Aus dem Institut für Neuroradiologie der
der Medizinischen Fakultät – Charité Universitätsmedizin Berlin

DISSERTATION

Sex related differences in clinical disability, MRI lesion load and atrophy of subcortical deep grey matter in patients with multiple sclerosis

Geschlechterspezifische Unterschiede in klinischer Beeinträchtigung, MRT-Läsionslast und Atrophie von subkortikaler grauer Hirnsubstanz bei Patienten mit Multipler Sklerose

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Abbreviations

ARR	Annual relapse rate
CIS	Clinically isolated syndrome
CNS	Central nervous system
CSF	Cerebrospinal fluid
DMT	Disease-modifying treatment
DNA	Deoxyribonucleic acid
DOF	Degrees of Freedom
EAE	Experimental autoimmune encephalomyelitis
EBV	Epstein-Barr virus
EDSS	Expanded disability status scale
F:M	Female:Male
FAST	FMRIB's Automated Segmentation Tool
FDC	Follicular D cell
FIRST	FMRIB’s Integrated Registration & Segmentation Tool
FLIRT	FMRIB's Linear Image Registration Tool
FOXP3	Forkhead box P3
FSL	FMRIB Software Library
Gd	Gadolinium
GM	Gray matter
HERV	Human Endogenous Retroviruses
HERV-W	Type W Human Endogenous Retrovirus
HHV-6	Human herpes virus 6
HLA	Human leukocyte antigen
HSV-1	Herpes simplex virus 1

IFN	Interferon
IL	Interleukin
LC	Lesion count
LV	Lesion volume
RRMS	Relapse-remitting MS
mRNA	Messenger ribonucleic acid
MOG	Myelin oligodendrocyte glycoprotein
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
PBVC	Percentage brain volume change
PCR	Polymerase chain reaction
ROI	Region of interest
RRMS	Relapse-remitting MS
SDGM	Subcortical deep grey matter
SIENA	Structural Image Evaluation, using Normalization, of Atrophy
SIENAX	Structural image evaluation with normalization of atrophy cross-sectional
SNPs	Single nucleotide polymorphisms
SPMS	Secondary progressive multiple sclerosis
T2	T2-weighted imaging
TH	T-helper
TReg	Regulatory T-cell
VCA	Viral capside antigens

Abstract (EN)

Background: Sex differences in patients with multiple sclerosis have been widely described. However, with respect to clinical disability, lesion burden, and atrophy of gray matter, these differences were inconsistently reported.

Objective: Quantitative comparison of expanded disability status scale (EDSS) scores, T2 lesion burden, and subcortical gray matter volume between male and female MS patients.

Methods: Magnetic resonance images **(**MRI) and clinical data from 55 relapse-remitting multiple sclerosis (RRMS) patients (female n= 39, male n=16) were analyzed with respect to EDSS score, lesion burden and atrophy of subcortical gray matter. We performed semi-automatic lesion segmentation, brain volume estimation and subcortical gray volumetric measurements. Subsequently, data were compared cross-sectionally and longitudinally for a mean of 18.5 months between sexes. Multivariate linear regression models were used, and subgroup analysis of subcortical gray matter volume at different time points of the disease were performed, to identify predictors of atrophy.

Results: Male patients accumulate more clinical disability at early stages of multiple sclerosis (MS) than female patients ($p = 0.035$ at 3 years after disease onset). During this time span, a higher T2 lesion volume was also observed in male MS patients compared to female MS patients (10 ml and 3.1 ml respectively; $p = 0.03$). However, these sex differences disappear later in the disease course.

Sex was no predictor for atrophy of subcortical gray matter in the multivariate linear regression model. Also, no sex differences were found in the cross-sectional analysis of subcortical gray matter at any time point of comparison after post-hoc tests. In the longitudinal analysis men showed a thalamic volume loss of 0.41 ml (2%), and women of 0.18 ml (0.8%; p-value = 0.014). We found no sex differences concerning the atrophy of other SDGM structures during the observation time.

Conclusion: We conclude that male patients are more affected by MS than female patients regarding clinical disability and T2 lesion volume during the first years after MS onset. Male patients also develop a more pronounced atrophy of the thalamus in comparison to females during a mean observation time of 18.5 months.

Abstract (DE)

Hintergrund

Geschlechtsspezifische Unterschiede bei Patienten mit MS sind bereits untersucht worden. Dennoch ist der Einfluss des Geschlechtes auf klinische Beeinträchtigung, MRT-Läsionslast und Atrophie der grauen Hirnsubstanz noch nicht hinreichend untersucht.

Ziel

Durchführen eines quantitativen Vergleichs von EDSS-Werten, T2-Läsionslast und Volumen von subkortikaler grauer Substanz zwischen weiblichen und männlichen MS-Patienten.

Methoden

MRT- und klinische Daten von 55 Patienten (Frauen n = 39, Männer n = 16) wurden hinsichtlich EDSS, Läsionslast und Atrophie von grauer Hirnsubstanz untersucht. An MRT-Daten wurde eine halbautomatische Läsionssegmentierung und eine volumetrische Berechnung von Hirnvolumen und subkortikalen Strukturen durchgeführt. Die Ergebnisse wurden mittels Querschnitt- und Längsschnittanalyse auf Unterschiede zwischen den Geschlechtergruppen innerhalb eines Beobachtungszeitraumes von durchschnittlich 18,7 Monaten untersucht. Zur Identifizierung von prädiktiven Faktoren für die subkortikale Atrophie wurde eine multivariate lineare Regressionsanalyse durchgeführt.

Ergebnisse

Männliche MS-Patienten erkranken in den Frühstadien klinisch stärker als weibliche Patienten ($p<0,001$ nach einer Erkrankungsdauer von drei Jahren). In dieser Zeitspanne wiesen männliche Patienten eine mittlere höhere T2-Läsionslast als Frauen auf (10 ml bzw. 3,1 ml; $p=0,03$). Im späteren Krankheitsverlauf fanden sich diesbezüglich jedoch keine Unterschiede mehr zwischen den Geschlechtern. In der multivariaten Regressionsanalyse hatte das Geschlecht keinen Vorhersagewert für die subkortikale Atrophie grauer Substanz. Ebenso gab es in der Querschnittsanalyse keine vom Geschlecht abhängigen Volumenveränderungen von subkortikaler grauer Substanz zu den jeweiligen Vergleichszeitpunkten nach post-hoc-Tests. In der longitudinalen Analyse wiesen Männer einen Verlust von 0,39 ml (2%) und Frauen von 0,08 ml (0,4%) des Thalamusvolumens auf ($p=0,014$). Bezüglich der Atrophie anderer SDGM-Strukturen fanden sich im Beobachtungszeitraum keine geschlechter-spezifischen Unterschiede.

Schlussfolgerung

Innerhalb der ersten Erkrankungsjahre einer MS sind Männer bezüglich klinischer Beeinträchtigung und T2-Läsionsvolumen stärker betroffen als weibliche Patienten. Männliche Patienten entwickeln innerhalb eines Beobachtungszeitraums von durchschnittlich 18,7 Monaten zudem eine stärker ausgeprägte Thalamusatrophie als erkrankte Frauen.

1.- INTRODUCTION

1.1. Chapter 1: MS overview

1.1.1. Epidemiology

MS is an autoimmune disease of the central nervous system (CNS; Gharagozloo et al., 2018) characterized by inflammation, blood-brain barrier breakdown, demyelination, axonal/neuronal damage, and metabolic changes (Guan et al., 2019; Martin et al., 2016). It is the most common inflammatory neurological disease in young adults between 20 and 40 years of age, with a mean age of diagnosis of approximately 30 years (Martin et al., 2016; Wallin et al., 2019). In 2016, over 2.2 million people worldwide had multiple sclerosis, which corresponds to a prevalence of 30.1 cases per 100,000 persons.

A north to south decrease in prevalence by latitude gradient was recognized in North America and western Europe (Noonan et al., 2010; Simpson et al., 2011), and a reverse south to north increase in gradient was reported in Australia (Simpson et al., 2011). Thus, the prevalence increases by 1.03 times per degree of latitude. This distribution of multiple sclerosis can be generally described as three zones of frequency or risk: high prevalence rates in north-west Europe, Canada, and northern USA; medium frequency in southern Europe, southern USA and Australia; and low frequency (<5 per 100,000 persons) for the rest of the surveyed world (Kurtzke, 2013). The global prevalence of multiple sclerosis differs substantially by sex (Gold et al., 2019). Among preteen children, the prevalence of multiple sclerosis is similar in boys and girls. During adolescence the curves start to diverge, with prevalence increasing more strongly among girls than boys. This pattern continues until around the end of the sixth decade of life, when the sex ratio is 2:1 in favor of women. In older people, prevalence generally continues to climb for women, but an attenuation in prevalence is seen in men. In Germany the female to male ratio of MS incidence is 1.9 (Schmedt et al., 2017).

1.1.2. Etiology

MS seems unlikely to result from a single causative event. Instead, the disease seems to develop in genetically susceptible populations as a result of environmental exposures (Marrie, 2004; Ramagopalan et al., 2010). Sex differences regarding the etiology of MS have been widely described and will be explained in more detail in Chapter 2.

1.1.2.1 Genetic risk factors

Family members of affected individuals have a 10-25 times greater risk of disease than the general population (Ebers et al., 1995; Ramagopalan et al., 2010; Sadovnick et al., 1996). Half-siblings of affected persons have roughly half the risk of full siblings of developing MS, and adopted siblings have no greater risk than the general population (Sadovnick et al., 1996). This indicates that genetic factors contribute to an individual's risk of MS. Although monozygotic twins have a greater concordance (~30%) than dizygotic twins (~5%), the concordance is less than 100%, indicating that genetics alone cannot fully explain the development of the disease (Sadovnick et al., 1996).

Human leukocyte antigen (HLA) types exert the strongest genetic effect in MS, but an association with a single HLA-complex has not been described. The correlation with HLA-DR2 (HLA-DRB1*15) is well known in northern Europe, i.e. heterozygosity conferring an odds ratio (OR) of 2.7 and homozygosity of 6.7 (Barcellos et al., 2003; Jersild et al., 1973). In other regions, the association is predominantly seen with HLA-DRB1*0301, HLA-DRB1*0405, and HLA-DRB1*1303 (Marrosu, 2001).

1.1.2.2. Environmental risk factors

Although over 100 genes have been implicated in MS (Sawcer et al., 2014), there is strong evidence that environmental factors play a major role in determining MS risk (Ascherio and Munger, 2016; Ramagopalan et al., 2010). Factors with the strongest evidence for involvement in MS are Epstein-Barr virus (EBV), smoking, and probably latitude-dependent vitamin D serum levels. Reports on other factors such as geographical region, and data from migration studies, suggest that the timing of exposure is a crucial determinant of risk for MS, particularly at younger ages. People migrating from an area of high MS prevalence to an area with less prevalence have a lower disease risk, whereas people who migrate from areas of low risk to areas of high risk tend to preserve the lower risk. This susceptibility towards disease development is reported to be established in the first two decades of life (Marrie, 2004).

1.1.2.2.1. Infections

1.1.2.2.1.1. Epstein-Barr Virus

Nearly all individuals with MS (>99%) have been found to be infected with Epstein-Barr Virus (EBV), compared to approximately 94% of age-matched controls (Ascherio and Munger, 2007). The relationship seems to be temporal: plasma antibody titers against the EBV nuclear antigen 1 (EBNA1) increase several years before the onset of neurological symptoms of MS (Ascherio, 2008). In addition, individuals with a history of infectious

mononucleosis have an increased risk of developing MS (Guan et al., 2019; Levin, 2005; Sundstrom et al., 2004).

