentages for categorical variables. Quantitative

variables were synthesized through mean ± standard deviation (SD) and median and interquartile range (IQR) when the variable distribution was asymmetric. The analysis of data at study visits and last clinical assessments was performed considering the pregnancy status at the recruitment, while data at disease onset and diagnosis were analysed according to the pregnancy status of patients before disease onset.

ANOVA test were performed for comparisons of continuous variables and Chi-square test for trend were used to compare categorical variables between nulliparous women, women with at least one male son, and women with exclusively female daughters ($p < 0.05$). Bonferroni correction was applied to reduce the chances of obtaining type I errors. Multiple regression models with adjustments for age and disease duration were performed to evaluate the relationship between the sex of offspring and disease features, testing the possible effects of confounding or modification factors. Additional adjustments for age at disease onset, number of previous DMTs, and number of previous pregnancies were performed where applicable.

A mixed model was built for the analysis of ophthalmological data and OCT in both eyes; patient identifier was set as fixed-effect variable and age, disease duration, number of previous optic neuritis, and time-gap from the last optic neuritis were applied as covariates.

Pearson correlation coefficient and Spearman's rank correlation were used to evaluate the linear and non-linear correlations between disease features and microchimeric status. Correlation matrixes will be also applied to evaluate the relationships between multiple variables.

5.4. Results

5.4.1. Participant characteristics

Forty-three consecutive patients were recruited in the study. The NLp had a mean age of 33.8 ± 7.0 years and were younger than XXp (48.4 ± 8.0 years; coeff = 11.482, p-value = 0.001, 95%CI = 4.932 to 18.033) and XYp (45.3 ± 12.3 years; coeff = 14.500, p-value 0.003, 95%CI = 5.112 to 23.888). The disease duration was similar in the three groups (p-value >0.05) (***Table 5.1***). I did not find any difference comparing the prevalence of comorbidity and family history for MS or other autoimmune diseases between the three

groups (***Table 5.1***). No differences were detected in age at menarche, prevalence of irregular periods, age at menopause, and prevalence of drug-induced/surgical menopause (p-value >0.05) (***Table 5.1***). Comparing the obstetric history of patients with at least a child, I found that the mean number of pregnancies was non-significantly lower in XXp compared to XYp (1.50 ± 0.55 vs 2.32 ± 1.38; p-value >0.05), while the time-gap from last pregnancy was non-significantly lower in XYp (159.60 ± 148.8 vs 263.5 ± 131.0; p-value >0.05) (***Table 5.1***).

Characteristics	NLp (n=18)	XYp (n=19)	XXp (n=6)	p-value§
Demographic characteristics				
Age (years), mean ± sd	33.8 ± 7.0	45.3 ± 12.3	48.4 ± 8.0	**p = 0.001****
Disease duration (years), mean ± sd	10.7 ± 6.9	15.6 ± 11.3	14.1 ± 13.7	p = 0.329
Education (years), mean ± sd	17.4 ± 3.1	11.8 ± 4.3	10.2 ± 3.5	**p < 0.001****
Comorbidity, prevalence (%)	9/18 (50.0%)	15/19 (79.0%)	4/6 (66.7%)	p = 0.181
Family history for AI diseases, prevalence (%)	8/18 (44.4%)	11/19 (57.9%)	4/6 (66.7%)	p = 0.560
Obstetrical characteristics				
Age at menarche (years), mean ± sd	11.7 ± 1.0	12.2 ± 1.2	11.7 ± 1.0	p = 0.321
Irregular periods, prevalence (%)	1/17 (5.9%)	4/19 (21.1%)	2/6 (33.3%)	p = 0.236
Age at menopause (years), mean ± sd	–	44.1 ± 6.2	30.8 ± 20.8	p = 0.135
Drug-induced/Surgical menopause, prevalence (%)	0/1 (0.0%)	2/7 (28.6%)	2/4 (50.0%)	p = 0.585
Pregnancy (number), mean ± sd	–	2.32 ± 1.38	1.50 ± 0.55	p = 0.175
Time to last pregnancy (months), mean ± sd	–	159.6 ± 148.8	263.5 ± 131.0	p = 0.140

§Comparison between the three groups using ANOVA test

Table 5.1. *Characteristics of the enrolled population.*

5.4.2. Differences in clinical features at onset and diagnosis, and last clinical follow-up between the three groups

I collected clinical, radiological and paraclinical data at onset, diagnosis, and last clinical follow-up. At onset and diagnosis, 27 patients were nulliparous, 9 patients had at least a male son, and 7 patients had only female daughters (***Table 5.2*** and ***Table 5.3***). NLp were younger than XYp (22.2 ± 6.2 years vs 39.4 ± 7.5 years; coeff = 17.222, p-value <0.001, 95%CI = 11.909 to 22.535) and XXp (22.2 ± 6.2 years vs 32.9 ± 8.4 years; coeff = 10.635, p-value = 0.001, 95%CI = 4.780 to 16.489). XYp patients had experienced more pregnancies before onset when compared to XXp (2.56 ± 1.01 vs 1.43 ± 0.53; coeff = -1.127, p = 0.019, 95%CI = -2.038 to -0.216). The same group reported higher frequency

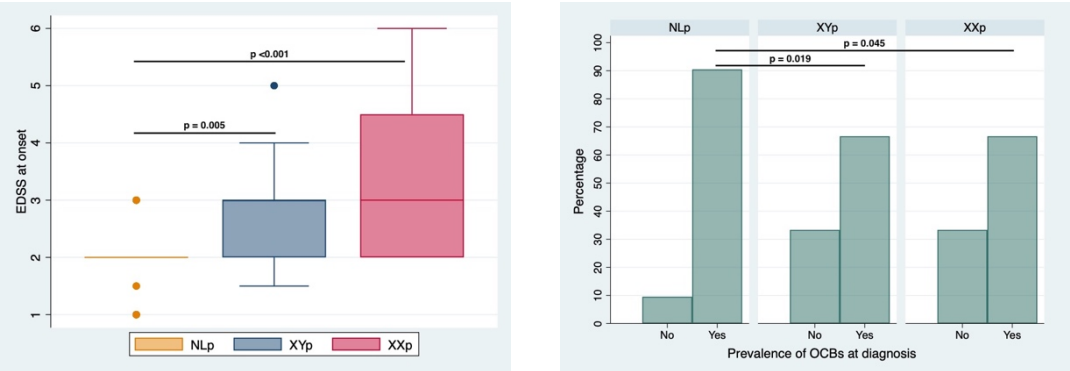
of onset within 6 months post-partum (2/9 [22.2%] vs 0/7 (0.0%); coeff = -0.292, p = 0.005, 95%CI = -0.490 to -0.095) and within 3 years post-partum (3/9 [33.3%] vs 1/7 [14.3%]; coeff = -0.325, p = 0.011, 95%CI = -0.571 to -0.080). These differences were confirmed adding the number of pregnancies before MS onset as covariate (*onset within 6 months post-partum*: coeff = -0.432, p-value = 0.041, 95%CI = -0.843 to -0.020; *within 3 years post-partum*: coeff = -0.589, p-value = 0.009, 95%CI = -0.999 to -0.178). The observations resulted too few to calculated odds ratio for the onset within 6 months post-partum, instead I found that the risk of onset within 3 years post-partum in XYp was 4.43 higher than in XXp (p-value = 0.043, 95%CI = 1.047 to 18.717). Comparing clinical symptoms at onset (***Table 5.2***), I found that the percentage of patients who experienced brainstem symptoms was higher in the XYp group than XXp group (5/9 [55.6%] vs 1/7 [14.3%]; coeff = -0.500, p-value = 0.047, 95%CI = -0.994 to -0.007). However, this difference became non-significant when the comparison was adjusted for the number of previous pregnancies (coeff = -0.489, p-value = 0.104, 95%CI = -1.083 to 0.105). Similarly, I found that XXp patients had higher spine lesion load at diagnosis when compared to XYp (*prevalence of ≥3 lesion*: 0/3 [0.0%] vs 3/5 [60.0%]; coeff = 0.914, p-value = 0.020, 95%CI = 0.155 to 1.673) (***Table 5.3***), although also this difference lost significance after adjusting for number of previous pregnancies (coeff = 0.635, p-value = 0.135, 95%CI = -0.216 to 1.486). I did not identify any other difference between the two groups comparing data at onset and diagnosis (all p-value >0.050).

Comparing NLp with XYp and XXp, I found that patients with no history of previous pregnancy had lower EDSS score at onset in comparison to XYp (2.0 [1.0 – 3.0] vs 3.0 [1.5 – 5.0]; coeff = 1.429, p-value = 0.005, 95%CI = 0.459 to 2.400) and XXp (2.0 [1.0 – 3.0] vs 3.0 [2.0 – 6.0]; coeff = 1.649, p-value <0.001, 95%CI = 0.791 to 2.508) (***Table 5.2*** and ***Graph 5.1***). At diagnosis, I found that XXp patients had higher infratentorial lesion load when compared to NLp (*prevalence of >1 lesion*: 2/3 [66.7%] vs 3/6 [50.0%]; coeff = -1.681, p-value = 0.041, 95%CI = -3.269 to -0.093) (***Table 5.3***). Finally, I found that NLp patients had higher prevalence of positive OCBs in CSF in comparison to XYp (19/21 [90.5%] vs 6/9 [66.7%]; coeff = -0.525, p-value = 0.019, 95%CI = -0.957 to -0.092) and XXp (19/21 [90.5%] vs 4/6 [66.7%]; coeff = -0.414, p-value = 0.045, 95%CI = -0.817 to -0.010) (***Table 5.3*** and ***Graph 5.2***). I did not identify any other difference comparing data at diagnosis (all p-value >0.050).

Disease features at onset	NLp (n=27)	XYp (n=9)	XXp (n=7)	NLp vs XYp^	NLp vs XXp^	XYp vs XXp^
Age at onset (years), mean ± sd	22.2 ± 6.2	39.4 ± 7.5	32.9 ± 8.4	**p < 0.001****	**p = 0.001****	p = 0.063
Pregnancy before onset (number), mean ± sd	–	2.56 ± 1.01	1.43 ± 0.53	–	–	**p = 0.019***
Onset within 6 months post-partum, prevalence (%)	–	2/9 (22.2%)	0/7 (0.0%)	–	–	**p = 0.005****
Onset within 3 years post-partum, prevalence (%)	–	3/9 (33.3%)	1/7 (14.3%)	–	–	**p = 0.011***
Progression at onset, prevalence (%)	0/27 (0.0%)	1/9 (11.1%)	0/7 (0.0%)	p = 0.780	p = 0.454	p = 0.323
Polysymptomatic onset, prevalence (%)	6/27 (22.2%)	3/9 (33.3%)	3/7 (42.9%)	p = 0.278	p = 0.176	p = 0.899
Functional system involved at onset, prevalence (%)						
Visual FS	6/27 (22.2%)	2/9 (22.2%)	2/7 (28.6%)	p = 0.236	p = 0.260	p = 0.840
Brainstem FS	8/27 (29.7%)	5/9 (55.6%)	1/7 (14.3%)	p = 0.064	p = 0.959	**p = 0.047***
Pyramidal FS	2/27 (7.4%)	1/9 (11.1%)	3/7 (42.9%)	p = 0.748	p = 0.082	p = 0.052
Cerebellum FS	3/27 (11.1%)	0/9 (0.0%)	0/7 (0.0%)	p = 0.968	p = 0.759	p = 0.743
Sensory FS	13/27 (48.2%)	4/9 (44.4%)	3/7 (42.9%)	p = 0.595	p = 0.626	p = 0.916
Sphincter FS	0/27 (0.0%)	1/9 (11.1%)	1/7 (14.3%)	p = 0.565	p = 0.269	p = 0.666
Ambulation involvement at onset, prevalence (%)	1/27 (3.7%)	1/9 (11.1%)	1/7 (14.3%)	p = 0.329	p = 0.254	p = 0.970
Spinal cord involvement at onset, prevalence (%)	6/27 (22.2%)	3/9 (33.3%)	5/7 (71.4%)	p = 0.825	p = 0.086	p = 0.068
EDSS at onset, median (range)	2.0 (1.0 – 3.0)	3.0 (1.5 – 5.0)	3.0 (2.0 – 6.0)	**p = 0.005****	**p < 0.001****	p = 0.632
Recovery after the first relapse. prevalence (%)				p = 0.053	p = 0.756	p = 0.078
Complete	9/26 (34.6%)	6/9 (66.7%)	2/7 (28.6%)			
Incomplete	17/26 (65.4%)	3/9 (33.3%)	5/7 (71.4%)			
Relapses within the first 3 years from onset (number), mean ± sd	2.96 ± 1.70	1.89 ± 1.05	2.29 ± 1.38	p = 0.100	p = 0.240	p = 0.512

^Comparison between the groups using a linear regression model with adjustment for age at onset

Table 5.2. *Disease features at onset in the three groups.*



Graphs 5.1 and 5.2. *EDSS at disease onset (left) and prevalence of positive oligoclonal bands at diagnosis (right) in the three groups.*

Disease features at diagnosis	NLp (n=27)	XYp (n=9)	XXp (n=7)	NLp vs XYp^	NLp vs XXp^	XYp vs XXp^
Time-gap from onset to diagnosis (months), mean ± sd	28.44 ± 36.91	16.22 ± 26.98	29.14 ± 49.79	p = 0.402	p = 0.965	p = 0.498
MRI T2w brain lesions (number), prevalence (%)						
0 lesions	0/20 (0.0%)	0/8 (0.0%)	0/5 (0.0%)	p = 0.968	p = 0.373	p = 0.340
1-2 lesions	1/20 (10.0%)	0/8 (0.0%)	1/5 (20.0%)			
3-8 lesions	9/20 (45.0%)	2/8 (25.0%)	1/5 (20.0%)			
≥9 lesions	9/20 (45.0%)	6/8 (75.0%)	3/5 (60.0%)			
MRI T2w cortical brain lesions (number), prevalence (%)						
0 lesions	5/8 (62.5%)	1/3 (33.3%)	2/3 (66.7%)	p = 0.984	p = 0.616	p = 0.621
1 lesion	0/8 (0.0%)	1/3 (33.3%)	0/3 (0.0%)			
>1 lesions	3/8 (37.5%)	1/3 (33.3%)	1/3 (33.3%)			
MRI T2w periventricular brain lesions (number), prevalence (%)						
0 lesions	0/12 (0.0%)	0/6 (0.0%)	0/3 (0.0%)	p = 0.248	p = 0.379	p = 0.885
1-2 lesions	2/12 (16.7%)	1/6 (16.7%)	0/3 (0.0%)			
≥3 lesions	10/12 (83.3%)	5/6 (83.3%)	3/3 (100.0%)			
MRI T2w infratentorial brain lesions (number), prevalence (%)						
0 lesions	2/6 (33.3%)	1/3 (33.3%)	1/3 (33.3%)	p = 0.063	**p = 0.041***	p = 0.632
1 lesion	1/6 (16.7%)	1/3 (33.3%)	0/3 (0.0%)			
>1 lesions	3/6 (50.0%)	1/3 (33.3%)	2/3 (66.7%)			
MRI T1w brain lesions (number), mean ± sd	1.00 ± 1.172	1.14 ± 2.19	0.34 ± 0.58	p = 0.207	p = 0.076	p = 0.374
MRI T1w Gd+ brain lesions (number), mean ± sd	0.55 ± 1.10	0.71 ± 0.76	1.25 ± 1.26	p = 0.972	p = 0.342	p = 0.385
MRI T2w spine lesions (number), prevalence (%)						
0 lesions	7/17 (41.2%)	1/5 (20.0%)	1/5 (20.0%)	p = 0.090	p = 0.350	**p = 020***
1-2 lesions	8/17 (47.1%)	4/5 (80.0%)	1/5 (20.0%)			
≥3 lesions	2/17 (11.8%)	0/5 (0.0%)	3/5 (60.0%)			
MRI T1w spine lesions (number), prevalence (%)						
0 lesions	11/13 (84.6%)	2/3 (66.7%)	2/4 (50.0%)	p = 0.502	p = 0.742	p = 0.735
1-2 lesions	1/13 (7.7%)	1/3 (33.3%)	2/4 (50.0%)			
≥3 lesions	1/13 (7.7%)	0/3 (0.0%)	0/4 (0.0%)			
MRI T1w Gd+ spine lesions (number), mean ± sd	0.24 ± 0.56	0.00 ± 0.00	0.40 ± 0.55	p = 0.401	p = 0.655	p = 0.263
Positive OCBs, prevalence (%)	19/21 (90.5%)	6/9 (66.7%)	4/6 (66.7%)	**p = 0.019***	**p = 0.045***	p = 0.598
Abnormal VEP, prevalence (%)	14/21 (66.7%)	2/6 (33.3%)	2/3 (66.7%)	p = 0.481	p = 0.763	p = 0.370
Abnormal BAEP, prevalence (%)	2/11 (18.2%)	0/6 (0.0%)	0/4 (0.0%)	p = 0.977	p = 0.946	p = 0.917
Abnormal SEP, prevalence (%)	3/16 (18.8%)	0/6 (0.0%)	0/3 (0.0%)	p = 0.177	p = 0.249	p = 0.874
Abnormal MEP, prevalence (%)	2/12 (16.7%)	1/5 (20.0%)	2/3 (66.7%)	p = 0.837	p = 0.167	p = 0.167

Table 5.3. *Disease features at diagnosis in the three groups.*

At last clinical follow-up, 18 subjects were nulliparous, 19 subjects had at least a male son, and 6 subjects had only female offspring (***Table 5.4***). NLp were younger than XYp (22.2 ± 6.2 vs 39.4 ± 7.5; coeff = 8.926, p-value = 0.003, 95%CI = 3.123 to 14.730) and XXp (22.2 ± 6.2 vs 32.9 ± 8.4; coeff = 12.741, p-value = 0.003, 95%CI = 4.595 to 20.886). XXp patients had registered higher ARR both compared to NLp (0.59 ± 0.30 vs 0.89 ± 0.71; coeff = -0.460, p-value = 0.015, 95%CI = -0.825 to -0.094) and XYp (0.45 ± 0.30 vs 0.89 ± 0.71; coeff = -0.442, p-value = 0.009, 95%CI = -0.768 to -0.116) (***Graph 5.3***). These differences were confirmed applying as covariates age, disease duration, age at disease onset, and number of previous DMTs (*XXp vs NLp*: coeff = 0.438, p-value =

0.021, 95%CI = 0.069 to 0.806; *XXp vs XYp*: coeff = 0.412, p-value = 0.016, 95%CI = 0.080 to 0.743). No differences were detected neither comparing the number of relapses before reaching EDSS 4.0 nor the diseases duration at EDSS 4.0 (all p-value >0.050) (***Graph 5.4*** and ***Graph 5.5***). No difference either was found comparing the diseases duration at EDSS 6.0 (p-value >0.050) (***Graph 5.6***). On contrary, the number of relapses before reaching EDSS 6.0 was higher in NLp than XYp (13.00 ± 11.31 vs 8.80 ± 10.55; coeff = -29.750, p-value = 0.047, 95%CI = -58.799 to -0.701) and XXp (13.00 ± 11.31 vs 5.00 ± 0.00; coeff = -34.721, p-value = 0.040, 95%CI = -66.415 to -3.027) (***Graph 5.7***). This difference was lost after adding age at onset and previous number of previous DMTs as covariates.

I found that, adjusting for age, disease duration, age at disease onset, and number of previous DMTs, XYp patients had more frequently progressive MS than XXp (4/18 [22.22%] vs 0/6 [0.00%]; coeff = -0.314, p-value = 0.035, 95%CI = -0.603 to -0.024).

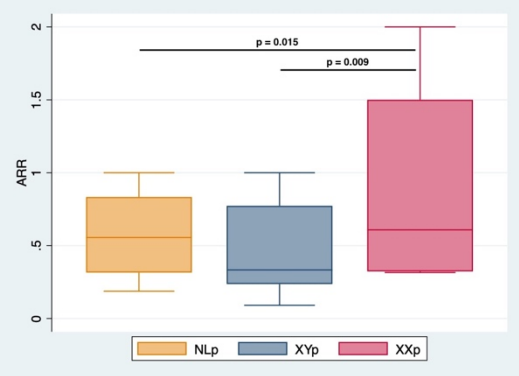
Comparing the number of previous inflammatory episodes in the optic nerve, I found a statistical significance comparing NLp vs XXp (13/18 [72.2%] vs 0/5 [0.0%]; coeff = 0.788, p-value = 0.004, 95%CI = 0.267 to 1.310), but not comparing XYp vs XXp (9/19 [47.4%] vs 0/5 [0.0%]; coeff = 0.455, p-value = 0.062, 95%CI = -0.024 to 0.934). None of the comparisons were significant after adding age at onset and number of previous DMTs as covariates.

I did not identify any other difference comparing data at last clinical follow-up (all p-value >0.050).

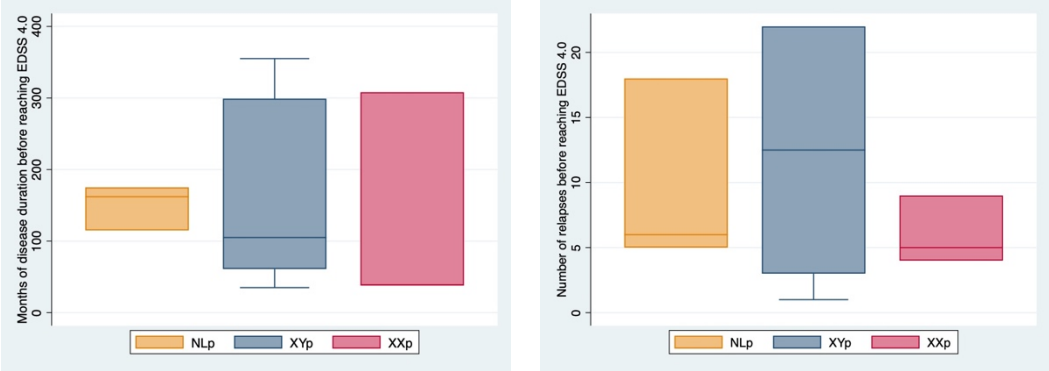
Disease features at last clinical visit	NLp (n=18)	XYp (n=19)	XXp (n=6)	NLp vs XYp^	NLp vs XXp^	XYp vs XXp^
Age (years), mean ± sd	22.2 ± 6.2	39.4 ± 7.5	32.9 ± 8.4	**p < 0.001****	**p = 0.001****	p = 0.063
Relapse during pregnancy, mean ± sd	–	0.17 ± 0.51	0.00 ± 0.00	–	–	p = 0.562
Relapse within 6 months post-partum, mean ± sd	–	0.47 ± 0.77	0.00 ± 0.00	–	–	p = 0.221
Progressive phenotype, prevalence (%)	2/18 (11.1%)	4/18 (22.2%)	0/6 (0.0%)	p = 0.698	p = 0.383	p = 0.202
Disease duration at progression (months), mean ± sd	158.0 ± 60.8	173.7 ± 216.3	–	p = 0.703	–	–
Patients with EDSS ≥4.0, prevalence (%)	3/18 (16.7%)	7/19 (36.8%)	3/6 (50.0%)	p = 0.895	p = 0.718	p = 0.610
Disease duration at reaching EDSS 4.0 (months), mean ± sd	150.7 ± 31.6	156.6 ± 123.3	128.3 ± 155.6	p = 0.076	p = 0.262	p = 0.425
Relapse at reaching EDSS 4.0 (number), mean ± sd	9.67 ± 7.23	12.17 ± 9.41	6.00 ± 2.65	p = 0.946	p = 0.585	p = 0.327
Patients with EDSS ≥6.0, prevalence (%)	2/18 (11.1%)	5/19 (26.3%)	1/6 (16.7%)	p = 0.728	p = 0.363	p = 0.456
Disease duration at reaching EDSS 6.0 (months), mean ± sd	763.5 ± 897.3	205.0 ± 171.7	98.0 ± 0.0	p = 0.568	p = 0.492	p = 0.765
Relapse at reaching EDSS 6.0 (number), mean ± sd	13.00 ± 11.31	8.80 ± 10.55	5.00 ± 0.00	**p = 0.047***	**p = 0.040***	p = 0.419
Total relapses (number), mean ± sd	5.50 ± 4.60	6.53 ± 7.85	5.83 ± 3.97	p = 0.417	p = 0.494	p = 0.904
Annual relapse rate, mean ± sd	0.59 ± 0.30	0.45 ± 0.30	0.89 ± 0.71	p = 0.891	**p = 0.015***	**p = 0.009****
Months on 1 DMT, mean ± sd	28.00 ± 27.00	41.65 ± 60.02	36.33 ± 43.94	p = 0.680	p = 0.945	p = 0.793
DMTs (number), mean ± sd	2.39 ± 1.54	2.79 ± 2.10	3.33 ± 2.25	p = 0.652	p = 0.540	p = 0.298
Optic neuritis, prevalence (%)	13/18 (72.2%)	9/19 (47.4%)	0/5 (0.0%)	p = 0.066	**p = 0.004****	p = 0.062
Bilateral optic neuritis, prevalence (%)	2/12 (16.7%)	5/8 (62.5%)	–	p = 0.589	–	–
Relapsing optic neuritis, prevalence (%)	5/12 (41.7%)	4/8 (50.0%)	–	p = 0.531	–	–
Total optic neuritis (number), mean ± sd	1.53 ± 1.87	1.39 ± 2.48	0.00 ± 0.00	p = 0.844	p = 0.242	p = 0.257
Time-gap from last optic neuritis (months), mean ± sd	89.5 ± 77.15	144.2 ± 86.3	–	p = 0.116	–	–