The mechanism underlying this association remains unexplained, but recent findings denote that it may be mediated by enhanced blood-brain barrier permeability triggered by the acute primary EBV infection (Engelhardt and Ransohoff, 2012). The presence of EBV in B cells in active and chronic MS brain lesions was reported recently (Moreno et al., 2018). Even though they found viral proteins and viral RNA transcripts in patients with MS, as well as in control brains, they demonstrated that the EBV cycle is modified by the course of MS, as they did not find viral immediate-early proteins in chronic active MS plaques.

1.1.2.2.1.2. Human Herpes Virus 6

Initial evidence supporting a possible pathogenic role for human herpes virus 6 (HHV-6) in MS was based on cerebrospinal fluid (CSF) detection of viral deoxyribonucleic acid (DNA) using polymerase chain reaction (PCR; Wilborn et al., 1994). Subsequent studies showed similar results in control subjects (Challoner et al., 1995). However, immunocytochemical staining did detect differences in viral antigen distribution. Detection of viral messenger ribonucleic acid (mRNA) and protein expression in oligodendrocytes further contributed to the hypothesis of HHV-6 as a driver of MS (Leibovitch and Jacobson, 2014).

1.1.2.2.1.3. Human Endogenous Retroviruses

Human endogenous retroviruses are a part of human DNA representing approximately 8% of the human genome. Under physiological conditions these elements are frequently inactive or non-functional due to deactivating mutations and epigenetic control. They may undergo reactivation under certain pathological conditions and produce viral transcripts and proteins (Marrodan et al., 2019). *In vitro* and *in vivo* studies showed that common viruses such as herpes simplex virus type 1 (HSV-1), HHV- 6, influenza or EBV can activate human endogenous retrovirus-W (HERV-W) sequence amplification in cells involved in MS pathogenesis, including B cells, macrophages, microglia, and astrocytes (Brütting et al., 2016). Isolation of HERV was observed in leptomeningeal cells shedding into CSF, and in monocytes from a patient with progressive MS, supporting a link between HERVs and MS (Perron et al., 1991).

1.1.2.2.1.4. Bacterial infections

Helicobacter pylori: Lower prevalence of *H. pylori* infection was found in various MS patient cohorts when compared to controls (Park et al., 2017). Furthermore, mice infected with *H. pylori* and sensitized with myelin oligodendrocyte glycoprotein (MOG) for experimental autoimmune encephalomyelitis (EAE) induction showed fewer clinical signs of disease, decreased levels of MOG-specific lymphoproliferation, as well as reduced numbers of type 1 T-helper cells (Th1) and type 17 T-helper cells (Th17) in the CNS and spleen when compared to controls (Cook et al., 2015). Based on these findings, a protective role was proposed for *H. pylori* in MS (Marrodan et al., 2019).

1.1.2.2.2. Smoking

Many studies showed that smoking increased the risk of MS (Hedström et al., 2016; Hernan, 2001; O'Gorman et al., 2014). At present, tobacco smoking is one of the best-confirmed environmental factors contributing to MS, influencing MS development mainly through autoimmune progression and CNS damage (Wang et al., 2019).

1.1.2.2.3. Vitamin D and light exposure

The duration and intensity of sunlight were strongly correlated with MS prevalence in early ecological studies (Acheson et al., 1960; Leibowitz et al., 1967). Therefore, the higher incidence of MS at higher latitudes may be directly related to vitamin D deficiency (Agranoff and Goldberg, 1974). The first nested case-control study to examine pre-onset 25(OH)-D_3 vitamin levels and MS risk was conducted on active duty U.S. military personnel (Munger et al., 2006). Analyses were conducted separately on non-Hispanic whites, non-Hispanic blacks and Hispanics, because higher skin pigmentation lowers the amount of vitamin D produced by sun exposure. Among non-Hispanic whites, MS risk declined with increasing levels of 25(OH)-D_3: the risk was 62% lower among individuals in the highest quintile [25(OH)-D_3 > 99.2 nmol/L] as compared with those in the lowest quintile [25(OH)-D_3 < 63.2 nmol/L] (Koduah et al., 2017).

1.1.2.2.4. Other environmental factors

Other potentially modifiable MS risk factors have been proposed, but the evidence so far has been insufficient to draw final conclusions. These include: salt intake (Farez et al., 2015), levels of stress (Warren et al., 1982), childhood obesity (Chitnis et al., 2016; Gianfrancesco et al., 2014; Langer-Gould et al., 2013), and occupational exposures and toxins (Casetta et al., 1994; Stenager et al., 2003; Zorzon et al., 2003).

1.1.3. Pathophysiology

Without a known predominant exogenous risk factor, it is an open question whether multiple sclerosis is triggered in the periphery or in the CNS (Dendrou et al., 2015). In the CNS-extrinsic (peripheral) model, autoreactive T cells that are activated at peripheral sites traffic to the CNS, along with activated B cells and monocytes. This activation occurs potentially through molecular mimicry (Harkiolaki et al., 2009; Münz et al., 2009; Olson et al., 2001), bystander activation or the co-expression of T-cell receptors (TCRs) with different specificities (Ji et al., 2010). Alternatively, CNS-intrinsic events may trigger disease development, with the infiltration of autoreactive lymphocytes occurring as a secondary phenomenon (Dendrou et al., 2015). To date, it is unclear what these specific-CNS-intrinsic events are. Postulated mechanisms include inflammatory responses to an as yet unknown CNS viral infection or to processes leading to primary neurodegeneration, similar to those that have been implicated in Alzheimer's disease or Parkinson's disease (Heneka, 2014).

1.1.3.1. T cells

The presence of T cells within CNS lesions is detectable in the early stages of multiple sclerosis (Popescu and Lucchinetti, 2012). The long-appreciated HLA associations with the disease are thought to reflect the presentation of specific CNS autoantigens to autoreactive T cells. As demyelination is a key feature of multiple sclerosis neuropathology, myelin protein-derived antigens have been hypothesized to be the main autoreactive targets (Dendrou et al., 2015). TH1 cells and TH17 cells are the main CD4+ T cell subsets implicated in disease. Thus, skewing of T cell differentiation away from these subsets and towards a type 2 T helper (TH2) cell phenotype has been a prominent therapeutic concept.

1.1.3.2. B cells

CD8+ T cells are found in higher frequency than CD4+ T cells in the white and in gray matter demyelinating lesions. Their numbers closely correlate with axonal damage (Frischer et al., 2009). Clonally expanded B cells can be found in the meninges, parenchyma and CSF. Intrathecal B cells produce antibodies that are detectable in the CSF and are of diagnostic value. The meninges of patients with secondary progressive disease often contain tertiary lymphoid structures of aggregated plasma cells, B cells, T cells and follicular dendritic cells (FDCs) (Howell et al., 2011), which are a product of long-

term inflammation, as observed in other chronic inflammatory or infectious diseases (Drayton et al., 2006).

1.1.3.3. Defective regulatory cells

The emergence and action of autoreactive B cells and T cells in multiple sclerosis may be due to the defective functions of regulatory cells, such as forkhead Box B3 (FOXP3)-expressing CD4+ capable of infiltrating and promoting damage within the CNS (Dendrou et al., 2015), regulatory T cells (T-Regs) and interleukin 10 (IL-10) producing T regulatory type 1 (TR1) cells (Martinez-Forero et al., 2008). Similarly, disease-associated HLA class II variants could incorrectly influence thymic selection of T-Regs, leading to inadequately suppression of autoreactive effector T cells (Venken et al., 2008).

1.1.3.4. Demyelination and neurodegeneration

Four different patterns of pathology resulting in demyelination were identified in MS lesions (Hernández-Pedro et al., 2013; Sriram, 2011): i) Type I is T cell mediated where demyelination is induced by macrophages either directly or by macrophage toxins; ii) Type II involves both T cells and antibodies, and is the most common pathology observed in MS lesions. In this case, demyelination is caused by specific antibodies and complement; iii) Type III is related to distal oligodendrocytopathy, where degenerative changes occur in distal processes that are followed by apoptosis; iv) Type IV results in primary oligodendrocyte damage followed by secondary demyelination (Sriram et al., 1998).

1.1.4. Disease course and symptomatology

Early multiple sclerosis is usually characterized by acute episodes of neurological deficits known as relapses, that depend on the location of the CNS region affected (Alan J Thompson et al., 2018). If the affected brain area has a motor or sensory function, clear symptoms can be identified when acute inflammation occurs (***Table 1***). However, other brain areas responsible for hormone release, behavior and executive functions may also be affected, leading to more unspecific or subjective symptoms such as fatigue, cognitive dysfunction, sleep disorders, depression or pain that strongly impact the quality of life of MS patients (Hasselmann et al., 2016; Paul, 2016; Penner and Paul, 2017; Veauthier and Paul, 2014; von Bismarck et al., 2018).

In the majority of patients with MS (85%), the disease starts with the RRMS phenotype (Krieger et al., 2016; Miller et al., 2012). They develop relapses (defined as a subacute

onset of new neurologic symptoms that last for at least 24 hours in the absence of fever or infection) followed by symptom recovery. Thus, this clinical recovery does not imply periodical absence of the disease. As Krieger et al., (2016) described, patients with the relapsing-remitting form of MS demonstrate evidence of disease progression below a subclinical threshold.

Localization of affected region	Symptoms
Optic nerve	Monocular painful vision loss
Spinal cord	Hemiparesis, mono/paraparesis, hypoesthesia, dysesthesia, paresthesia, urinary and/or fecal sphincter dysfunction
Brainstem and cerebellum	Diplopia, oscillopsy, vertigo, ataxia, dysmetria, facial paresis, dysarthria/dysphagia, intentional/postural tremor, and/or hypoesthesia
Cerebral hemisphere	Facio–brachial–crural hemiparesis, facio–brachial–crural hemihypoesthesia
Other clinical manifestations	Painful spasms/spasticity, neuropathic pain, sexual dysfunction, fatigue, cognitive impairment, depression.

Table 1 | Neurologic symptoms of multiple sclerosis (Adapted from Thompson et al., 2018).

Clinically isolated syndrome (CIS) is a term that refers to the first clinical manifestation of the disease that by definition is isolated in time or not preceded by any neurologic event. It usually affects the optic nerves (20%), the brainstem (10%–20%), or the spinal cord (40%), causing an optic neuritis, a brainstem syndrome, or an incomplete transverse myelitis, respectively (Miller, 2012, 2012; Miller et al., 2005).

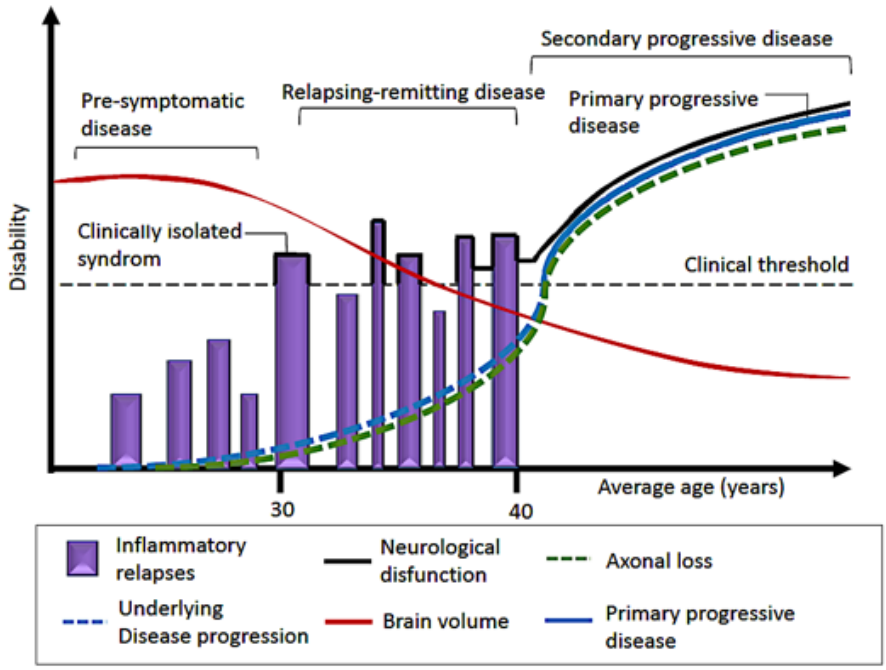

Figure 1 | Disease courses of multiple sclerosis (Adapted from Dendrou et al., 2015).