^Comparison between the groups using a linear regression model with adjustment for age and disease duration

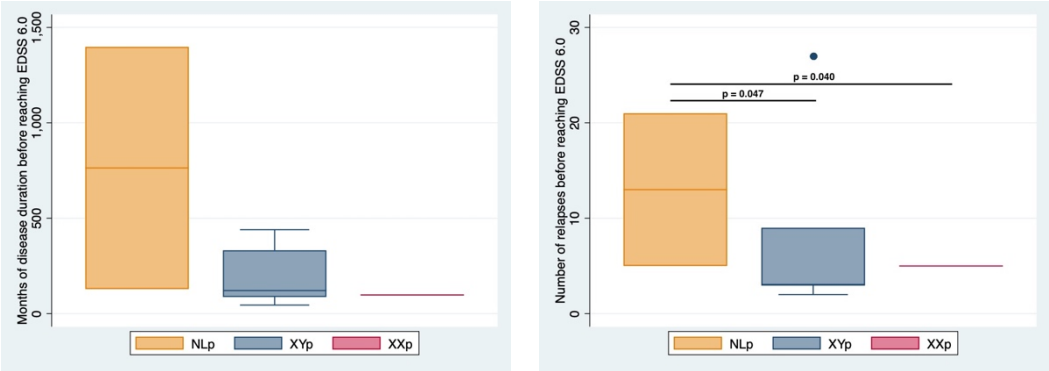
Table 5.4. *Disease features at last clinical follow-up in the three groups.*



Graphs 5.3. *ARR in the three groups.*



Graphs 5.4 and 5.5. *Disease duration at reaching EDSS 4.0 (left) and number of relapses before reaching EDSS 4.0 in the three groups.*



Graphs 5.6 and 5.7. *Disease duration at reaching EDSS 6.0 (left) and number of relapses before reaching EDSS 6.0 in the three groups.*

5.4.3. Differences in clinical and ophthalmological features at study visit

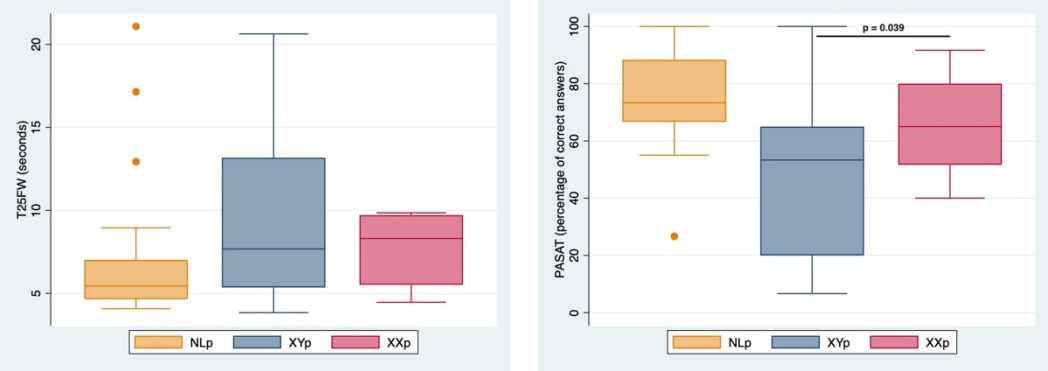
At study visit, 18 subjects were nulliparous, 19 subjects had at least a male son, and 6 subjects had only female offspring (***Table 5.5***). NLp were younger than XYp (22.2 ± 6.2 vs 39.4 ± 7.5; coeff = 8.926213, p-value = 0.003, 95%CI = 3.123 to 14.730) and XXp (22.2 ± 6.2 vs 32.9 ± 8.4; coeff = 12.741, p-value = 0.003, 95%CI = 4.595 to 20.886); while no difference was detected comparing disease duration between the three groups. I did not find any difference comparing either the functional system (FS) scores at neurostatus or the EDSS score. Similarly, the groups did not show any difference in T25FW and 9HPT scores. I noticed that, despite being younger, NLp had a higher score at T25FW; I have then excluded outliers (±3.0 standard deviation, SD) and I have noticed that indeed the differences between the groups disappeared (*NLp*: 7.42 ± 4.95; *XYp*: 9.72

± 5.37; *XXp*: 7.69 ± 2.30; *NLp vs XYp*: coeff = 0.530, p-value = 0.779, 95%CI = -3.278 to 4.339; *NLp vs XXp*: coeff = -1.988, p-value = 0.446; 95%CI = -7.228 to 3.251) (***Graph 5.8***). At cognitive test, I found that PASAT score was higher in XXp patients when compared to XYp (50.26 ± 28.39 vs 65.56 ± 19.11; coeff = 20.583, p-value = 0.039, 95%CI = 1.108 to 40.057) (***Graph 5.9***). The result was confirmed also when the comparison was adjusted for age at onset and number of previous DMTs as additional covariates (coeff = 23.633, p-value = 0.019; 95%CI = 4.189 to 43.077). No difference was found at SDMT, CVLT-II and BVMT (all p-value >0.050).

Clinical data at baseline	NLp (n=18)	XYp (n=19)	XXp (n=6)	NLp vs XYp^	NLp vs XXp^	XYp vs XXp^
Age (years), mean ± sd	33.8 ± 7.0	45.3 ± 12.3	48.4 ± 8.0	**p = 0.003****	**p = 0.003****	p = 0.344
Disease duration (years), mean ± sd	10.7 ± 6.9	15.6 ± 11.3	14.1 ± 13.7	p = 0.715	p = 0.343	p = 0.442
FS at EDSS, median (range)						
Visual FS	0.5 (0.0 – 3.0)	1.0 (0.0 – 2.0)	1.5 (0.0 – 2.0)	p = 0.677	p = 0.639	p = 0.845
Brainstem FS	0.0 (0.0 – 2.0)	1.0 (0.0 – 3.0)	1.0 (0.0 – 2.0)	p = 0.084	p = 0.224	p = 0.977
Pyramidal FS	1.0 (0.0 – 3.0)	1.0 (0.0 – 4.0)	2.0 (1.0 – 3.0)	p = 0.527	p = 0.750	p = 0.391
Cerebellum FS	0.0 (0.0 – 3.0)	1.0 (0.0 – 3.0)	1.0 (0.0 – 3.0)	p = 0.735	p = 0.729	p = 0.905
Sensory FS	0.0 (0.0 – 2.0)	0.0 (0.0 – 5.0)	2.0 (0.0 – 2.0)	p = 0.591	p = 0.564	p = 0.827
Sphincter FS	0.0 (0.0 – 3.0)	1.0 (0.0 – 3.0)	2.5 (0.0 – 3.0)	p = 0.672	p = 0.305	p = 0.140
Cerebral FS	0.0 (0.0 – 2.0)	1.0 (0.0 – 4.0)	1.5 (0.0 – 2.0)	p = 0.876	p = 0.665	p = 0.718
Ambulation	0.0 (0.0 – 9.0)	0.0 (0.0 – 8.0)	0.0 (0.0 – 5.0)	p = 0.721	p = 0.361	p = 0.458
EDSS, median (range)	1.5 (0.0 – 6.5)	2.5 (0.0 – 6.5)	4.0 (1.5 – 6.0)	p = 0.952	p = 0.534	p = 0.516
T25FW (seconds), mean ± sd	14.11 ± 28.78	9.72 ± 5.37	7.69 ± 2.30	p = 0.234	p = 0.268	p = 0.778
9HPT dH (seconds), mean ± sd	23.47 ± 8.72	23.34 ± 5.57	25.87 ± 5.50	p = 0.668	p = 0.685	p = 0.427
9HPT ndH (seconds), mean ± sd	23.94 ± 6.42	26.83 ± 10.05	28.68 ± 7.17	p = 0.886	p = 0.678	p = 0.725
9HPT (seconds), mean ± sd	0.05 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	p = 0.993	p = 0.388	p = 0.330
PASAT (percentage of right answers), mean ± sd	74.07 ± 17.32	50.26 ± 28.39	65.56 ± 19.11	p = 0.554	p = 0.202	**p = 0.039***
SDMT (right answers), mean ± sd	61.78 ± 14.56	45.79 ± 15.35	38.50 ± 16.13	p = 0.955	p = 0.627	p = 0.583
CVLT-II (right answers), mean ± sd	59.67 ± 7.79	45.74 ± 13.20	40.50 ± 13.08	p = 0.533	p = 0.357	p = 0.560
BVMT (right answers), mean ± sd	28.00 ± 6.63	19.84 ± 8.17	17.83 ± 10.61	p = 0.867	p = 0.958	p = 0.933

^Comparison between the groups using a linear regression model with adjustment for age and disease duration

Table 5.5. *Clinical features at study visit in the three groups.*



Graphs 5.8 and 5.9. *T25FW scores excluding outliers (left) and PASAT scores in the three groups.*

OCT data (***Table 5.6***) revealed that RNFL thickness was higher in XYp when compared to NLp (89.88 ± 18.26 µm vs 97.22 ± 13.65 µm; coeff = -13.444, p-value = 0.049, 95%CI = -26.802 to -0.087) and XXp (62.48 ± 9.29 µm vs 97.22 ± 13.65 µm; coeff = -14.747, p-value = 0.115, 95%CI = -33.096 to 3.603), although the latter comparison was not statistically significant (***Graph 5.10***). The difference between XYp and NLp was confirmed also when the number of previous optic neuritis and the time-gap from the last optic neuritis were set as covariates in the mixed model (coeff = -23.233, p-value = 0.030, 95%CI = -44.275 to -2.191). Similar trends were observed in GCIPL, although the comparisons did not reach the statistical significance (***Graph 5.11***). I found that the comparisons between NLp and XYp were borderline when the regression was adjusted for age, disease duration, age at onset, number of previous DMTs, and number of previous optic neuritis (*RNFL*: coeff = -13.489, p-value = 0.055, 95%CI = -27.245 to 0.267; *GCIPL*: coeff = 9.718, p-value = 0.063, 95%CI = -0.526 to 19.963).

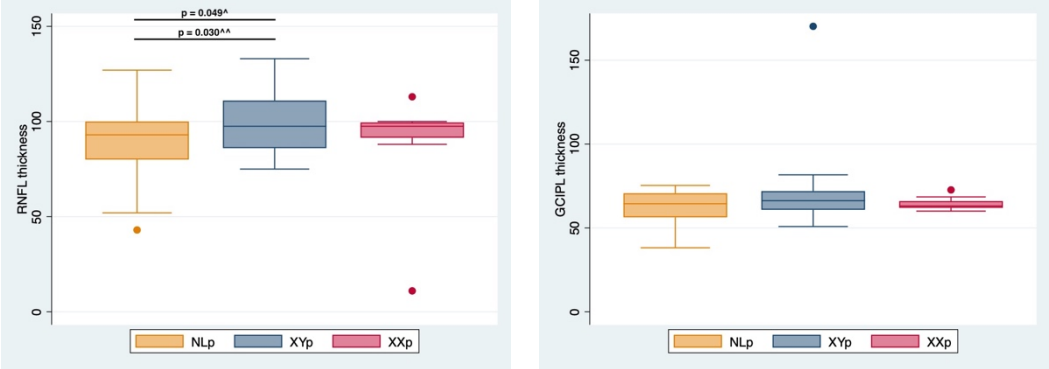
The mixed model regression adjusted for age, disease duration, age at onset, number of previous DMTs, number of previous optic neuritis, and time-gap from last optic neuritis revealed that there was a significant difference between XYp and NLp in RNFL (coeff = 26.919, p-value = 0.021, 95%CI = 4.072 to 49.766), but not in GCIPL.

OCT data at baseline	NLp (n=17)	XYp (n=15)	XXp (n=4)	NLp vs XYp^	NLp vs XXp^	XYp vs XXp^
RNFL (µm), mean ± sd	89.88 ± 18.26	97.22 ± 13.65	62.48 ± 9.29	**p = 0.049*** **p = 0.030* ^^**	p = 0.895	p = 0.115
GCIPL (µm), mean ± sd	87.63 ± 31.74	98.92 ± 7.39	64.35 ± 4.15	p = 0.059 p = 0.343 ^^	p = 0.742	p = 0.308

^Comparison between the groups using a linear regression model with adjustment for age, disease duration, and number of previous optic neuritis

^^Comparison between the groups using a linear regression model with adjustment for age, disease duration, number of previous optic neuritis, and time-gap from the last optic neuritis

Table 5.6. *RNFL and GCIPL at study visit in the three groups.*



Graphs 5.10 and 5.11. *RNFL thickness (left) and GCIPL thickness in the three groups.*

5.5. Discussion

The aim of this prospective, case-control study was to investigate the role of fetal microchimerism in MS. The study is still on-going and the recruitment is still open. However, my preliminary data revealed some interesting results. I found that the risk of MS onset in post-partum was higher in XYp when compared to XXp. I also found that XXp patients had higher spine lesion load at diagnosis and registered higher ARR, while XYp had more frequently brainstem involvement at onset, presented more frequently with progressive MS phenotype at last clinical follow-up, and reached lower scores at PASAT. OCT revealed that, despite having a similar age and disease duration, XXp patients had lower RNFL and GCIPL thickness when compared with XYp, although the difference was not significant. I also found similar trends in XYp and XXp when these groups were compared to NLp. However, the RNFL and GCIPL was again higher in XYp when compared to NLp, while non-significant differences were detected between NLp and XXp. Overall, my results support the hypothesis that XY and XX fMCs could differently modulate the inflammatory and degenerative processes underlying MS.

The results of my third project, albeit preliminary, seemed to confirm some findings obtained in the previous studies and to highlight several interesting differences between XXp and XYp, supporting the hypothesis that fMCs could modulate the disease processes pregnancy-related.

Firstly, I observed that the risk of MS onset in post-partum was higher in XYp when compared to XXp. This result suggested that XY and XX fMCs could act differently in maternal brain. Post-partum is a delicate moment for the immune system (McCombe and Greer, 2013). During pregnancy, the immune system of the mother is partially suppressed to host the foetus, an organism who contains also antigens that are half foreign allograft to the mother: Th2-related activity is increased, while Th1- and Th17-related activities are reduced (McCombe and Greer, 2013). After the pregnancy, these changes are reversed. At present, there is a scientific consensus on the increase frequency of fMCs in maternal blood as gestation progresses (Vernochet, Caucheteux and Kanellopoulos-Langevin, 2007; Fujiki *et al.*, 2008; Adams Waldorf *et al.*, 2010) and several authors reported that the number of fMCs in peripheral blood reaches its peak after delivery (Adams Waldorf *et al.*, 2010). Also, Tan et al. observed that the largest numbers of fMCs in murine maternal brain was observed 4 weeks post-partum (Tan *et al.*, 2005). These findings suggested that during post-partum the concentration of fMCs is at its peak, a condition that is even more relevant considering that microchimeric cells were found present at levels with potential for immunological effects also long after delivery (Loubière *et al.*, 2006; Nelson, 2012). Proceeding from these data, it is possible to speculate that during post-partum fMCs are at their highest potential to induce an immune response in the mother. Indeed, current evidence agrees that the risk of MS relapses increases during the post-partum period and a systematic review confirmed the observation that the risk of relpase is reduced during pregnancy and rises in post-partum (Finkelsztejn *et al.*, 2011; McCombe and Greer, 2013). It is then possible to speculate that fMCs couls play a role in increasing the risk of inflammatory activity during post-partum, when their number is high and the immuno-tolerance versus these *non-self* cells is reduced due to the loss of syncytiotrophoblast cells (Kolialexi *et al.*, 2004; Adams Waldorf *et al.*, 2010).

I hypothesised that the difference detected between women with male and female pregnancies could be related to the phenomenon of *multiple* or *multi-generational*

microchimerism. Being the feto-maternal cellular flow bidirectional, the mother is at the same time carrier of maternal microchimeric cells (mMCs) and fMCs (Guettier *et al.*, 2005; Loubière *et al.*, 2006; Adams Waldorf *et al.*, 2010; Chan *et al.*, 2012; Boddy *et al.*, 2015; Müller *et al.*, 2015). My hypothesis behind the greater risk of onset in male pregnancy is that the maternal immune system could better tolerate XX fMCs as maternal XX microchimeric cells survive and proliferate in each individual since birth. Therefore, after pregnancy, the loss of immunotolerance against *non-self* antigens would be stronger versus XY fMCs than versus XX fMCs, determing an higher risk of onset in patient with male pregnancy.

On contrary, in the long term, patients with male pregnancy and supposedly carrier of XY fMCs reported a disease course characterised by less inflammation and more neurodegeneration than the XX counterpart. Indeed, I found that XXp group had higher ARR and had experienced more relapses before reaching EDSS 6.0, while XYp had higher prevalence of progressive phenotype and lower score at PASAT. Interestingly, during the early disease stage, XXp showed higher spine lesion load at diagnosis, while XYp had experienced more frequently brainstem involvement at onset, both variables considered negative prognostic factors.

Investigating the role of sex chromosomes in a concordant hormonal background on a murine model, Smith-Bouvier et al. found that the production of Th2 cytokines IL-13 and IL-5 were significantly higher in XY genotypes (p-value <0.050). The group also reported a trend for higher production of the Th2 cytokine IL-10 and Th1 cytokines in XY–mice than in XX-mice, although these comparisons did not reach statistical significance (Smith-Bouvier *et al.*, 2008). Th2 cytokines, including IL-13, IL-4, and IL-10, have been previously associated with protection from disease in Experimental Autoimmune Encephalomyelitis (EAE) (Young *et al.*, 2000). Therefore, an increased in Th2 cytokine production could induce the decreased of EAE disease severity. The difference between patients with male and female previous pregnancy, and therefore supposedly carriers of XY fMCs and XX fMCs, could be explained by the different modulation that X and Y sexual chromosomes could exert on the immune system: the female immune response would be more robust that the male one (Nicot, 2009; Voskuhl, Sawalha and Itoh, 2018), while XY genotype could be associated to neurodegeneration and a subsequent more severe disability (Smith-Bouvier *et al.*, 2008; Voskuhl, Sawalha and Itoh, 2018).

Finally, the OCT data revealed that, despite having similar age and disease duration and lower visual relapse, XXp had lower RNFL and GCIPL thicknesses. The difference did not reach the statistical difference, but this is likely due to the unbalance sample between the two groups. Optic nerve is considered an extension of brain parenchyma more than a *true* cranial nerve. In fact, it is a white matter tract surrounded by a dural sheath and formed by the axons of the retinal ganglion cells, which start being myelinated by oligodendrocytes as they leave the optic disc (Freddi and Ottaiano, 2022). After penetrating the lamina cribrosa sclerae, the axons of retinal ganglion cells form the RNFL, whose thickness is considered a valuable measure to investigate *in vivo* the optic nerve damage. The GCIPL is composed of ganglion cell layer and inner plexiform layer and it is another good marker of optic nerve integrity. In neurological disorders, due to the strong neural connection between optic nerve and central nervous system (CNS), any insult to one of the organ could result in a retrograde and anterograde degeneration of the neural pathways (Gupta *et al.*, 2016). Therefore, degenerative neurological disorders could manifest into a damage of the optic nerve that could be assessed using OCT.
My results suggested that XXp had experienced a more sever accrual of damage than XYp both in RNFL and GCIPL. This condition could be explained by (1) higher inflammatory disease activity and/or (2) more severe degenerative processes. Considering the available literature on the effect of X chromosomes on immune system (Smith-Bouvier *et al.*, 2008), it is possible to speculate that the damage might be associated to a more severe inflammatory activity in the brain. However, further investigation would be necessary to test this hypothesis.

Overall, these preliminary data suggested that there was a difference between patients with and without male sons. Being most of the changes occurring during pregnancy not different in male and female pregnancies, I speculated that the sex chromosome of fMCs could play a role in the observed unbalances and that fMCs are the trigger of this sex-associated differences. However, being the study still on-going, the findings reported are only preliminary and laboratory analysis will be essential to (1) demonstrate the presence and frequency of fMCs in the three groups, (2) evaluate their effect on MS pathological processes, and (3) formulate a definite hypothesis on the role of microchimerism in MS.

CHAPTER VI

Can microchimerism open a new perspective in sex differences in Neurology? Conclusions and future works

6.1. Conclusions

After 180 years of Research and several hypotheses, multiple sclerosis (MS) is still an *unveiled* disease as for researchers as for the 2.3 million people affected by the disease. Over the last 30 years, the definition of the disease itself has been completely turned upside down. Currently, we know that MS is not only an inflammatory, but also a degenerative disease, which involves both white matter and grey matter, and whose pathogenesis is associated to a global activation of the immune systems. The available literature reports that more than 200 genes and at least 40 environmental factors have been associated to the natural history of the disease with different level of evidence. Nonetheless, its *primum movens* is still unknown and, in a so undefined background, it is suggestive to hypothesise that each identified factor could only partially influence the biological processes associated to MS, and that an interaction between all these factors is more likely to determine the disease onset and course.

Microchimerism is a fascinating phenomenon still not completely understood and investigated. During pregnancy, the mother regulates her immune system in a so sophisticated way that her body is able to host a *non-self* organism. The theory that the *non-self* cells of the foetus could survive and modulate the maternal immune system is equally intriguing, as these processes would represent one the highest expression of human adaptation. Recently, Kinder et al. has demonstrated that maternal microchimeric cells (mMCs) could survive in the offspring and establish regulatory T cells (T_{reg} cells) specific for non-inherited maternal antigens that could have the aim to protect the next generation of offspring from fetal wastage, suggesting the existence of a *cross-*

generational protection and reproductive advantages actively maintained by tolerogenic microchimeric cells that could be involved in the *self* versus *non-self* antigen distinction (Kinder *et al.*, 2015). Being the loss of this adaptative property to differentiate between *self* and *non-self* a milestone in immune-mediate diseases, such as MS, it is tempting to theorise that the phenomenon of fetal microchimerism, so deeply involved in maternal immune system modulation during pregnancy, could play a role in influencing the disease onset and course.

Starting from these assumptions, during my PhD course, I have investigated the role the sex of offspring as an indirect marker of fetal microchimeric cells (fMCs) with the aim to define the role of these cells in MS and to evaluate whether there is any correlation between the presence of XX or XY genotype and the clinical, paraclinical, and radiological disease features of the disease. The data obtained during the last three years, although not without limitations, seem to support my hypothesis that a relationship between these *non-self* cells and disease characteristics may exist. Over the previous Chapters, I have reported my results supporting my hypothesis that these fMCs could, although marginally and likely interacting with other factors, be involved in MS inflammation and axonal degeneration, influencing the immune system activation and inducing mechanism of repair. Proceeding from my findings, I have also speculated that fMCs could modulate the maternal immune system differently depending on their sexual chromosomes, opening to a new point of view in the gender-specific medicine.