The accumulation of disability can be quantified with the Expanded Disability Status Scale (EDSS; Kurtzke et al., 1977). The EDSS is an ordinal scale ranging from 0 (normal neurologic examination) to 10 (death owing to MS). EDSS mostly relies on motor function, and important milestones include requiring unilateral assistance for walking 100 m (EDSS score of 6.0), requiring bilateral assistance for walking 20 m (EDSS score of 6.5), or requiring a wheelchair for most parts of the day (EDSS score of 8.0), (Vidal-Jordana and Montalban, 2017) .

Eventually, improvement during each remission tends to wane as disability accumulates, and approximately 80% of patients go on to develop secondary progressive multiple sclerosis (SPMS), one to two decades post diagnosis (Vidal-Jordana and Montalban, 2017). In secondary progressive disease, inflammatory lesions are no longer characteristic, and progressive neurological decline is instead accompanied by CNS atrophy (Dendrou et al., 2015; ***Figure* 1**: Blue solid line and green dashed lines). Risk factors associated with the development of neurologic disability, and risk of conversion to progressive types, include male sex (See Chapter 2), an older age at CIS onset (Scalfari et al., 2014; Tintore et al., 2015), a higher annual relapse rate (ARR; Degenhardt et al., 2009; Scalfari et al., 2014), a short time to the second relapse (Degenhardt et al., 2009), intrathecal IgM production (Pfuhl et al., 2019), presence of oligoclonal bands in the cerebrospinal fluid, and a greater number of white matter lesions in the baseline brain magnetic resonance image (Tintore et al., 2015).

1.1.5. Diagnosis

To date, a specific test for the diagnosis of MS does not exist. Therefore, the diagnosis is established by the fulfilment of diagnostic criteria. The diagnostic criteria are based on demonstrating the involvement of 2 or more areas of the CNS (dissemination in space) at different timepoints (dissemination in time; Vidal-Jordana and Montalban, 2017). The most recent criteria incorporate magnetic resonance imaging to establish the presence of dissemination in space and in time (McDonald Criteria; see ***Table 2***), which allows for an earlier diagnosis. After the occurrence of a CIS, the diagnosis of MS can be established with a single magnetic resonance image demonstrating dissemination in space and time and excluding other neurological disorders that can clinically and radiologically mimic MS. This means that for the diagnosis of MS the concept of “no better explanation” after a typical CIS plays a major role. (Charil et al., 2006; Filippi et al., 2019; Geraldes et al., 2018; Montalban et al., 2010; Polman et al., 2011).

	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 space 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.

Table 2 | The 2017 McDonald criteria for the diagnosis of MS (Adapted from Thompson et al., 2018).

1.1.6. Treatment

Over the last few years, an increasing number of disease-modifying treatments have been developed and brought to market for treating relapsing MS. All of these therapies were demonstrated to be effective in reducing clinical and radiologic disease activity, and also in modifying the natural history of MS (Dörr and Paul, 2015; Vidal-Jordana and Montalban, 2017). Treatments mainly target neuroinflammation, and could have an indirect effect on neurodegeneration. However, their efficacy for reducing the development of brain atrophy in clinical trials was moderate at best (Alan J. Thompson et al., 2018).

Two therapeutic approaches are available in the clinical setting, namely escalation strategy and induction strategy: a) Escalation strategy consists of starting with a first-line treatment (a moderately effective medication) and escalating to a more effective (but potentially less safe and more expensive) medication in case of continuous relapses (Alan J. Thompson et al., 2018). This strategy may not be effective for patients with highly active or rapidly evolving disease; b) Induction strategy involves starting with a highly effective therapy with the aim of either obtaining a persistent disease remission, or to continue with a long-term maintenance therapy with a less effective disease-modifying treatment (Wiendl et al., 2017).

1.2. Chapter 2: Sex differences in MS

1.2.1. Environmental risk factors

In individuals with MS, a number of genetic, environmental and lifestyle factors have potentially sexually dimorphic effects on MS disease susceptibility and progression. These include environmental, behavioral, metabolic (such as obesity), genetic and epigenetic risk factors (Bove and Chitnis, 2014; ***Table 3***). Emerging factors of potentially considerable importance include sex differences in the regulation of the gut microbiome (Markle et al., 2013) and sex-specific signaling pathways that control central nervous system autoimmunity or repair (Krementsov and Teuscher, 2013).

Behavioral risk factors	
Westernizing gender norms	1. In areas where the F:M ratio in MS is increasing, girls have experienced rapid increases in the time spent indoors as a result of rapid urbanization, education and participation in the workforce. 2. There has been a dramatic shift in women's reproductive choices and trajectories in the past century.
Smoking	1. Smoking may increase the risk of MS in women only. 2. Potential mechanisms may include an interaction between sex/gender and smoking, yielding increased levels of mature peripheral functioning T cells in female smokers. 3. The increasing F:M ratio in MS parallels that in smoking; but in smoking, a higher F:M ratio may be driven both by a decrease in male's rates, as well as by an increase in female's smoking rates.
Sunlight	Sunlight deprivation has worse consequences in females than in males.
Dietary risk factor	
Vitamin D	A functional synergy between 1,25(OH)D_3 and 17-β estradiol is observed, mediated through estrogen receptor α, mainly in females and secondarily in males. As a consequence, vitamin D_3 may play a more important immunomodulatory role in females with MS than in males.
Diet and metabolism	1. Overweight/obesity at 18 – 20 years of age may double MS risk. 2. Only in female adults, obesity at MS onset may be associated with a 2-fold increase OR of a relapsing course at onset. 3. Estrone produced by adipocytes may represent an important source of inflammatory signaling in both females and males. 4. The potential interaction between obesity and vitamin D status in mediating MS is unexplored in females.
Infectious risk	
EBV exposure	Female sex and HLA DR2 status may correlate with anti-EBV VCA IgG levels.
Genetic risk	
Genetics / Epigenetics	1. By controlling for sex, genome-wide association studies risk overlooking an effect (if present) of SNPs on MS risk. 2. Mothers may be more likely to transmit the risk of MS, and of the HLA-DRB1*1501 risk allele, even when the mother is not affected. 3. The HLA-DRB5*0101 – HLA-DRB1*1501 – HLA-DQA1*0102 – HLA-DQB1*0602, extended haplotype is more common in females than in male patients, has a higher F:M ratio in MS subjects than in controls and in families with two generations of MS, the females in the latest generation have an increased frequency of HLA-DRB1*15.
Other risk factors	
Uric acid	Urate, an antioxidant is significantly lower in females than males in all types of MS.

Table 3 | MS risk factors might be differentially regulated in males and females (Adapted from Bove and Chitnis, 2014).

1.2.2. Clinical course

1.2.2.1. Disease susceptibility

The sex ratio in MS appears to be rising, from the 1:1 F:M ratio reported by the National Multiple Sclerosis Society (NMSS) in the 1940s to a ratio approaching or even exceeding 3:1 today in northern countries (Bove and Chitnis, 2013; Gold et al., 2019; Trojano et al., 2012). A possible explanation for this might be the intensification of the effect in these latitudes of sex-related risk factors such as decreased solar ultraviolet radiation exposure and vitamin D_3 levels, together with a genetic susceptibility and/or hormonal dysregulation (Trojano et al., 2012).

1.2.2.2. Age

There is a 1 or 2 year difference in the average age of disease onset between male and female patients, but there is evidence that in young patients (disease onset before the age of 20 years) the female to male ratio is greater than in the general MS population (3.2:1 versus 2:1; Duquette et al., 1992).

1.2.2.3. Disease course type

The female preponderance for RRMS has been established for many years (Runmarker and Andersen, 1993), whereas men are more likely to have progressive onset of MS compared to women (Compston, 2006). Female sex is associated with an increased risk of developing clinical definite MS after a first demyelinating event, including after optic neuritis (Optic Neuritis Study Group, 2008). Additionally, sex-specific reproductive exposure to an altered hormonal state after a clinically isolated syndrome, such as a pregnancy, may increase the risk of clinical definite MS (Lebrun et al., 2012). The fact that females have a 2.1 relative risk for developing a clinically isolated syndrome compared to males, but only a 1.2 relative risk for developing MS after CIS than males, indicates that sexual dimorphism has a strong influence on factors acting early on in disease pathogenesis (Dobson et al., 2012).

1.2.2.4. Disease activity and progression

Early predictors of future disability in the major subtypes of MS (relapsing–remitting MS and secondary progressive MS) include sex, age of disease onset, and degree of recovery from the first episode (Confavreux et al., 2003; Runmarker and Andersen, 1993). While females are at a higher risk for MS, males are more likely to display a more progressive disease onset, poor recovery after initial attacks, more rapid accrual of disability, more rapid EDSS progression, and an overall more malignant course, even

after controlling for sex differences in the age at onset and other confounders (Bove and Chitnis, 2014). A natural history study of untreated patients with MS found that male sex was associated with a shorter time, and a younger age, for conversion to SPMS (Koch et al., 2010). Another study showed that males had a more severe disease phenotype with faster accumulation of disability (Tomassini and Pozzilli, 2009), with yet another showing that male sex and older age at onset were predictive of more rapid progression from disease onset of RRMS (Shirani et al., 2012).

Conversely, females seem more likely to manifest RRMS with very mild attacks, separated by long periods with no symptoms, which has been described as “benign MS” (Bove and Chitnis, 2013; Reynders et al., 2017). Some, but not all, studies found that females had a higher relapse rate (Compston, 2006; Confavreux and Vukusic, 2006) and that the location of relapses (e.g. sensory versus motor) differed by sex, implying that there are sex differences in oligodendrocyte or neuronal vulnerability or repair. However, the possibility that males are less likely to report new symptoms, particularly if they are sensory, was not adequately addressed (Tremlett et al., 2008).

Impaired cognition is a specific disability that appears to be worse in men. An early study showed that male patients with MS performed worse on several cognitive subtests compared with female patients with MS, who were matched for age, education, and other neurologic and emotional measures (Beatty and Aupperle, 2002).

1.2.2.5 Heritability

Support for potential sex biases in the transmissibility of MS derives from observations that: 1) Women were more likely to carry the HLA DRB1 risk allele than men; 2) The HLA DRB1*15 risk allele was more often transmitted by unaffected mothers than by unaffected fathers; and 3) Transmission of HLA DRB1*15 was more likely to show transmission disequilibrium among female-female relatives in collateral (same generation affected) rather than throughout multi-generational families (Sadovnick, 2013).

1.2.2.6. Responsiveness to disease-modifying treatments

In general, sex differences in the effectiveness of first-line disease-modifying treatments (DMTs), including interferon (IFN) and glatiramer acetate, were not noted (Rudick and Goelz, 2011; Wolinsky et al., 2009). In SPMS, there were some hints that women may preferentially benefit from IFNβ-1a (Secondary Progressive Efficacy Clinical Trial of Recombinant Interferon-Beta-1a in MS (SPECTRIMS) Study Group, 2001). The subgroup analyses of the pivotal natalizumab trials found that natalizumab therapy

decreased the relapse rate in both men and women, but only delayed disability progression in women (Hutchinson et al., 2009).