The hypothesis that a small percentage of cells with an XX or XY genotype could, through the expressions of sexual chromosome genes, regulate the maternal immune system and the repair mechanisms activated in the mother is fascinating and lends a fresh perspective to the sexual differences in neurological diseases. So far, several studies have suggested that sexual chromosomes may influence the morphology and functioning of the central nervous system (CNS) both during the intra-uterine life and throughout subject's life, being involved also in the pathological processes. Discovering whether the presence of fetal *non-self* chimeric cells, and their chromosomes, may play a role in the modulation of the nervous system and its pathology is surely one of the more interesting challenges for the future.

6.2. Future works

Since 1977, when Liégeois et al. proposed the definition of "microchimerism", several authors have confirmed the existence of fetal cells in maternal blood and tissues (Nelson, 2012; Boddy *et al.*, 2015; Stevens, 2016). The available literature on the role of the microchimerism in multiple sclerosis (MS) are still limited. Three previous study have reported a higher percentage of microchimeric cells in patients with MS when compared to healthy controls (Willer *et al.*, 2006; Bloch *et al.*, 2011; Jafarinia *et al.*, 2020). However, to-date, only one tissue study has been conducted to evaluate the role of maternal microchimeric cells (mMCs) in brain tissue of male MS patients and no data are available on the presence and distribution of fMCs in MS patients.

Considering the available literature and the data obtained in the three studies reported in this dissertation, I designed a tissue-based study to investigate the role of fMCs and their interaction with the neurological and immunological cells and molecules in maternal brain. To my knowledge, the project would be the first study investigating the role of fMCs in brain tissue of female MS patients. Proceeding from the assumption that fMCs could have a role in modulating the pathological mechanisms underlying the MS, and particularly that XY and XX fMCs could act differently in the inflammatory and neurodegenerative processes, I designed a tissue-based study with the following aims.

The primary aim of this study will be to evaluate the presence and distribution of fMCs in the MS brain tissue. I will analyse 40 human brain tissue samples to evaluate the presence and distribution of XY fMCs. Snap frozen tissue specimens obtained from adult women who met clinical and pathologic criteria for MS according to revised McDonald criteria (Thompson *et al.*, 2018) and without other neurologic diseases will be used. Firstly, genomic DNA will be extracted from brain tissues using the specific kit and PCR-based techniques will be used to amplify Y chromosome-specific sequences, such as AMELY gene or the recently proposed TSPY gene (Dash, Rawat and Das, 2020), as better markers to check the presence of male DNA in women. The research of male fMCs will be performed in different brain areas, including white matter lesions, normal appearing white matter, grey matter lesions, normal appearing grey matter, and basal

ganglia. Brain specimens form 20 healthy controls will be obtained and analysed to evaluate the presence and distribution of microchimeric cells in unaffected patients. This finding would allow us to investigate whether the presence of injured brain tissue and an affected blood-brain barrier (BBB) could influence the migration and colonisation of XY fMCs, as reported in previous studies (Tan *et al.*, 2005; Zeng *et al.*, 2010; Chan *et al.*, 2012).

The second aim of the study will be to evaluate the role of microchimeric cells in modulating the inflammation and neurodegeneration processes underlying the disease. To address this purpose, I will analyse tissue samples from MS patients to evaluate (1) the activation of resident CNS glial cells (such as microglia) and CD8+T cells, (2) the role of CD4+T-helper 1 (TH1) cells and CD4+T-helper 2 (TH2) cells and (3) their release of pro-inflammatory cytokines, including interferon-gamma, interleukin-2 (IL-2), and tumour necrosis factor-a (TNF-a), and anti-inflammatory molecules, including interleukin-4 (IL-4), interleukin-5 (IL-5), and interleukin-10 (IL-10). Finally, (3) the contribution of B cells and their relationship with XY microchimeric cells will be evaluated in meningeal inflammation and B cell follicle-like structures adjacent to subpial cortical lesions. Cell-specific stains will be used to evaluate these phenomena in brain tissue. The same analysis will be performed on tissue from healthy controls.

Finally, considering the interesting results obtained comparing the cortical thickness and the possible role of fMCs in the chronic inflammation associated to cortical damage, the tertiary aim of the study will be to analyse the cerebrospinal fluid (CSF) of MS patients for evaluating (1) the presence and frequency of fMCs and (2) the immunological profile of patients. To fulfil this aim, I will analyse the CSF of 120 female patients affected by MS, including 40 nulliparous patients, 40 patients with at least a male son, and 40 with only daughters. Genomic DNA will be extracted from CSF using the specific kit and PCR-based techniques will be used to amplify Y chromosome-specific sequences, such as AMELY gene or the recently proposed TSPY gene (Dash, Rawat and Das, 2020), as better markers to check the presence of male DNA in women. Patients will be then classified according to the presence of male fMCs and their CSF inflammatory profile will be examinated to evaluate the presence and density of pro-inflammatory cytokines, including IFNγ, TNF, IL2, and IL22, and molecules related to sustained B-cell activity

and lymphoid-neogenesis, including CXCL13, CXCL10, LTα, IL6, IL10 (Magliozzi *et al.*, 2018).

The overarching aim of the study will be to focus on microchimerism phenomenon to investigate its role in MS, and more specifically to study the role of the sexual chromosomes in the microchimeric cells in modulating the maternal immune system. I hypothesise that, in a multifactorial background, these *non-self* fMCs and their sexual chromosome could influence the activation of the immune system and modulate the inflammatory and neurodegenerative processes underlying the disease. The study of this phenomenon in brain tissues and CFS would provide information on the role of these cells in the pathogenic mechanism underlying MS, increasing our knowledge on both the biological role of fetal-maternal microchimerism and the immunological response to these *non-self* cells in MS patients. The project will help to take a step further in defining the disease biology and, as recently highlighted by Kuhlmann et al., a more profound knowledge of the pathological bases of the disease is essential to build a future in which clinical benefit accrues directly from biomarker-based, biologically informed treatment decisions (Kuhlmann *et al.*, 2022).

APPENDICES

Appendix 1. Areas included in Aseg, Aparc, Wmparc Free Surfer Atlases.

Atlante *Aseg*	Atlante *Aparc*	Atlante *WMparc*
Left lateral ventricle	Bankssts	Bankssts
Left inferior lateral ventricle	Caudal anterior cingulate	Caudal anterior cingulate
Left cerebellum WM	Caudal middle frontal	Caudal middle frontal
Left cerebellum cortex	Cuneus	Cuneus
Left thalamus	Entorhinal	Entorhinal
Left caudate	Fusiform	Fusiform
Left putamen	Inferior parietal	Inferior parietal
Left pallidum	Inferior temporal	Inferior temporal
3rd ventricle	Isthmus cingulate	Isthmus cingulate
4th ventricle	Lateral occipital	Lateral occipital
Brainstem	Lateral orbitofrontal	Lateral orbitofrontal
Left hippocampus	Lingual	Lingual
Left amygdala	Medial orbitofrontal	Medial orbitofrontal
CSF	Middle temporal	Middle temporal
Left accumbens area	Parahippocampal	Parahippocampal
Left ventral DC	Paracentral	Paracentral
Left vessel	Pars opercularis	Pars opercularis
Left choroid plexus	Pars orbitalis	Pars orbitalis
Right lateral ventricle	Pars triangularis	Pars triangularis
Right inferior lateral ventricle	Pericalcarine	Pericalcarine
Right cerebellum WM	Postcentral	Postcentral
Right cerebellum cortex	Posterior cingulate	Posterior cingulate
Right thalamus	Precentral	Precentral
Right caudate	Precuneus	Precuneus
Right putamen	Rostral anterior cingulate	Rostral anterior cingulate
Right pallidum	Rostral middle frontal	Rostral middle frontal
Right hippocampus	Superior frontal	Superior frontal
Right amygdala	Superior parietal	Superior parietal
Right accumbens area	Superior temporal	Superior temporal
Right ventral DC	Supramarginal	Supramarginal
Right vessel	Frontal pole	Frontal pole
Right choroid plexus	Temporal pole	Temporal pole
5th Ventricle	Transverse temporal	Transverse temporal
WM hypointensities	Insula	Insula
Left WM hypointensities		Unsegmented
Right WM hypointensities		
Non-WM hypointensities		
Left non-WM hypointensities		
Right non-WM hypointensities		
Optic chiasm		
CC Posterior		
CC Middle posterior		
CC Central		
CC Middle anterior		
CC Anterior		

Appendix 2. Subcortical volumes (cm3, mean ± sd) in the three groups.