1.2.3. Hormonal influence

The higher risk of women to develop MS may theoretically be a result of either the negative effect of female sex hormones, or the protective effect of male sex hormones (Voskuhl and Gold, 2012). The immunologic and neuroprotective effects of estrogens and androgens were extensively described and reviewed by Voskuhl and Gold, (2012). They also reported that in EAE, estrogen appears to have a biphasic dose effect on inflammation. At lower levels, estrogens such as estradiol may promote inflammation, but at higher levels, estrogens such as the pregnancy hormone estriol may induce a shift in the immune response from a TH1 response to a TH2 response, thereby muting inflammation. Previous studies demonstrated that the activation of the CD4+ lymphocytes differs between males and females (Pennell et al., 2012). Females show a preponderance toward Th2 immune responses and B cell activation, whereas males predominantly generate Th1 CD4+ and CD8+ lymphocytes (Ghazeeri et al., 2011). Low doses of estrogens accompanying menstruation and during the luteal phase invoke Th1-mediated immunity, whereas higher doses during the follicular phase invoke Th2-mediated immunity. Estrogen is an effective treatment for EAE, as it regulates Th1, Th2 differentiation and Th17 lineage polarization implicated in autoimmunity. One regulatory mechanism of estradiol treatment in EAE is associated with an increased expression of programmed death-1 in T-regs, which promotes suppressive activity (Wang et al., 2009). Conversely, Voskuhl and Gold, (2012) demonstrated that testosterone may have anti-inflammatory effects, as is evidenced by the more inflammatory milieu and increased disease activity in states of androgen deficiency following castration. In support thereof, *ex vivo* exposure of encephalitogenic T cells to testosterone was shown to significantly change the secreted cytokine profile from IFNγ to IL-10, and the pathogenic potential of these T cells (Gubbels Bupp and Jorgensen 2018). Furthermore, myelin-basic protein-primed female T cells and T cells from gonadectomized males expressed significantly higher levels of the VLA-4 integrin β1 subunit, and secreted higher levels of pro-inflammatory cytokines, such as IL-1β, than male-derived cells, thereby promoting T cell infiltration into the brain and brain pathogenesis (Brahmachari and Pahan, 2010).

1.3. Chapter 3: MS and MRI

1.3.1. MRI in the diagnosis of MS

Demonstration of dissemination in space and time is pivotal for the diagnosis of MS. MRI complements clinical and laboratory evaluation, allowing an early diagnosis whilst helping to rule out other conditions (Filippi et al., 2019; Geraldes et al., 2018; Alan J. Thompson et al., 2018). According to the McDonald criteria from 2017, dissemination in space can be demonstrated by the presence of at least one T2 lesion in two or more typical regions (periventricular, cortical/juxtacortical, infratentorial, and spinal cord), with no distinction between symptomatic and asymptomatic MRI lesions. Dissemination in time can be demonstrated by**:** a) a simultaneous presence of gadolinium-enhancing and non-enhancing lesions at any time; b) a new T2-hyperintense and/or gadolinium-enhancing lesion on follow-up MRI with reference to a baseline scan, irrespective of the timing of the baseline MRI; or c) the presence of cerebrospinal fluid-specific oligoclonal bands. According to the 2017 McDonald criteria, to minimize the risk of oversimplification of MS diagnosis, more distinctive MRI features of MS need to be identified. From this perspective, the central vein sign is one of the most promising (Sati et al., 2016). In line with pathological data, the use of T2-weighted magnitude and phase imaging at 3.0 and 7.0 T showed that many MS lesions form around small vessels. The proportion of lesions showing a central vein was found to be higher in MS compared with other conditions, e.g. neuromyelitis optica spectrum disorders, systemic autoimmune diseases, cerebral small vessel disease, Susac syndrome and migraine (Mistry et al., 2016; Sati et al., 2016**)**. Recently, Sinnecker et al., (2019) reported a 68% sensitivity and a 83% specificity for distinguishing MS from non-MS using a 35% central vein sign proportion threshold, suggesting that a central vein sign-based criteria could fill a gap in specificity when diagnosing MS.

Sizes, shapes, and locations of MS lesions vary. Typically, they have an ovoid shape, a diameter greater than or equal to 3 mm, and cluster close to the ventricles and in the corpus callosum, although juxtacortical and infratentorial regions are other common sites of involvement. On sagittal images, lesions can appear as “fingers” stemming from the ventricular borders and reaching the corona radiata. A well-defined nodular enhancement usually occurs in acute small lesions, whereas a ring-like appearance may be present in subacute large lesions, which have a higher level of tissue destruction, and tend to

resolve more slowly (Giorgio and De Stefano, 2018). Filippi et al., (2019) reviewed and described typical MRI features from MS to enhance the proper recognition of MS lesions, depicting “green flags” as typical MS lesions and “red flags” as atypical MS lesions that highlight alternative diagnoses. In the last decade, several studies have demonstrated that atrophy, a measure of neurodegeneration, occurs even in the earliest MS stages (Azevedo et al., 2015). The clinical relevance of brain atrophy, especially of the GM, stems from a better association, compared with WM lesion measures, with clinical progression, in terms of both disability and cognitive impairment (De Stefano et al., 2014). Both grey matter (GM) compartments, the cortex and deep GM (especially the thalamus) are affected (Giorgio and De Stefano, 2018; Pasquier et al., 2019; Solomon et al., 2017).

1.3.1.1. MRI sequences

Standardized brain MRI protocols for MS diagnosis have been proposed. The MRI should be performed at a magnetic field strength of at least 1.5T (preferably 3.0T) with a maximum slice thickness of 3 mm and an in-plane spatial resolution of 1×1 mm (voxel size 3×1×1 mm), and using defined pulse sequences, one of them being the T2-weighted sequence (Rovira et al., 2015).

Conventional or fast spin-echo proton-density and T2-weighted sequences are considered to be the reference standard as they have shown a high sensitivity for detecting focal MS lesions regardless of location (Rovira et al., 2015). Contrast is not required if no lesions are detected; however, when lesions are seen on T2-weighted sequences, gadolinium-enhanced (single dose, 0.1 mmol/kg body weight) T1-weighted spin-echo sequences are mandatory in the initial study, as they distinguish acute lesions from chronic ones and may demonstrate dissemination in time (Rovira et al., 2015).

1.3.2. T2 lesion volume and count

Longitudinal studies of clinical disease phenotypes or therapy efficacy commonly use changes in global T2 lesion burden as an outcome measure (Molyneux et al., 1998). Large-scale clinical trials and cross-sectional studies generally use global T2 lesion volume as surrogate for disease severity and long-term trends therein as indicators of disease activity and therapeutic effect (Filippi et al., 1995). Lesion evolution is likely to be not only patient-specific (Minneboo et al., 2005), but also a phenomenon related to the disease stage (Meier et al., 2007). Lesion evolution patterns are good candidates for markers that may stage disease progression and subtype. The emphasis on evolution is

important. Changes in enhancing lesion number and T2 lesion volume correlated with clinical activity (i.e. attacks) and clinical progression (Lee et al., 2000), as well as with markers of immunologic activity (Khoury et al., 2000). Different levels not only of hypointensity, but also of the rate of temporal change, were observed when comparing remyelinating and inactive demyelinating T1 lesions (Bitsch et al., 2001).

1.3.3. Subcortical deep gray matter atrophy

Demyelination is variably associated with axonal transection, degeneration, volume loss and eventual overall CNS atrophy (Trapp and Stys, 2009). The involvement of deep GM structures in MS is of particular interest because the thalamus, limbic and striatal structures are involved in all the major functional circuits in the brain and provide points of convergence across multiple cortical, limbic, brain stem and cerebellar systems (Debernard et al., 2015). Deep GM hypointensity measures on T2-weighted scans and lesions on double inversion recovery were correlated with disability (Calabrese et al., 2013; Zhang et al., 2007) and cognitive impairment. Several automated methods for segmentation of deep GM structures exist, e.g. FMRIB Software library (FSL) (Patenaude et al., 2011) or FreeSurfer (Fischl et al., 2002). Studies using these tools to investigate atrophy in MS demonstrated significant deep GM volume loss, particularly in the thalamus (Calabrese et al., 2010; Houtchens et al., 2007; Minagar et al., 2013; Schoonheim et al., 2012). This volume decrease probably reflects neuronal loss, and provides a plausible marker of neurodegeneration in deep GM, which may be due to either local pathology or Wallerian degeneration along white matter pathways that traverse the deep GM (Haider et al., 2014). In RRMS, reduced deep GM volume was associated with fatigue (Calabrese et al., 2010), and decreased thalamic volume with cognitive impairment (Houtchens et al., 2007; Paul, 2016; Schoonheim et al., 2012). In 2015, Debernand et al. identified atrophy in the thalamus, hippocampus and putamen, and associations between deep GM atrophy in these structures with impaired cognitive function, particularly information processing speed. Atrophy in the thalamus, hippocampus and putamen could result from focal demyelinated lesions, diffuse oxidative injury and neurodegeneration, all of which have been observed in the deep GM of MS patients (Haider et al., 2014). Although more likely found in MS patients with long-standing disease, histopathologically confirmed neurodegeneration (axonal and neuronal loss) may be a driver for MRI-measured cortical atrophy (Popescu et al., 2015). The findings from Debernand et al., (2015) in a RRMS cohort added to the growing literature

stating that thalamic atrophy is an especially prominent and early site of deep GM atrophy in MS (Minagar et al., 2013). They also supported the hypothesis that anatomically distant white matter (WM) lesions, through anterograde and retrograde (Wallerian) axonal degeneration and loss, also contribute to deep GM abnormalities (Mühlau et al., 2013), as they found a significant association between WM lesion load and both thalamus and putamen volume. The association between thalamic volume and cognition reflects the role of the thalamus in the control of cortical information processing and cognition (Schoonheim et al., 2012). With respect to the putamen, as part of striatum this structure receives inputs from prefrontal cortex, particularly from dorsolateral prefrontal cortex, highly implicated in executive functions and working memory, and from the orbito-frontal cortex, which is known to be involved in decision making and reward-seeking behavior (Tziortzi et al., 2014). These connections are likely to account for the associations between the putamen volume with executive function, working memory, attention and processing speed. Additionally, the putamen receives input from the frontal eye fields, which can also account for these associations, especially when performance depends on visual searching (Batista et al., 2012).

1.4. Chapter 4: Sex differences in MS, from an MRI perspective

1.4.1. Sex differences in lesion burden

A T2 lesion is a persistent tissue abnormality differing from a Gadolinium (Gd)-enhancing lesion, as the latter indicates acute inflammation that can chronically evolve to a T2 lesion (Dunn et al., 2015). T2 lesions per se are rather unspecific, and reflect a variety of underlying tissue pathologies including inflammatory demyelination, axonal injury, gliosis, and edema (Sahraian and Radü 2007). Lesion burden in MS commonly refers only to the T2 lesion volume, independent of the number of lesion counts (Li et al., 2006). To date, the analysis of T2 lesion volume and count focusing on sex differences has been limited, and results are conflicting. Some studies evaluated sex-based differences in the number of Gd-enhancing lesions in MS and most (Pozzilli et al., 2003; Tomassini et al., 2005; Weatherby et al., 2000), but not all (Barkhof et al., 2005), of these observed a higher lesion count in women. A weak correlation of this parameter with concurrent EDSS scores was reported (Barkhof and van Walderveen, 1999). Additionally, sex differences in T2 lesion volume have been inconsistently described. Antulov et al., (2009) and Tedeschi et al., (2005), did not find sex-related differences, whereas Schoonheim et al., (2014), Rojas et al., (2013) and Li et al., (2006) reported a higher T2 lesion volume in males. These studies indicate that despite exhibiting a lower extent of CNS inflammation (less count of Gd-enhancing lesions) than women, men may exhibit worse white matter damage, i.e. more T2 lesion volume. These data hint that either the underlying biology of inflammation or the vulnerability of tissue to inflammatory insults differs between the sexes. In contrast to lesion count, T2 lesion volume is considered to be a sensitive, but nonspecific marker of the total white matter damage that has accumulated in MS patients (Sahraian and Radü 2007). In regard to the correlation of T2 lesion volume with EDSS, studies reported that this association ranged from weak ($r = 0.13$) to strong ($r = 0.66$), as reviewed in Barkhof and van Walderveen (1999). One of the largest reviews to date that examined the relationship between T2 lesion volume and EDSS in 1,312 placebo treated MS patients from 11 randomized controlled trials in the Sylvia Lawry Centre for MS research database reported that T2 lesion volume correlated with EDSS scores up until an EDSS of 4 (Li et al., 2006). After this point, there was a plateau in this relationship, denoting a disconnection between inflammatory disease activity and disability progression after this disability landmark (Li et al., 2006).