Subcortical volumes	NLp (n=26)	XXp (n=8)	XYp (n=20)	NLp vs XXp p-value (CI)	NLp vs XYp p-value (CI)	XXp vs XYp p-value (CI)
Left lateral ventricle	10.84 ± 9.02	13.31 ± 9.73	9.62 ± 5.59	0.302 (-8.197 – 2.601)	0.095 (-7.544 – 0.625)	0.502 (-8.402 – 4.249)
Left inferior lateral ventricle	0.42 ± 0.20	0.47 ± 0.21	0.43 ± 0.24	0.971 (-0.177 – 0.183)	0.666 (-0.165 – 0.107)	0.649 (-0.275 – 0.175)
Left cerebellum WM	11.91 ± 1.60	12.18 ± 1.81	12.30 ± 1.92	0.289 (-0.647 – 2.122)	0.469 (-0.668 – 1.428)	0.687 (-1.977 – 1.328)
Left cerebellum cortex	50.07 ± 5.12	47.90 ± 5.59	48.91 ± 3.87	0.587 (-5.167 – 2.961)	0.565 (-3.959 – 2.189)	0.920 (-3.912 – 4.315)
Left thalamus	6.30 ± 0.94	5.88 ± 1.03	6.41 ± 0.91	0.750 (-0.938 – 0.681)	0.423 (-0.366 – 0.859)	0.390 (-0.526 – 1.293)
Left caudate	3.03 ± 0.45	3.06 ± 0.41	3.02 ± 0.44	0.738 (-0.329 – 0.461)	0.944 (-0.309 – 0.288)	0.659 (-0.497 – 0.321)
Left putamen	3.92 ± 0.81	3.64 ± 0.74	4.05 ± 0.62	0.900 (-0.665 – 0.586)	0.326 (-0.240 – 0.707)	0.249 (-0.267 – 0.975)
Left pallidum	1.54 ± 0.25	1.54 ± 0.19	1.65 ± 0.25	0.652 (-0.161 – 0.255)	0.152 (-0.044 – 0.271)	0.381 (-0.123 – 0.309)
3rd ventricle	1.16 ± 0.46	1.44 ± 0.83	1.15 ± 0.50	0.709 (-0.371 – 0.542)	0.389 (-0.494 – 0.196)	0.459 (-0.779 – 0.364)
4th ventricle	2.02 ± 0.59	1.95 ± 0.50	1.70 ± 0.41	0.233 (-0.688 – 0.172)	**0.022 (-0.708 – -0.057)**	0.545 (-0.490 – 0.266)
Brainstem	18.23 ± 2.20	17.85 ± 2.76	19.04 ± 2.23	0.740 (-1.566 – 2.188)	0.191 (-0.483 – 2.357)	0.430 (-1.389 – 3.146)
Left hippocampus	3.49 ± 0.39	3.46 ± 0.36	3.58 ± 0.35	0.872 (-0.302 – 0.354)	0.394 (-0.142 – 0.354)	0.617 (-0.253 – 0.416)
Left amygdala	1.25 ± 0.13	1.24 ± 0.18	1.30 ± 0.27	0.949 (-0.167 – 0.178)	0.659 (-0.102 – 0.159)	0.835 (-0.203 – 0.249)
Left accumbens area	0.47 ± 0.13	0.41 ± 0.13	0.49 ± 0.10	0.883 (-0.106 – 0.091)	0.200 (-0.026 – 0.122)	0.201 (-0.038 – 0.169)
Left ventral DC	3.35 ± 0.43	3.30 ± 0.45	3.45 ± 0.45	0.525 (-0.235 – 0.455)	0.339 (-0.136 – 0.386)	0.771 (-0.356 – 0.474)
Left vessel	0.03 ± 0.03	0.03 ± 0.03	0.04 ± 0.04	0.890 (-0.032 – 0.027)	0.444 (-0.014 – 0.031)	0.290 (-0.015 – 0.048)
Left choroid plexus	0.62 ± 0.39	0.61 ± 0.19	0.53 ± 0.23	0.133 (-0.412 – 0.056)	0.094 (-0.327 – 0.027)	0.645 (-0.248 – 0.157)
Right lateral ventricle	9.87 ± 7.99	11.75 ± 8.27	8.81 ± 5.19	0.273 (-7.537 – 2.180)	0.105 (-6.697 – 0.653)	0.580 (-7.164 – 4.115)
Right inferior lateral ventricle	0.46 ± 0.22	0.48 ± 0.22	0.51 ± 0.32	0.582 (-0.286 – 0.162)	0.915 (-0.160 – 0.178)	0.707 (-0.233 – 0.337)
Right cerebellum WM	11.41 ± 1.59	11.67 ± 1.76	12.10 ± 1.75	0.197 (-0.454 – 2.140)	0.126 (-0.221 – 1.742)	0.947 (-1.564 – 1.466)
Right cerebellum cortex	50.28 ± 5.50	47.83 ± 5.75	49.21 ± 4.03	0.573 (-5.529 – 3.095)	0.720 (-3.847 – 2.677)	0.769 (-3.568 – 4.762)
Right thalamus	5.76 ± 0.86	5.51 ± 0.81	5.99 ± 0.91	0.995 (-0.743 – 0.738)	0.272 (-0.251 – 0.869)	0.399 (-0.481 – 1.159)
Right caudate	3.14 ± 0.47	3.19 ± 0.48	3.12 ± 0.45	0.454 (-0.252 – 0.554)	0.878 (-0.281 – 0.328)	0.479 (-0.567 – 0.275)
Right putamen	4.00 ± 0.77	3.87 ± 0.55	4.07 ± 0.76	0.738 (-0.517 – 0.724)	0.564 (-0.334 – 0.605)	0.795 (-0.572 – 0.737)
Right pallidum	1.54 ± 0.23	1.63 ± 0.24	1.68 ± 0.22	0.146 (-0.052 – 0.340)	**0.049 (0.001 – 0.297)**	0.877 (-0.196 – 0.228)
Right hippocampus	3.60 ± 0.44	3.63 ± 0.36	3.66 ± 0.44	0.625 (-0.283 – 0.466)	0.662 (-0.221 – 0.346)	0.922 (-0.400 – 0.364)
Right amygdala	1.42 ± 0.19	1.42 ± 0.17	1.44 ± 0.29	0.854 (-0.187 – 0.225)	0.718 (-0.128 – 0.184)	0.973 (-0.255 – 0.247)
Right accumbens area	0.44 ± 0.10	0.42 ± 0.09	0.46 ± 0.09	0.646 (-0.057 – 0.091)	0.208 (-0.020 – 0.091)	0.556 (-0.055 – 0.100)
Right ventral DC	3.31 ± 0.44	3.28 ± 0.42	3.43 ± 0.44	0.409 (-0.204 – 0.493)	0.194 (-0.091 – 0.436)	0.712 (-0.318 – 0.457)
Right vessel	0.04 ± 0.04	0.04 ± 0.03	0.04 ± 0.04	0.812 (-0.028 – 0.035)	0.597 (-0.030 – 0.017)	0.763 (-0.039 – 0.029)
Right choroid plexus	0.64 ± 0.26	0.66 ± 0.19	0.59 ± 0.27	0.245 (-0.293 – 0.077)	0.181 (-0.234 – 0.046)	0.803 (-0.263 – 0.206)
Optic chiasm	0.20 ± 0.04	0.22 ± 0.04	0.19 ± 0.04	0.802 (-0.030 – 0.039)	0.066 (-0.051 – 0.002)	0.108 (-0.070 – 0.008)
CC Posterior	0.86 ± 0.26	0.87 ± 0.13	0.80 ± 0.14	0.448 (-0.234 – 0.105)	0.181 (-0.215 – 0.042)	0.217 (-0.199 – 0.048)
CC Middle posterior	0.48 ± 0.16	0.43 ± 0.12	0.42 ± 0.11	0.194 (-0.203 – 0.042)	0.135 (-0.162 – 0.023)	0.789 (-0.121 – 0.093)
CC Central	0.44 ± 0.12	0.39 ± 0.08	0.42 ± 0.11	0.421 (-0.141 – 0.060)	0.745 (-0.088 – 0.064)	0.660 (-0.076 – 0.118)
CC Middle anterior	0.42 ± 0.12	0.34 ± 0.11	0.41 ± 0.13	0.155 (-0.190 – 0.031)	0.734 (-0.098 – 0.069)	0.332 (-0.060 – 0.169)
CC Anterior	0.76 ± 0.15	0.66 ± 0.21	0.78 ± 0.14	0.151 (-0.245 – 0.039)	0.684 (-0.086 – 0.129)	0.211 (-0.056 – 0.240)

Appendix 3. Cortical thickness (mm, mean ± sd) in the three groups.

Cortical thickness	NLp (n=26)	XXp (n=8)	XYp (n=20)	NLp vs XXp p-value (CI)	NLp vs XYp p-value (CI)	XXp vs XYp p-value (CI)
Left bank of the superior temporal sulcus	2.42 ± 0.15	2.42 ± 0.16	2.40 ± 0.19	0.282 (-0.062 – 0.208)	0.995 (-0.102 – 0.102)	0.610 (-0.224 – 0.135)
Left caudal anterior cingulate	2.57 ± 0.31	2.61 ± 0.33	2.54 ± 0.27	0.927 (-0.272 – 0.248)	0.547 (-0.256 – 0.137)	0.579 (-0.362 – 0.207)
Left caudal middle frontal	2.52 ± 0.16	2.49 ± 0.17	2.53 ± 0.13	0.544 (-0.085 – 0.160)	0.454 (-0.058 – 0.127)	0.736 (-0.116 – 0.162)
Left cuneus	1.84 ± 0.15	1.80 ± 0.14	1.93 ± 0.10	0.684 (-0.135 – 0.089)	0.057 (-0.003 – 0.167)	**0.042 (0.005 – 0.225)**
Left entorhinal	3.19 ± 0.26	3.10 ± 0.31	3.23 ± 0.37	0.968 (-0.276 – 0.265)	0.491 (-0.134 – 0.275)	0.734 (-0.254 – 0.354)
Left fusiform	2.66 ± 0.16	2.70 ± 0.25	2.65 ± 0.17	0.357 (-0.085 – 0.230)	0.824 (-0.132 – 0.106)	0.289 (-0.279 – 0.087)
Left inferior parietal	2.40 ± 0.10	2.44 ± 0.13	2.41 ± 0.15	0.144 (-0.027 – 0.179)	0.673 (-0.062 – 0.094)	0.289 (-0.193 – 0.061)
Left inferior temporal	2.77 ± 0.17	2.74 ± 0.19	2.79 ± 0.21	0.743 (-0.200 – 0.144)	0.856 (-0.118 – 0.142)	0.714 (-0.166 – 0.238)
Left isthmus cingulate	2.14 ± 0.22	2.21 ± 0.15	2.11 ± 0.14	0.211 (-0.058 – 0.256)	0.686 (-0.143 – 0.095)	0.119 (-0.245 – 0.030)
Left lateral occipital	2.10 ± 0.12	2.17 ± 0.09	2.14 ± 0.14	0.091 (-0.014 – 0.187)	0.494 (-0.050 – 0.103)	0.311 (-0.157 – 0.052)
Left lateral orbitofrontal	2.69 ± 0.22	2.77 ± 0.28	2.66 ± 0.23	0.354 (-0.114 – 0.311)	0.798 (-0.181 – 0.140)	0.228 (-0.381 – 0.096)
Left lingual	2.02 ± 0.13	1.98 ± 0.09	2.07 ± 0.08	0.993 (-0.090 – 0.089)	0.135 (-0.017 – 0.119)	0.088 (-0.011 – 0.144)
Left medial orbitofrontal	2.52 ± 0.17	2.58 ± 0.28	2.50 ± 0.15	0.248 (-0.067 – 0.254)	0.836 (-0.134 – 0.109)	0.222 (-0.307 – 0.075)
Left middle temporal	2.79 ± 0.14	2.80 ± 0.17	2.77 ± 0.16	0.210 (-0.044 – 0.193)	0.760 (-0.103 – 0.076)	0.336 (-0.215 – 0.077)
Left parahippocampal	2.76 ± 0.25	2.69 ± 0.39	2.80 ± 0.35	0.971 (-0.262 – 0.272)	0.421 (-0.120 – 0.283)	0.847 (-0.287 – 0.346)
Left paracentral	2.34 ± 0.16	2.39 ± 0.17	2.46 ± 0.17	**0.043 (0.004 – 0.258)**	**0.004 (0.047 – 0.238)**	0.741 (-0.130 – 0.180)
Left cprs opercularis	2.55 ± 0.15	2.57 ± 0.15	2.51 ± 0.16	0.177 (-0.040 – 0.211)	0.542 (-0.124 – 0.066)	0.112 (-0.250 – 0.028)
Left pars orbitalis	2.72 ± 0.19	2.72 ± 0.28	2.73 ± 0.24	0.453 (-0.120 – 0.264)	0.663 (-0.114 – 0.177)	0.648 (-0.284 – 0.181)
Left pars triangularis	2.44 ± 0.14	2.48 ± 0.21	2.44 ± 0.14	0.059 (-0.004 – 0.217)	0.847 (-0.075 – 0.092)	0.123 (-0.229 – 0.029)
Left pericalcarine	1.64 ± 0.14	1.59 ± 0.19	1.72 ± 0.12	0.571 (-0.152 – 0.085)	**0.041 (0.004 – 0.184)**	**0.032 (0.014 – 0.282)**
Left postcentral	2.07 ± 0.12	2.03 ± 0.16	2.09 ± 0.10	0.669 (-0.076 – 0.117)	0.141 (-0.019 – 0.128)	0.396 (-0.064 – 0.154)
Left posterior cingulate	2.42 ± 0.15	2.50 ± 0.23	2.40 ± 0.16	0.169 (-0.044 – 0.244)	0.662 (-0.132 – 0.085)	0.057 (-0.309 – 0.005)
Left precentral	2.50 ± 0.19	2.46 ± 0.18	2.52 ± 0.14	0.460 (-0.084 – 0.182)	0.319 (-0.050 – 0.151)	0.697 (-0.113 – 0.166)
Left precuneus	2.27 ± 0.11	2.34 ± 0.16	2.34 ± 0.17	**0.046 (0.002 – 0.234)**	0.079 (-0.009 – 0.166)	0.599 (-0.187 – 0.112)
Left rostral anterior cingulate	2.79 ± 0.19	2.76 ± 0.31	2.88 ± 0.34	0.804 (-0.200 – 0.257)	0.140 (-0.044 – 0.302)	0.391 (-0.165 – 0.405)
Left rostral middle frontal	2.37 ± 0.14	2.34 ± 0.19	2.41 ± 0.14	0.801 (-0.108 – 0.139)	0.267 (-0.041 – 0.146)	0.631 (-0.112 – 0.180)
Left superior frontal	2.66 ± 0.18	2.61 ± 0.21	2.71 ± 0.14	0.564 (-0.099 – 0.180)	0.092 (-0.015 – 0.196)	0.412 (-0.092 – 0.215)
Left superior parietal	2.14 ± 0.10	2.13 ± 0.14	2.17 ± 0.14	0.508 (-0.066 – 0.131)	0.489 (-0.049 – 0.100)	0.852 (-0.134 – 0.112)
Left superior temporal	2.70 ± 0.16	2.63 ± 0.22	2.68 ± 0.19	0.780 (-0.118 – 0.156)	0.940 (-0.100 – 0.108)	0.898 (-0.180 – 0.159)
Left supramarginal	2.52 ± 0.13	2.51 ± 0.17	2.54 ± 0.16	0.258 (-0.050 – 0.184)	0.274 (-0.040 – 0.137)	0.856 (-0.162 – 0.135)
Left frontal pole	2.68 ± 0.25	2.70 ± 0.16	2.75 ± 0.20	0.579 (-0.140 – 0.248)	0.285 (-0.068 – 0.226)	0.746 (-0.156 – 0.215)
Left temporal pole	3.57 ± 0.33	3.60 ± 0.49	3.52 ± 0.40	0.716 (-0.280 – 0.404)	0.613 (-0.324 – 0.193)	0.449 (-0.567 – 0.260)
Left transverse temporal	2.37 ± 0.25	2.30 ± 0.26	2.35 ± 0.17	0.291 (-0.072 – 0.234)	0.410 (-0.068 – 0.163)	0.880 (-0.179 – 0.155)
Left insula	3.05 ± 0.17	3.10 ± 0.08	3.00 ± 0.16	0.095 (-0.019 – 0.231)	0.656 (-0.116 – 0.074)	0.133 (-0.237 – 0.033)
Right bank of the superior temporal sulcus	2.51 ± 0.17	2.51 ± 0.19	2.51 ± 0.16	0.543 (-0.097 – 0.182)	0.685 (-0.084 – 0.127)	0.604 (-0.198 – 0.118)
Right caudal anterior cingulate	2.44 ± 0.28	2.43 ± 0.26	2.49 ± 0.32	0.617 (-0.186 – 0.310)	0.384 (-0.106 – 0.269)	0.648 (-0.227 – 0.358)
Right caudal middle frontal	2.51 ± 0.17	2.43 ± 0.15	2.50 ± 0.17	0.934 (-0.117 – 0.127)	0.729 (-0.076 – 0.108)	0.793 (-0.121 – 0.156)
Right cuneus	1.90 ± 0.16	1.87 ± 0.12	1.95 ± 0.11	0.922 (-0.126 – 0.114)	0.199 (-0.032 – 0.150)	0.169 (-0.035 – 0.186)
Right entorhinal	3.29 ± 0.35	3.11 ± 0.43	3.25 ± 0.34	0.546 (-0.411 – 0.220)	0.860 (-0.260 – 0.218)	0.726 (-0.265 – 0.374)
Right fusiform	2.65 ± 0.15	2.68 ± 0.24	2.65 ± 0.14	0.501 (-0.093 – 0.188)	0.833 (-0.118 – 0.095)	0.343 (-0.232 – 0.084)
Right inferior parietal	2.44 ± 0.10	2.43 ± 0.15	2.42 ± 0.15	0.401 (-0.056 – 0.136)	0.671 (-0.088 – 0.057)	0.253 (-0.183 – 0.051)
Right inferior temporal	2.72 ± 0.14	2.73 ± 0.26	2.80 ± 0.22	0.739 (-0.194 – 0.139)	0.618 (-0.095 – 0.158)	0.676 (-0.174 – 0.264)
Right isthmus cingulate	2.19 ± 0.18	2.21 ± 0.18	2.15 ± 0.23	0.489 (-0.109 – 0.224)	0.581 (-0.161 – 0.091)	0.218 (-0.304 – 0.074)
Right lateral occipital	2.14 ± 0.11	2.25 ± 0.08	2.18 ± 0.13	**0.006 (0.041 – 0.234)**	0.409 (-0.043 – 0.103)	**0.027 (-0.207 – -0.014)**
Right lateral orbitofrontal	2.67 ± 0.24	2.71 ± 0.32	2.66 ± 0.25	0.819 (-0.201 – 0.253)	0.860 (-0.187 – 0.157)	0.602 (-0.331 – 0.197)
Right lingual	2.05 ± 0.11	1.98 ± 0.15	2.07 ± 0.12	0.318 (-0.140 – 0.046)	0.944 (-0.073 – 0.068)	0.372 (-0.059 – 0.151)
Right medial orbitofrontal	2.57 ± 0.15	2.58 ± 0.28	2.53 ± 0.21	0.820 (-0.152 – 0.192)	0.464 (-0.178 – 0.082)	0.626 (-0.275 – 0.169)
Right middle temporal	2.80 ± 0.15	2.84 ± 0.13	2.78 ± 0.19	0.206 (-0.048 – 0.215)	0.519 (-0.132 – 0.067)	0.095 (-0.269 – 0.023)
Right parahippocampal	2.69 ± 0.23	2.65 ± 0.30	2.72 ± 0.30	0.989 (-0.232 – 0.235)	0.604 (-0.131 – 0.222)	0.726 (-0.234 – 0.330)
Right paracentral	2.34 ± 0.17	2.31 ± 0.23	2.42 ± 0.14	0.407 (-0.078 – 0.189)	**0.015 (0.026 – 0.228)**	0.245 (-0.066 – 0.246)
Right cprs opercularis	2.51 ± 0.15	2.48 ± 0.18	2.51 ± 0.20	0.336 (-0.063 – 0.181)	0.710 (-0.075 – 0.110)	0.545 (-0.196 – 0.107)

Cortical thickness	NLp (n=26)	XXp (n=8)	XYp (n=20)	NLp vs XXp p-value (CI)	NLp vs XYp p-value (CI)	XXp vs XYp p-value (CI)
Right pars orbitalis	2.74 ± 0.19	2.71 ± 0.22	2.71 ± 0.19	0.897 (-0.159 – 0.181)	0.753 (-0.149 – 0.108)	0.379 (-0.233 – 0.092)
Right pars triangularis	2.46 ± 0.19	2.48 ± 0.20	2.46 ± 0.19	0.162 (-0.042 – 0.245)	0.991 (-0.108 – 0.109)	0.147 (-0.257 – 0.041)
Right pericalcarine	1.68 ± 0.17	1.64 ± 0.14	1.69 ± 0.12	0.806 (-0.144 – 0.112)	0.696 (-0.078 – 0.116)	0.270 (-0.051 – 0.174)
Right postcentral	2.05 ± 0.12	2.00 ± 0.15	2.08 ± 0.08	0.924 (-0.100 – 0.091)	0.084 (-0.009 – 0.135)	0.201 (-0.037 – 0.165)
Right posterior cingulate	2.43 ± 0.18	2.49 ± 0.21	2.37 ± 0.18	0.446 (-0.101 – 0.226)	0.244 (-0.196 – 0.051)	0.089 (-0.336 – 0.026)
Right precentral	2.48 ± 0.18	2.44 ± 0.17	2.51 ± 0.16	0.385 (-0.075 – 0.192)	0.102 (-0.017 – 0.185)	0.531 (-0.108 – 0.203)
Right precuneus	2.31 ± 0.12	2.31 ± 0.17	2.34 ± 0.17	0.295 (-0.056 – 0.181)	0.262 (-0.039 – 0.140)	0.868 (-0.167 – 0.142)
Right rostral anterior cingulate	2.89 ± 0.26	2.75 ± 0.32	2.91 ± 0.25	0.542 (-0.296 – 0.158)	0.416 (-0.102 – 0.241)	0.198 (-0.085 – 0.389)
Right rostral middle frontal	2.36 ± 0.13	2.38 ± 0.15	2.38 ± 0.17	0.215 (-0.045 – 0.195)	0.455 (-0.057 – 0.125)	0.558 (-0.192 – 0.107)
Right superior frontal	2.66 ± 0.17	2.60 ± 0.17	2.67 ± 0.18	0.787 (-0.118 – 0.155)	0.466 (-0.066 – 0.141)	0.722 (-0.133 – 0.189)
Right superior parietal	2.14 ± 0.09	2.10 ± 0.14	2.18 ± 0.14	0.765 (-0.110 – 0.081)	0.367 (-0.039 – 0.105)	0.464 (-0.077 – 0.163)
Right superior temporal	2.70 ± 0.18	2.69 ± 0.22	2.70 ± 0.20	0.143 (-0.036 – 0.242)	0.427 (-0.063 – 0.147)	0.434 (-0.226 – 0.101)
Right supramarginal	2.50 ± 0.15	2.47 ± 0.19	2.52 ± 0.12	0.467 (-0.072 – 0.155)	0.280 (-0.039 – 0.132)	0.988 (-0.124 – 0.126)
Right frontal pole	2.61 ± 0.26	2.74 ± 0.28	2.65 ± 0.25	0.241 (-0.093 – 0.361)	0.761 (-0.146 – 0.198)	0.574 (-0.317 – 0.181)
Right temporal pole	3.50 ± 0.44	3.53 ± 0.49	3.46 ± 0.37	0.761 (-0.146 – 0.198)	0.889 (-0.297 – 0.259)	0.375 (-0.536 – 0.210)
Right transverse temporal	2.39 ± 0.21	2.33 ± 0.18	2.41 ± 0.20	0.410 (-0.087 – 0.209)	0.170 (-0.034 – 0.189)	0.686 (-0.129 – 0.192)
Right insula	3.01 ± 0.14	3.04 ± 0.25	2.98 ± 0.18	0.368 (-0.080 – 0.211)	0.418 (-0.155 – 0.065)	0.242 (-0.301 – 0.080)

Appendix 4. White matter volumes (cm^3, mean ± sd) in the three groups.