Another conventional measure used to evaluate the extent of permanent tissue damage is the ratio of T2 lesions that evolve into T1 hypointensities or “black holes” on MRI (Carass et al., 2017). T1-weighted lesions represent areas of extensive, potentially irreversible axonal damage. Although one study did note that men had a higher ratio of T1/T2 lesions than women , the difference was not reported in other studies (Antulov et al., 2009; Riccitelli et al., 2012; Schoonheim et al., 2014, 2012; Tomassini et al., 2005; van Walderveen et al., 2001).

1.4.2. Sex differences in subcortical deep gray matter atrophy

The most recent cross-sectional study that evaluated sex differences in deep gray matter atrophy by Schoonheim et al., (2012) showed a significantly smaller volume in the left and right caudate nucleus and right putamen from male patients when compared to females. Volume reductions in the thalamus, pallidum, hippocampus, amygdala and accumbens were more pronounced in male patients but not significantly reduced in comparison to females. Similarly, in 2012 Rojas et al. reported a significant decrease of total brain volume and GM volume in males when compared to females. Finally, in 2009 Antulov et al. described overall more advanced GM and central atrophy (i.e. deep gray nuclei) in male patients in comparison to females, whereas WM atrophy was larger in females than in males. Conversely, some studies reported no sex differences in the atrophy of subcortical deep gray matter structures (Dolezal et al., 2013; Giorgio et al., 2008). Further research is needed to elucidate these conflicting findings. The identification of sex-associated differences in the atrophy of subcortical deep gray matter as well as T2 lesion burden should be prioritized, because, as described previously, there is a worse disease course and prognosis for males when compared to females, and these clinical features may be directly related to sex-preferential inflammatory and neurodegenerative mechanisms.

2.- AIMS AND HYPOTHESES

MS is a very complex multifactorial disease where sex-related differences have been reported. More information about sex-related differences in these aspects is required to elucidate previous conflicting findings. If it is true that sex is a key factor that influences disease course and severity, treatment strategies should target such sex-driven disparities to improve the quality of life of susceptible patients. The aim of this work was therefore to further explore these differences in three aspects: extent of disability, i.e. EDSS score, T2 lesion burden, and subcortical deep gray matter atrophy in a cohort of RRMS patients using a cross-sectional and longitudinal approach during a median observation time of 18.5 months (range: 12 to 25).

Regarding EDSS score, we aimed at replicating previous findings about disability progression, where males were found to accumulate disabilities faster than females (Fazekas et al., 2009; Giorgio et al., 2014; Leray et al., 2010). Similarly, we aimed at identifying the T2 lesion burden for each sex to elucidate previous conflicting findings, where sex did not play a role in T2 lesion load (Antulov et al., 2009; Tedeschi et al., 2005), or males showed to have a slightly greater T2 burden (Li et al., 2006). Finally, we sought to reveal differences in subcortical gray matter volume and identify sex differences, taking disease duration into account, as this aspect of MS has scarcely been explored.

Our hypotheses were:

1. Males develop a more severe disease course and accumulate faster disability, expressed by a higher EDSS score, compared to females.
2. Males display greater T2 lesion burden than females.
3. Males show more subcortical deep gray matter atrophy than females.

3.- METHODS

3.1. Patients

We evaluated 55 RRMS patients (female n = 39, male n= 16) from a database acquired by the NeuroCure Clinical Research Centre, Charité-Universitätsmedizin Berlin. Inclusion criteria were at least 18 years of age, confirmed diagnosis of RRMS, and at least one additional MRI acquisition after the baseline scanning, with a more than one-year span. Disease duration was calculated by determining the time elapsed between initial symptoms and the baseline MRI scan or follow-up MRI visit.

3.2. Statistical analysis

We took both a cross-sectional and a longitudinal approach for our analysis. In the cross-sectional approach we compared EDSS score, T2 lesion burden and subcortical deep gray matter volume at baseline corrected for disease duration between sexes using a Wilcoxon-Mann-Whitney test. In addition, we investigated the association of total disease duration, sex, and T2 lesion burden with subcortical deep gray matter volume for both sexes using a linear regression model. To further explore associations of subcortical volumes with disease duration, we additionally performed a subgroup analysis and compared the volume of each subcortical deep gray matter between males and females, dividing the sample into three different time periods: 1) less than 5 years of disease duration, 2) 5 to 10 years of disease duration, and 3) more than 10 years of disease duration. A post-hoc Bonferroni correction was performed to correct for multiple comparisons. In the longitudinal approach, we assessed changes in EDSS, T2 lesion burden, and subcortical deep gray matter volume between the baseline visit and a follow-up visit (12-25 months later, mean = 18.5). We calculated both the absolute increase and percentage change in these variables for both sexes. All group comparisons were performed using a Wilcoxon-Mann-Whitney test. All statistical analyses and plots were performed using R version 1.1.447. Statistical significance in all tests was set to a p-value of less than 0.05.

3.3. MRI Acquisition and Analysis

MRI scans were performed on a 3-Tesla (Siemens MAGNETOM Trio Tim, Erlangen, Germany) scanner. The MRI protocol for this work included the following: (1) a T1-weighted 3D magnetization-prepared rapid gradient echo (MPRAGE) brain MRI (1 x 1 x 1 mm resolution, repetition time (TR) = 1,900 ms, time to echo (TE) = 3.03 ms), (2) a T2-weighted 3D fluid-attenuated inversion recovery (FLAIR) brain MRI (1 x 1 x 1 mm resolution, TR = 6,000 ms, TE = 388 ms) and (3) 2D-sagittal T2-weighted sequence (slice thickness = 2 mm, TR = 3,500 ms, TE = 101 ms, in-plane resolution = 0.91 mm Å~ 0.91 mm). No patient was imaged during acute relapse.

3.3.1. Preprocessing of MRI sequences

Preprocessing and processing of MRI sequences was performed using FSL software ("FSL - FslWiki,") and the following description of steps refers to the use of this tool unless otherwise specified. Initial processing starts converting MRI acquired sequences to format DICOM using tools such as mricron/dicom2nii or freesurfer/mri_convert. Following this, the tool fslreorient2std was used. This tool is designed to reorient an image to match the orientation of the standard template images (MNI152) so that they appear in the same position and orientation (Smith et al., 2004; ***Figure 2***). This is not a registration tool, so it will not align the image to standard space, it will only apply 90-, 180- or 270-degree rotations about the different axes as necessary to get the labels in the same position as the standard template. MNI152 stands for Montreal Neurological Institute and is a brain template created by combining data from the brains of many different individuals to create an "average" brain. This is used to compare brain activations between subjects as well as individual functional and anatomical images, which must be first transformed to match a common template (See "Anatomical Preprocessing — C-PAC 1.4.3 Beta documentation").

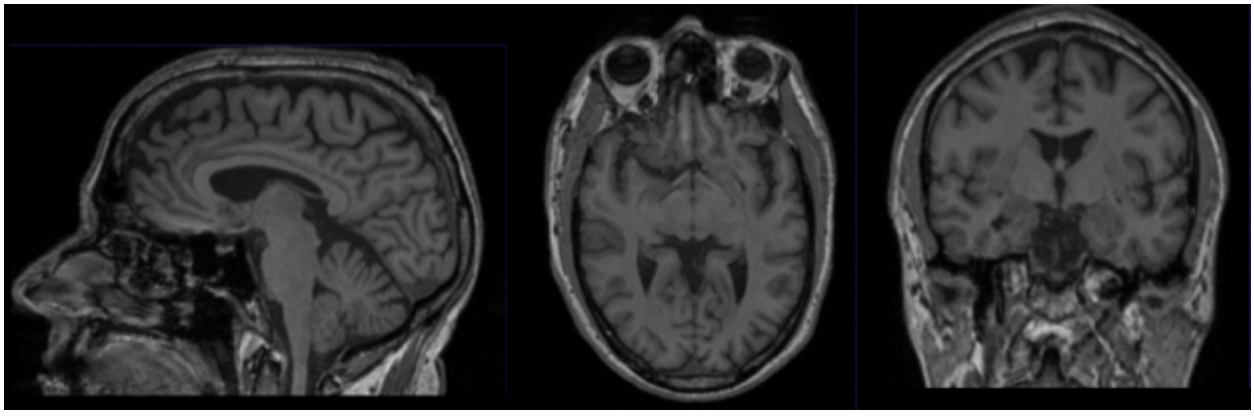

Figure 2 | FSL view orientation to MNI152 (AG Paul, Charité-Universitätsmedizin Berlin).

3.3.1.1. Registration

Image registration (also known as image fusion, matching or warping), can be defined as the process of aligning two or more images (Oliveira and Tavares, 2013). This step is achieved by using FLIRT (Jenkinson et al., 2002; Jenkinson and Smith, 2001). A spatial transformation is required to change the position and orientation of the shape of structures in the MR images. Mathematically it is expressed as a set of equations relating the old image positions (coordinates) to the new ones (Jenkinson et al., 2002). These equations need to be restricted in some way in order to limit the possible deformations of the images. These restricted models of transformations (e.g. rigid-body, affine, viscous-fluid) determine the physical model for the deformations – either due to changes in the anatomy or in the imaging process (Jenkinson et al., 2002). Each model has different characteristics, for example, rigid-body transformations do not allow the size or shape of any structures to change, and are useful for intra-subject registrations, but not for inter-subject registrations where size and shape are different. The transformation model is often described by its degrees of freedom (DOF), which is the number of independent ways that the transformation can be changed. For example, when considering translations (shifts) in 3D, there are three independent translations (one in x, one in y and one in z) making this a 3 DOF transformation model. In general, increasing the number of DOF allows the transformations greater scope to make one image match the other. The three most common models of transformations in 3D are: rigid-body (6 DOF), affine (12 DOF), and non-linear (anything from 12 to millions of DOF; Jenkinson et al., 2002). FSL uses rigid body transformations which only permit rotations and translations. In 3D it has 6 DOF: three rotations (one about each axis) and three translations. This fully describes the type of movements that a rigid body (one that does not change shape) can undergo, and so it is a good model of how a rigid body part (e.g. brain) can move (***Figure 3***). This transformation model does not allow any structures within the image to change size or shape, and is only used when this is known to be true, such as for images of the same subject where no anatomical changes are expected.

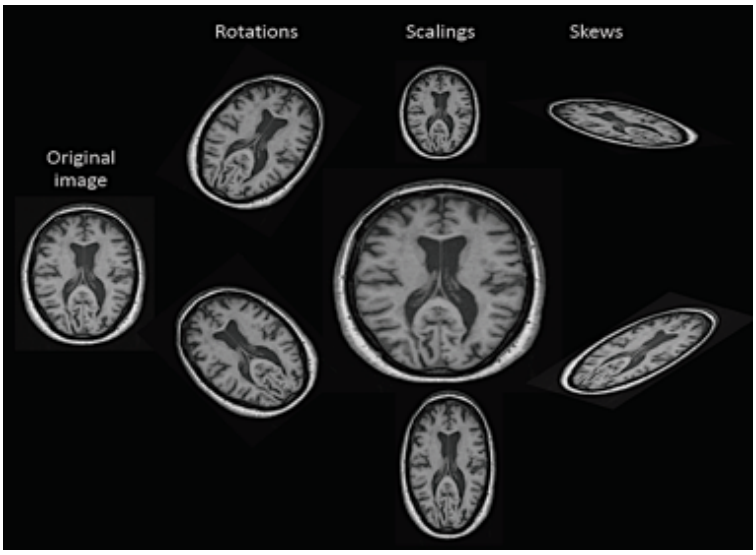

Figure 3 | Various examples of affine transformations of an original image (left). Rigid body transformations are a subset of affine transformations and include rotations, but not scalings or skews (Adapted from Jenkinson et al., 2002 with MRI data from AG Paul, Charité-Universitätsmedizin Berlin).

3.3.1.2. Brain extraction

Subsequently, brain extraction with neck removal (*bet* <*input*> <*output*>) was performed (***Figure 4***). Brain Extraction (BET; Smith, 2002) deleted non-brain tissue from an image of the whole head. It can also estimate the inner and outer skull surfaces, and outer scalp surface (Jenkinson et al., 2005).

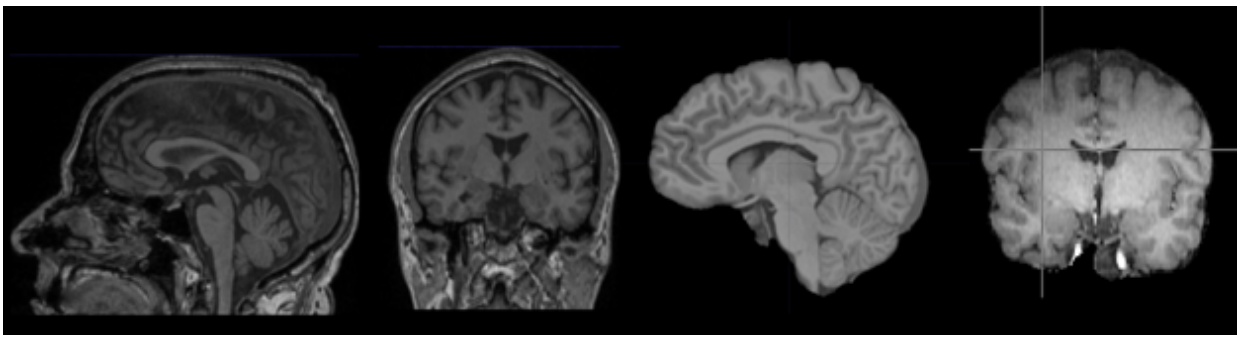

Figure 4 | Brain extraction performed by FSL BET (AG Paul, Charité-Universitätsmedizin Berlin).

3.3.1.3. Bias field correction and tissue type segmentation

After this step, bias field correction was applied to correct for the intensity inhomogeneity in MRI images. ***Figure 5*** shows on the left side the input image with intensity inhomogeneity, and on the right side the output image where the intensity inhomogeneity was corrected (no brain extraction performed).

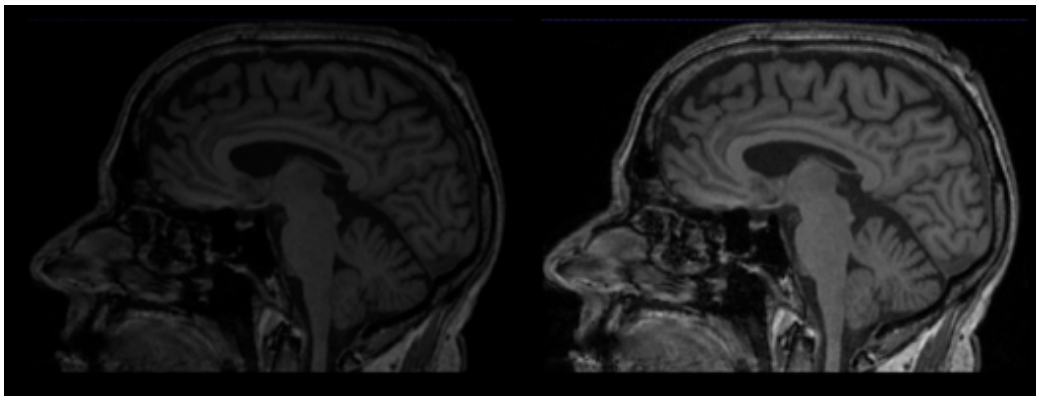

Figure 5 | FSL view bias field correction on the right figure (AG Paul, Charité-Universitätsmedizin Berlin).

Following brain/non-brain segmentation, tissue-type segmentation can be performed (***Figure 6***), that is, classification of each voxel into gray, white, or CSF and possible pathology (e.g. lesion). It is common now to segment purely on the basis of voxel intensity, since intensity thresholds were found to optimally distinguish between the different tissue classes (Zhang et al., 2001). This can be considered as an analysis of the image histogram, where the different classes appear (ideally) as separate peaks, which have a spread caused by factors such as image noise, motion artefacts, partial-volume effect, bias field (intensity fluctuations across the image caused by inhomogeneities in the radio-frequency field) and true within-class variation (Zhang et al., 2001).
FMRIB's Automated Segmentation Tool (FAST) (Zhang et al., 2001) uses mathematical models as a mixture of Gaussians (one for each class), giving each class' mean (and variance) intensity. Each voxel is then labelled by considering not just its intensity with respect to the estimated class means, but also the labelling of its local neighbors - a Markov random field (MRF) is placed on the labelling, causing spatial regularization (i.e. smoothness of segmentation; Zhang et al., 2001). This greatly reduces the effect of noise on the segmentation. The above approach easily generalizes to "multi-channel segmentation", i.e. if more than one input modality (image type) is available. For example, if both T1-weighted and proton density images are available, the input can be thought of as a vector image instead of just a scalar one. FAST allows for two or more input images, which can give improved results, e.g. in the deep gray structures where T1-only segmentation often has problems due to the intermediate (between white and cortical gray) intensities of some subcortical gray matter.

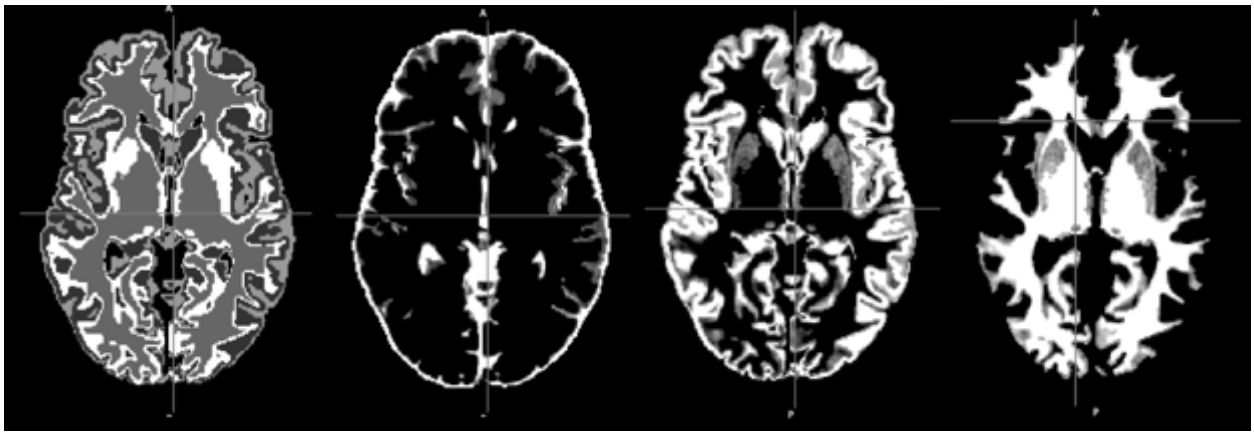

Figure 6 | Three-class segmentation (AG Paul, Charité-Universitätsmedizin Berlin).

3.3.2. Brain volume and V-scaling

Structural Image Evaluation, using Normalization, of Atrophy (SIENA) is a package for both single-time-point ("cross-sectional") and two-time-point ("longitudinal") analysis of brain change, in particular, the estimation of atrophy (volumetric loss of brain tissue). It estimates percentage brain volume change (PBVC) between two input images, taken of the same subject, at different points in time. It calls a series of FSL programs to strip the non-brain tissue from the two images, register the two brains (under the constraint that the skulls are used to hold the scaling constant during the registration), and analyze the brain change between the two time points. It is also possible to project the voxelwise atrophy measures into standard space in a way that allows for multi-subject voxelwise statistical testing (Smith et al., 2002).

Structural Image Evaluation with Normalization of Atrophy cross-sectional (SIENAX) estimates total brain tissue volume from a single image, normalized for skull size. It calls a series of FSL programs. It first strips non-brain tissue, and then uses the brain and skull images to estimate the scaling between the subject's image and standard space. It then runs tissue segmentation to estimate the volume of brain tissue, and multiplies this by the estimated scaling factor (V-scaling; See 3.4 Data processing), to reduce head-size-related variability between subjects.

3.3.3. Lesion segmentation

Semi-automatic segmentation of MS lesions was achieved using ITK-SNAP (http://itksnap.org). The lesion segmentation occurs in two steps. In the first stage, a probability map is computed, by applying a smooth threshold, which can be one-sided or two-sided, depending on whether the intensity range of the structure of interest lies at

one of the ends or in the middle of the histogram (Yushkevich et al., 2006). In this case the structure of interest is a T2-weghted hyperintensity that represents a MS lesion (***Figure 7***). In this way, an automatic mask is created from intensities and overlapped to the original MRI sequence. In a second step, lesions are individually analyzed and delineated by an expert grader, able to differentiate between a "true" MS lesion and other artifacts.

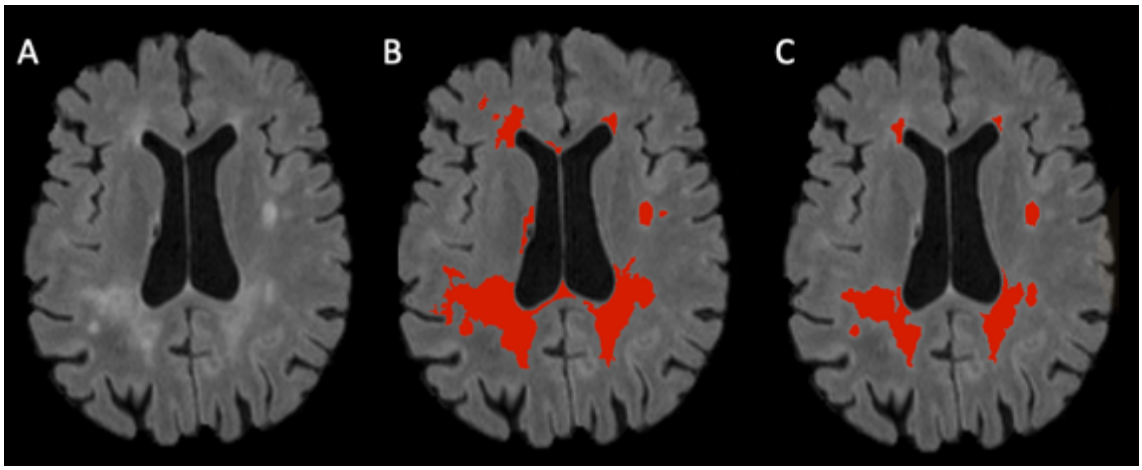

Figure 7 | Segmentation of MS lesions in white matter. A) FLAIR sequence MRI without lesion segmentation; B) Initial semiautomatic segmentation; C) Manual segmentation (AG Paul, Charité-Universitätsmedizin Berlin)

3.3.4. Subcortical deep gray matter segmentation

FMRIB's Integrated Registration & Segmentation Tool (FIRST) is a model-based segmentation/registration tool. The shape/appearance models used in FIRST are constructed from manually segmented images provided by the Center for Morphometric Analysis (CMA, training set), MGH, Boston (Patenaude et al., 2011). Segmentation is achieved by using a Bayesian Appearance Model that incorporates both shape and intensity information from the training set. The individual shapes are modelled by deformable meshes that consist of sets of vertices connected by edges, and which are each topologically equivalent to a tessellated sphere. To build the model, a mesh is fitted to each shape separately in each image of the training set, and the variation is modelled by a multi-variate Gaussian distribution of the concatenated vector of vertex coordinates and intensity samples (Patenaude et al., 2011).