White matter volumes (cm^3 volumes. mean ± sd)	NLp (n=26)	XXp (n=8)	XYp (n=20)	NLp vs XXp p-value (CI)	NLp vs XYp p-value (CI)	XXp vs XYp p-value (CI)
Left bank of the superior temporal sulcus	2.24 ± 0.51	2.43 ± 0.53	2.35 ± 0.45	0.305 (-0.212 – 0.662)	0.585 (-0.240 – 0.421)	0.598 (-0.559 – 0.330)
Left caudal anterior cingulate	1.79 ± 0.47	1.50 ± 0.35	1.78 ± 0.38	0.327 (-0.541 – 0.184)	0.773 (-0.235 – 0.313)	0.137 (-0.087 – 0.594)
Left caudal middle frontal	5.91 ± 0.96	5.40 ± 0.37	6.05 ± 1.06	0.245 (-1.333 – 0.349)	0.665 (-0.498 – 0.774)	0.170 (-0.258 – 1.368)
Left cuneus	2.36 ± 0.47	2.17 ± 0.27	2.25 ± 0.47	0.353 (-0.575 – 0.209)	0.315 (-0.446 – 0.147)	0.748 (-0.323 – 0.442)
Left entorhinal	0.55 ± 0.17	0.68 ± 0.25	0.59 ± 0.20	**0.028 (0.021 – 0.354)**	0.318 (-0.063 – 0.189)	0.158 (-0.336 – 0.058)
Left fusiform	5.19 ± 0.67	5.07 ± 0.67	5.36 ± 0.76	0.761 (-0.724 – 0.533)	0.471 (-0.304 – 0.647)	0.341 (-0.349 – 0.965)
Left inferior parietal	8.27 ± 1.16	8.81 ± 0.77	8.53 ± 1.33	0.131 (-0.245 – 1.821)	0.310 (-0.383 – 1.180)	0.405 (-1.543 – 0.648)
Left inferior temporal	5.50 ± 0.91	5.68 ± 1.01	5.38 ± 0.97	0.778 (-0.733 – 0.974)	0.671 (-0.783 – 0.508)	0.661 (-1.070 – 0.693)
Left isthmus cingulate	2.82 ± 0.61	2.69 ± 0.54	2.91 ± 0.34	0.670 (-0.321 – 0.495)	0.279 (-0.141 – 0.477)	0.272 (-0.180 – 0.608)
Left lateral occipital	9.17 ± 1.41	8.37 ± 1.06	8.91 ± 1.47	0.319 (-1.743 – 0.580)	0.458 (-1.205 – 0.552)	0.553 (-0.900 – 1.636)
Left lateral orbitofrontal	5.76 ± 0.84	5.49 ± 1.01	5.90 ± 0.95	0.598 (-1.019 – 0.593)	0.705 (-0.494 – 0.725)	0.406 (-0.509 – 1.210)
Left lingual	4.87 ± 0.78	4.66 ± 0.53	4.84 ± 0.83	0.828 (-0.696 – 0.560)	0.765 (-0.546 – 0.405)	0.874 (-0.620 – 0.724)
Left medial orbitofrontal	3.36 ± 0.65	3.37 ± 0.43	3.31 ± 0.58	0.997 (-0.534 – 0.536)	0.666 (-0.492 – 0.317)	0.818 (-0.519 – 0.414)
Left middle temporal	4.91 ± 0.85	4.71 ± 0.76	4.75 ± 1.05	0.548 (-1.053 – 0.567)	0.617 (-0.766 – 0.459)	0.745 (-0.713 – 0.983)
Left parahippocampal	1.04 ± 0.21	1.00 ± 0.21	1.11 ± 0.24	0.961 (-0.184 – 0.193)	0.287 (-0.066 – 0.218)	0.355 (-0.117 – 0.312)
Left paracentral	3.84 ± 0.30	3.82 ± 0.33	3.89 ± 0.60	0.881 (-0.409 – 0.352)	0.751 (-0.242 – 0.334)	0.761 (-0.400 – 0.540)
Left cprs opercularis	2.89 ± 0.39	2.60 ± 0.38	2.75 ± 0.57	0.300 (-0.617 – 0.194)	0.436 (-0.427 – 0.187)	0.657 (-0.387 – 0.600)
Left pars orbitalis	0.97 ± 0.18	0.99 ± 0.18	1.03 ± 0.20	0.821 (-0.147 – 0.185)	0.332 (-0.065 – 0.187)	0.395 (-0.102 – 0.249)
Left pars triangularis	3.06 ± 0.46	2.78 ± 0.27	2.90 ± 0.55	0.400 (-0.598 – 0.243)	0.520 (-0.421 – 0.216)	0.694 (-0.378 – 0.557)
Left pericalcarine	2.83 ± 0.61	2.98 ± 0.48	2.91 ± 0.60	0.524 (-0.357 – 0.692)	0.819 (-0.352 – 0.442)	0.719 (-0.620 – 0.434)
Left postcentral	6.60 ± 0.87	6.36 ± 0.73	6.87 ± 1.15	0.520 (-1.118 – 0.574)	0.561 (-0.454 – 0.826)	0.351 (-0.519 – 1.400)
Left posterior cingulate	2.98 ± 0.67	2.73 ± 0.53	3.09 ± 0.49	0.827 (-0.560 – 0.450)	0.320 (-0.191 – 0.573)	0.097 (-0.079 – 0.879)
Left precentral	12.03 ± 1.38	11.60 ± 1.29	12.71 ± 1.58	0.391 (-1.831 – 0.730)	0.184 (-0.320 – 1.618)	0.104 (-0.254 – 2.539)
Left precuneus	7.71 ± 1.08	7.59 ± 0.91	8.01 ± 1.29	0.801 (-1.138 – 0.883)	0.557 (-0.540 – 0.989)	0.544 (-0.722 – 1.330)
Left rostral anterior cingulate	2.03 ± 0.34	1.78 ± 0.53	2.04 ± 0.54	0.170 (-0.671 – 0.122)	0.906 (-0.318 – 0.282)	0.303 (-0.243 – 0.746)
Left rostral middle frontal	11.08 ± 1.76	10.03 ± 1.59	10.99 ± 1.98	0.119 (-2.880 – 0.341)	0.636 (-1.507 – 0.930)	0.240 (-0.660 – 2.493)
Left superior frontal	16.11 ± 2.32	15.27 ± 1.94	16.27 ± 2.50	0.404 (-2.969 – 1.218)	0.929 (-1.513 – 1.655)	0.335 (-1.065 – 2.988)
Left superior parietal	9.79 ± 0.94	10.49 ± 1.07	10.42 ± 1.43	0.146 (-0.266 – 1.730)	0.173 (-0.236 – 1.274)	0.699 (-1.253 – 0.856)
Left superior temporal	7.02 ± 0.88	6.77 ± 1.04	7.21 ± 1.00	0.590 (-1.083 – 0.623)	0.563 (-0.458 – 0.832)	0.292 (-0.456 – 1.444)
Left supramarginal	7.44 ± 1.34	7.06 ± 1.27	7.44 ± 1.08	0.589 (-1.384 – 0.796)	0.788 (-0.714 – 0.935)	0.522 (-0.746 – 1.426)
Left frontal pole	0.32 ± 0.06	0.31 ± 0.04	0.31 ± 0.05	0.557 (-0.063 – 0.035)	0.526 (-0.049 – 0.025)	0.870 (-0.039 – 0.046)
Left temporal pole	0.56 ± 0.11	0.65 ± 0.11	0.61 ± 0.15	0.186 (-0.036 – 0.180)	0.204 (-0.030 – 0.134)	0.645 (-0.163 – 0.103)
Left transverse temporal	0.92 ± 0.20	0.89 ± 0.11	0.82 ± 0.13	0.691 (-0.177 – 0.118)	0.146 (-0.194 – 0.030)	0.317 (-0.177 – 0.060)
Left insula	7.09 ± 0.79	6.84 ± 1.03	7.38 ± 1.09	0.633 (-1.038 – 0.638)	0.355 (-0.340 – 0.928)	0.292 (-0.487 – 1.541)
Right bank of the superior temporal sulcus	2.33 ± 0.43	2.34 ± 0.23	2.28 ± 0.32	0.994 (-0.322 – 0.325)	0.554 (-0.317 – 0.172)	0.513 (-0.315 – 0.162)
Right caudal anterior cingulate	1.87 ± 0.47	1.59 ± 0.32	1.84 ± 0.32	0.247 (-0.545 – 0.144)	0.849 (-0.285 – 0.236)	0.125 (-0.071 – 0.539)
Right caudal middle frontal	5.37 ± 0.78	4.99 ± 1.04	5.46 ± 0.84	0.299 (-1.131 – 0.356)	0.725 (-0.464 – 0.661)	0.256 (-0.358 – 1.274)
Right cuneus	2.54 ± 0.55	2.32 ± 0.36	2.41 ± 0.41	0.273 (-0.664 – 0.192)	0.310 (-0.489 – 0.159)	0.503 (-0.260 – 0.513)
Right entorhinal	0.52 ± 0.14	0.58 ± 0.18	0.51 ± 0.16	0.337 (-0.070 – 0.200)	0.871 (-0.110 – 0.094)	0.410 (-0.225 – 0.096)
Right fusiform	4.93 ± 0.80	4.90 ± 1.09	5.01 ± 0.82	0.926 (-0.808 – 0.736)	0.804 (-0.511 – 0.656)	0.578 (-0.588 – 1.027)
Right inferior parietal	9.50 ± 1.38	9.38 ± 1.15	9.86 ± 1.64	0.819 (-1.118 – 1.406)	0.329 (-0.487 – 1.423)	0.638 (-1.094 – 1.746)
Right inferior temporal	5.07 ± 0.69	4.94 ± 0.81	5.10 ± 0.84	0.703 (-0.819 – 0.557)	0.938 (-0.500 – 0.541)	0.609 (-0.546 – 0.910)
Right isthmus cingulate	2.53 ± 0.57	2.29 ± 0.44	2.61 ± 0.32	0.879 (-0.389 – 0.334)	0.246 (-0.114 – 0.433)	0.096 (-0.052 – 0.591)
Right lateral occipital	9.29 ± 1.55	8.54 ± 0.81	8.87 ± 1.09	0.410 (-1.581 – 0.657)	0.468 (-1.154 – 0.539)	0.700 (-0.754 – 1.103)
Right lateral orbitofrontal	6.00 ± 0.92	5.89 ± 1.03	6.04 ± 1.02	0.837 (-0.955 – 0.777)	0.967 (-0.669 – 0.642)	0.779 (-0.792 – 1.044)
Right lingual	5.04 ± 0.86	4.85 ± 0.78	5.12 ± 1.14	0.703 (-1.005 – 0.683)	0.996 (-0.640 – 0.637)	0.786 (-0.782 – 1.021)
Right medial orbitofrontal	3.64 ± 0.67	3.50 ± 0.48	3.50 ± 0.48	0.473 (-0.708 – 0.334)	0.368 (-0.572 – 0.216)	0.873 (-0.388 – 0.454)
Right middle temporal	5.62 ± 0.92	5.33 ± 0.64	5.51 ± 0.84	0.484 (-1.036 – 0.499)	0.848 (-0.636 – 0.525)	0.409 (-0.405 – 0.958)
Right parahippocampal	1.03 ± 0.18	1.02 ± 0.17	1.03 ± 0.21	0.800 (-0.190 – 0.148)	0.785 (-0.145 – 0.110)	0.939 (-0.163 – 0.152)
Right paracentral	4.62 ± 0.55	4.50 ± 0.35	4.88 ± 0.75	0.641 (-0.648 – 0.403)	0.360 (-0.215 – 0.580)	0.272 (-0.243 – 0.817)
Right cprs opercularis	2.62 ± 0.35	2.35 ± 0.25	2.60 ± 0.37	0.179 (-0.497 – 0.095)	0.938 (-0.215 – 0.233)	0.172 (-0.102 – 0.535
Right pars orbitalis	1.22 ± 0.21	1.17 ± 0.24	1.27 ± 0.22	0.758 (-0.226 – 0.165)	0.342 (-0.077 – 0.218)	0.232 (-0.085 – 0.331)
Right pars triangularis	3.03 ± 0.41	2.86 ± 0.48	3.07 ± 0.51	0.335 (-0.601 – 0.209)	0.964 (-0.299 – 0.313)	0.264 (-0.199 – 0.691)

White matter volumes (cm^3 volumes. mean ± sd)	NLp (n=26)	XXp (n=8)	XYp (n=20)	NLp vs XXp p-value (CI)	NLp vs XYp p-value (CI)	XXp vs XYp p-value (CI)
Right pericalcarine	2.85 ± 0.64	2.90 ± 0.68	2.89 ± 0.78	0.789 (-0.534 – 0.698)	0.986 (-0.470 – 0.462)	0.759 (-0.781 – 0.579)
Right postcentral	6.66 ± 0.86	6.69 ± 0.85	6.77 ± 0.95	0.772 (-0.910 – 0.680)	0.996 (-0.600 – 0.603)	0.737 (-0.681 – 0.947)
Right posterior cingulate	2.88 ± 0.55	2.70 ± 0.45	2.86 ± 0.47	0.908 (-0.461 – 0.411)	0.791 (-0.286 – 0.373)	0.478 (-0.285 – 0.588)
Right precentral	12.17 ± 1.21	12.06 ± 1.03	12.63 ± 2.11	0.750 (-1.610 – 1.168)	0.549 (-0.736 – 1.366)	0.629 (-1.254 – 2.028)
Right precuneus	7.88 ± 0.91	7.75 ± 1.20	8.00 ± 1.29	0.952 (-1.008 – 0.949)	0.707 (-0.601 – 0.879)	0.789 (-1.034 – 1.345)
Right rostral anterior cingulate	1.39 ± 0.30	1.32 ± 0.26	1.48 ± 0.40	0.793 (-0.325 – 0.250)	0.536 (-0.150 – 0.285)	0.488 (-0.226 – 0.459)
Right rostral middle frontal	11.27 ± 2.02	10.40 ± 2.10	11.42 ± 1.52	0.192 (-2.765 – 0.570)	0.962 (-1.232 – 1.291)	0.130 (-0.363 – 2.642)
Right superior frontal	15.80 ± 2.28	14.73 ± 2.06	16.08 ± 2.81	0.431 (-2.999 – 1.302)	0.695 (-1.308 – 1.945)	0.294 (-1.073 – 3.379)
Right superior parietal	9.72 ± 1.14	9.70 ± 1.30	9.93 ± 1.36	0.926 (-1.047 – 1.148)	0.708 (-0.675 – 0.986)	0.806 (-1.048 – 1.332)
Right superior temporal	5.98 ± 0.77	5.88 ± 0.80	5.93 ± 1.12	0.585 (-1.038 – 0.592)	0.674 (-0.746 – 0.487)	0.858 (-0.883 – 1.052)
Right supramarginal	7.08 ± 1.03	7.23 ± 0.77	7.40 ± 1.18	0.709 (-0.764 – 1.114)	0.438 (-0.434 – 0.986)	0.841 (-0.897 – 1.091)
Right frontal pole	0.39 ± 0.06	0.39 ± 0.05	0.40 ± 0.06	0.981 (-0.050 – 0.049)	0.849 (-0.0340 – 0.041)	0.969 (-0.051 – 0.050)
Right temporal pole	0.56 ± 0.11	0.58 ± 0.19	0.56 ± 0.12	0.791 (-0.122 – 0.093)	0.753 (-0.094 – 0.068)	0.788 (-0.114 – 0.148)
Right transverse temporal	0.57 ± 0.13	0.62 ± 0.08	0.67 ± 0.23	0.609 (-0.110 – 0.186)	0.104 (-0.020 – 0.204)	0.598 (-0.133 – 0.225)
Right insula	6.85 ± 0.84	6.52 ± 1.08	7.19 ± 0.98	0.632 (-1.030 – 0.632)	0.196 (-0.219 – 1.038)	0.173 (-0.311 – 1.624)

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