Patenaude et al., (2011) created a volumetric output from the mesh by the following steps: (i) identifying the voxels through which the mesh passes (i.e. partially filled voxels); (ii) marking these voxels in a volumetric image as the boundary voxels; (iii) filling the interior

of this boundary. Once this is done they classify whether each boundary voxel should remain part of the segmentation or not. For this step, they use a 3-class classification of the intensities (gray matter, white matter and CSF) using the FSL/FAST method (Zhang et al., 2001). A rectangular region of interest (ROI) that encompasses the structure of interest (extended by two voxels) is used as input to the FAST method, which models the intensity distribution as a Gaussian mixture model in addition to a Markov Random Field. ***Figure 8*** illustrates the changes based on the Bayesian Appearance Model. It shows the change in shape and intensity distribution as the shape parameters vary. It depicts three different modes of segmentation along variation intensities for the brainstem. The left figure shows an incomplete segmentation of the brainstem, due to an imprecise brain extraction. The central figure also depicts an incomplete brainstem segmentation, due to different intensities within the same structure, and the right figure shows a complete segmentation of the brainstem. These conditional and different shapes of brainstem capture the variance of the intensities in different surrounding structures (Patenaude et al., 2011) .

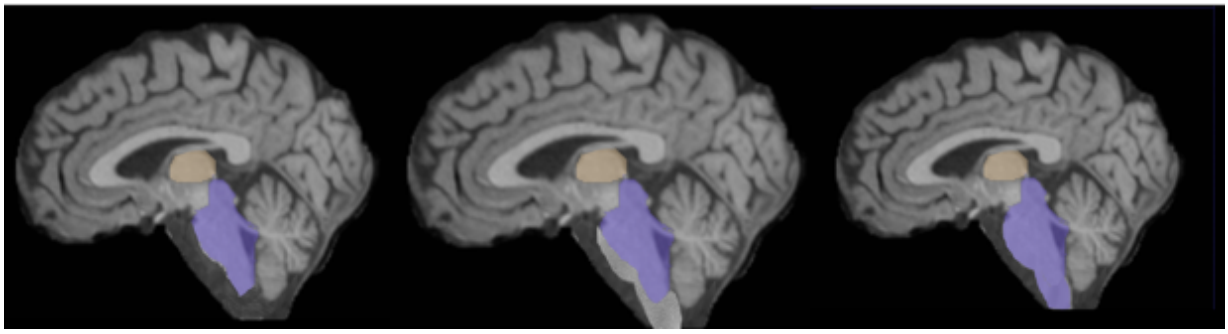

Figure 8 | Mode of variation for the brainstem (AG Paul, Charité-Universitätsmedizin Berlin)

3.4. Data processing

MRI data from each patient was processed individually through an automated pipeline which underwent each previously described step. The T2 lesion volume and count were obtained by calculating the total lesion volume and the number of lesions in the lesion masks created by ITK-SNAP.

Subcortical deep gray matter volumes were obtained using the output data from FIRST FSL. Given that FIRST performs segmentation from both sides of the brain, the volumes from the right and left side of every structure were acquired separately and consequently averaged and multiplied by the scaling factor to obtain normalized values for every structure. The scaling factor or V-scaling was taken from SIENAX output. This factor is a

ratio of each subject head size to a standard head size which allows us to weaken the head size effect between subjects (Smith et al., 2002).

4.- RESULTS

We included 55 RRMS patients (16 males and 39 females) in our study. Data were always compared between the female and male group using the corresponding statistical test. Cross-sectional data were analyzed comparing EDSS score, T2 lesion burden and subcortical deep gray matter (SDGM) volume at the beginning of the disease, which corresponded to the time of diagnosis or baseline MRI, respectively. The longitudinal data corresponded to 18 to 25 months after the initial MRI. As shown in ***Table 4***, there were no significant differences with respect to age, disease duration or EDSS between the groups at baseline. At the beginning of the observation period, patients had an average of 9 to 9.4 years of disease duration and an EDSS score of around 2 points.

Variable	**Female**	**Male**	**P value**
n (%)	39 (70)	16 (30)	NA
Age (years), mean ± SD	42 ± 10.3	44 ± 9.7	0.648
Disease duration (years), mean (range)	9.4 (1 – 29)	9.0 (1 – 21)	0.899
EDSS (Score), median (range)	2.12 (0.0 – 6)	2.41 (0.0 – 5)	0.603

Table 4 | Demographics at baseline

4.1. MRI output

Figure 9 illustrates deep gray mater structures segmented using FMRIB's FIRST software. It depicts the SDGM segmentation obtained after performing the steps for MRI processing described in the Methods section. In ***Figure 9A*** skull, skin and blood vessels are preserved (SDGM segmentation without MRI-preprocessing), whereas in ***Figure 9B***, ***9C*** and ***9D*** they were brain-extracted and bias corrected. The images correspond to the same patient.

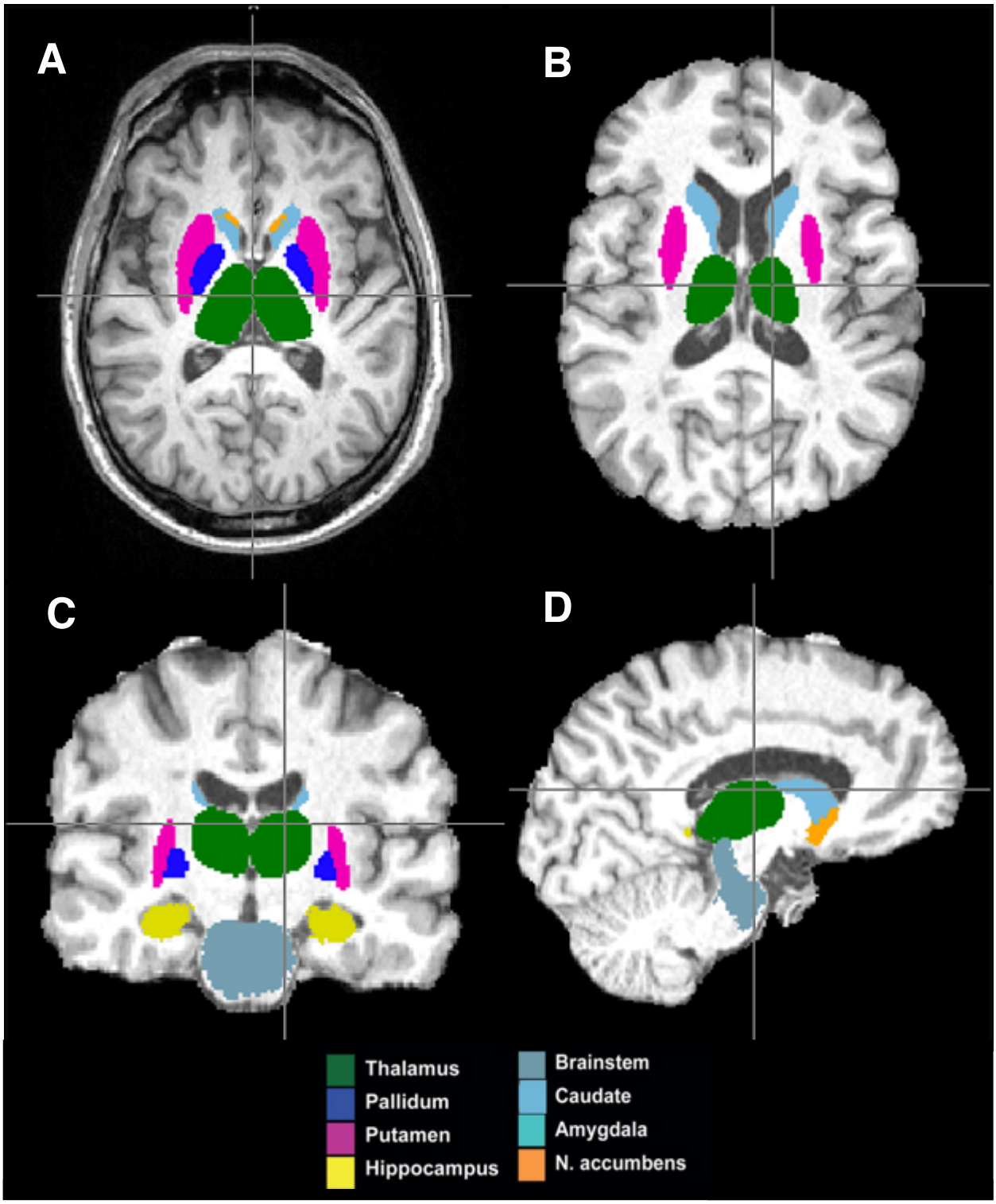

Figure 9 **|** T1 weighted MRI from a patient. A) Axial view of segmented structures without BET (transverse section at a middle thalamus level). B) Axial view of segmented structures with BET (transverse section at the superior border of thalamus). C) Coronal view of segmented structures with BET (section at the dorsal lateral thalamic nucleus). D) Sagittal view of segmented structures (section at the dorsal lateral thalamic nucleus (AG Paul, Charité-Universitätsmedizin Berlin).

4.2. Sex differences in disability

In our cohort men were demonstrated to have a higher EDSS score, i.e. more disability than women in the early stages of the disease (***Figure 10***). At 3 years of disease duration males had an average of 1.3 more points in their EDSS score compared to females (p = 0.03). As disease progressed, the male EDSS score remained relatively stable, whereas females accumulated further disability. ***Figure 10*** depicts this increase of clinical disability in female MS patients, which surpasses the initially higher EDSS scores from males as disease duration increases.

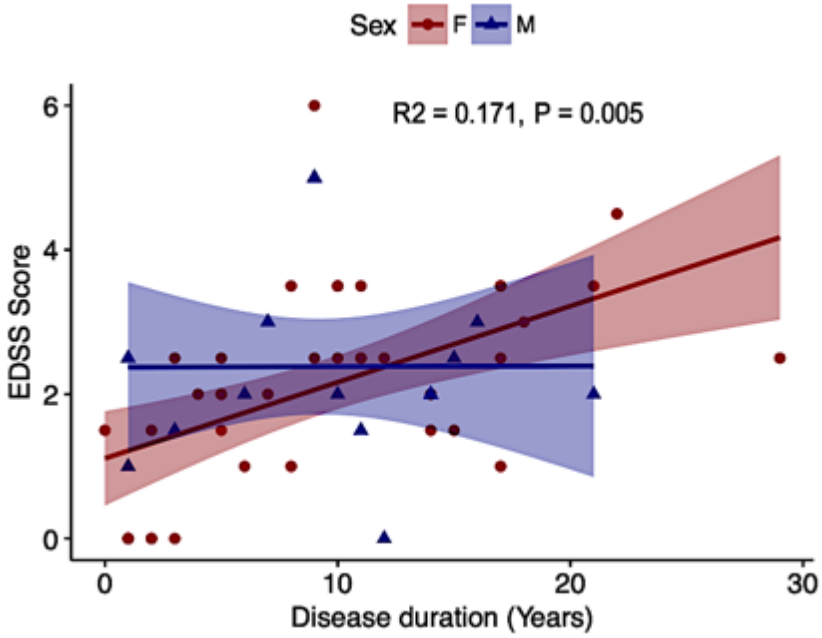

Figure 10 | Correlation of EDSS score and disease duration for each sex.

Figure 11A illustrates the absolute change in EDSS score when comparing the first visit with the last one. During the observation time, the mean change in EDSS score for females and males was 0.12 and -0.06 respectively, i.e. in our analysis we could see only small changes in disability progression and did not detect differences between sexes (p = 0.55).

Figure 11B depicts the percentage of change in EDSS score during the time of observation. The mean percentage of change in disability was 18% for females and -9% for males (p-value = 0.05). This indicates that the average disability in females increased more sharply compared to males in a timespan of approximately 1.5 years.

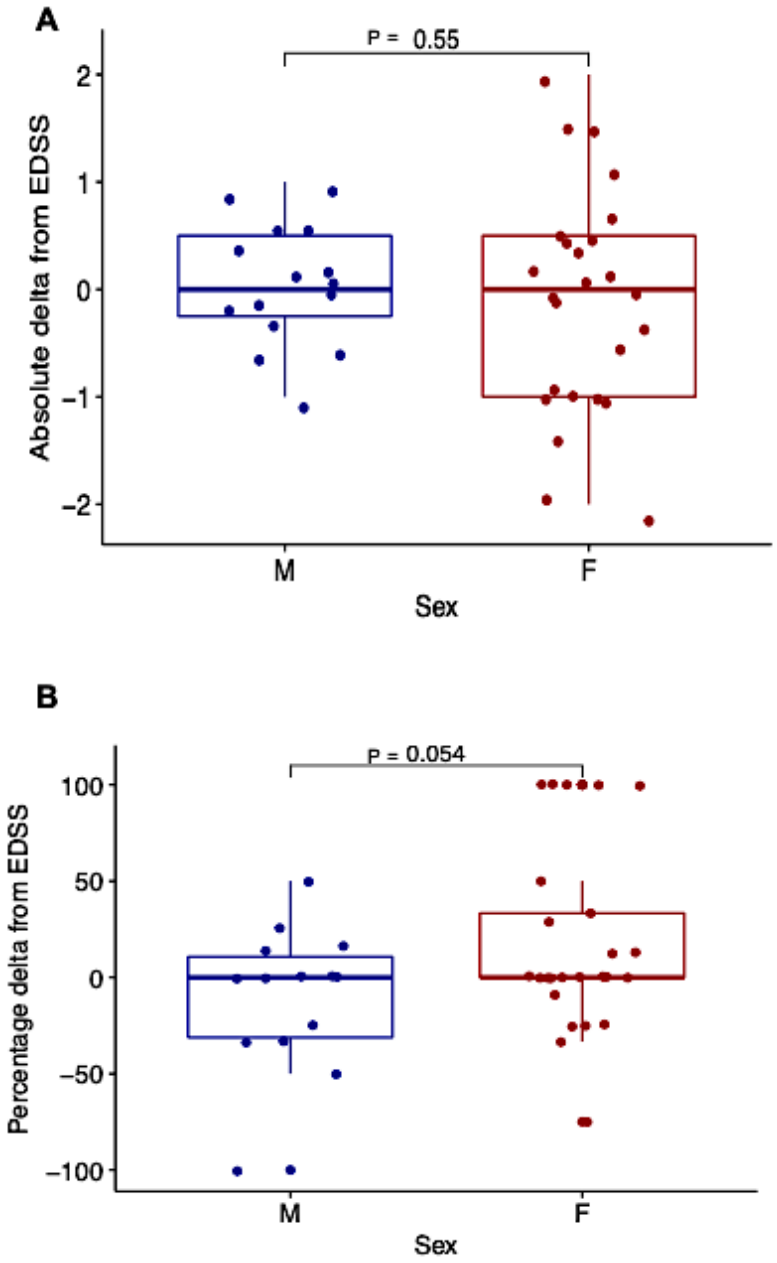

Figure 11 | Longitudinal analysis of EDSS change over 18 months expressed in absolute values and percentage.

In the longitudinal analysis we also found that 5 women had a 100% increase of the EDSS score during the time of observation, but males did not show an increase above 50% during this period of time. On the contrary, most of them showed a reduction in EDSS score from 0 to -100%. This result is in line with the findings from the cross-sectional approach that females accumulated more disability throughout disease evolution, whereas males remained relatively stable over time.

4.3. Sex differences in T2 lesion burden

We found that during the initial 3 years of disease evolution, men had a significantly higher T2 lesion volume than females (10 ml and 3.1 ml respectively; p-value = 0.03). After 15 years of disease duration, females had larger T2 lesion volume than males but no significant sex effect was found (9.4 ml and 7.9 ml respectively; p-value = 0.85). ***Figure 12*** illustrates this correlation of T2 lesion volume and T2 lesion count with disease duration according to sex.

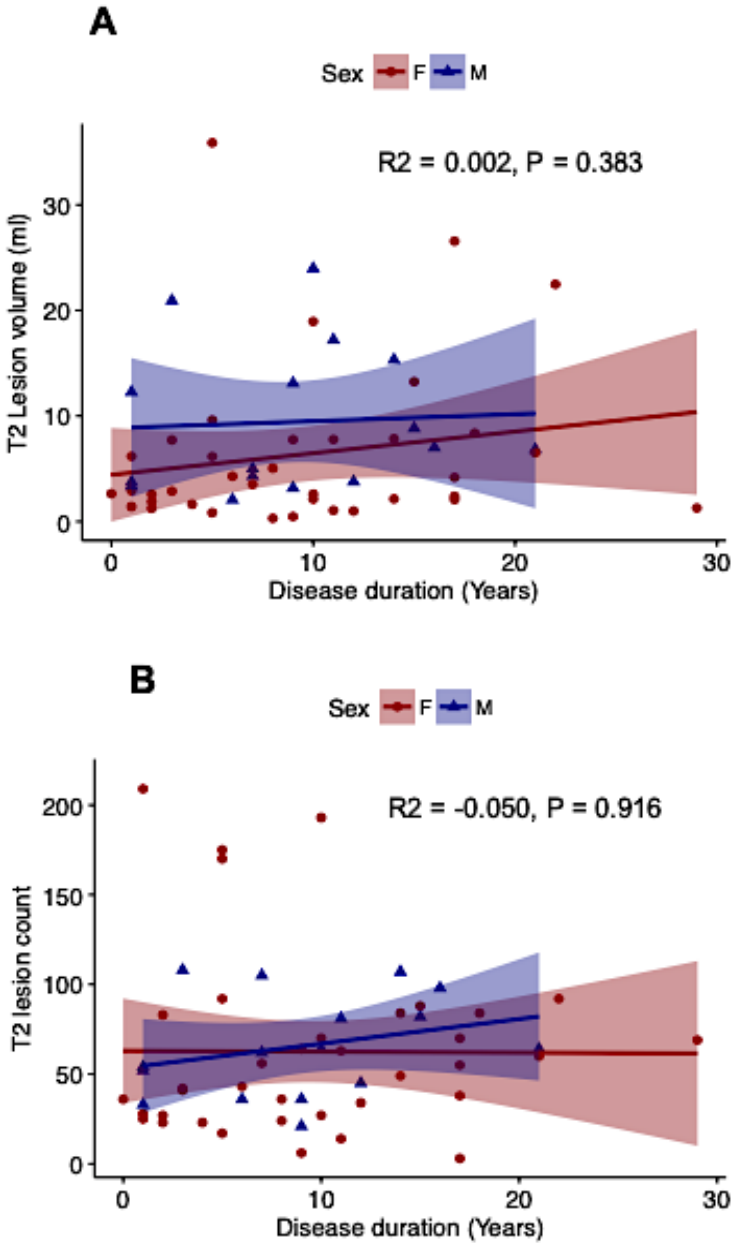

Figure 12 | A) Correlation between T2 lesion volume and disease duration in both sexes. B) Correlation between T2 lesion count and disease duration in both sexes.

Regarding lesion count (***Figure 12B***), we observed that at early stages of the disease men had lower T2 lesion counts than women, whereas at later stages they had higher counts. However, these differences were not significant (61 and 70 lesion counts at early stages, respectively, p-value 0.48; 90 and 56 counts at later stages, respectively, p-value = 0.285).

During the observation time, no effect of sex was found. Men had an increase of 0.28 ml in T2 lesion volume and women an increase of 0.04 ml (p-value 0.43). This corresponds to a 5.1 % increase in lesion volume over 18 months for males and a 1.7 % increase for females (p-value 0.35).

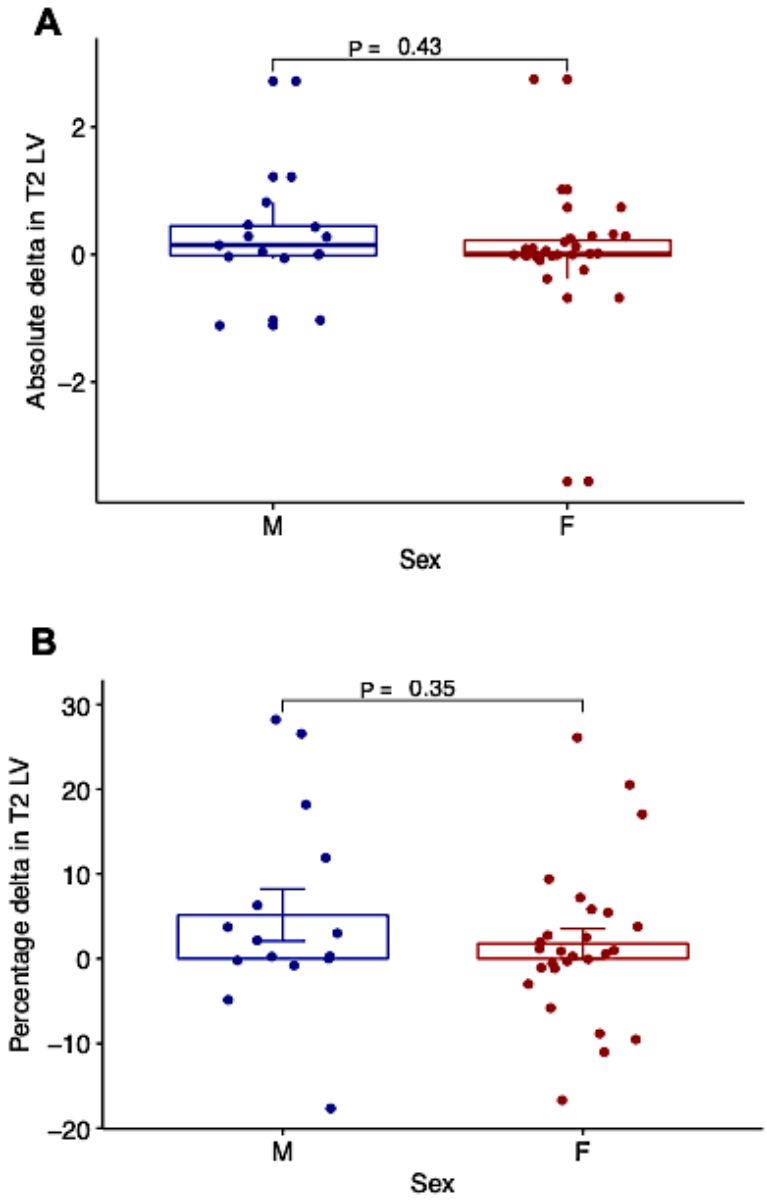

Figure 13 | A) Longitudinal analysis of T2 lesion volume change over 18 months. B) Longitudinal analysis of T2 lesion volume change over 18 months expressed as percentages.

In ***Figure 13*** the results of the longitudinal analysis are illustrated. ***Figure 13A*** shows the absolute difference in T2 lesion volume during the observation time in both sexes and ***Figure 13B*** depicts this difference expressed as percentages.

Regarding the longitudinal analysis of T2 lesion count, we found that during the observation period men had an increase in lesion count of 1.3, whereas women had an increase of 2 (p-value = 0.54), which correspond to 1.7% and 2%, respectively (p-value = 0.85).

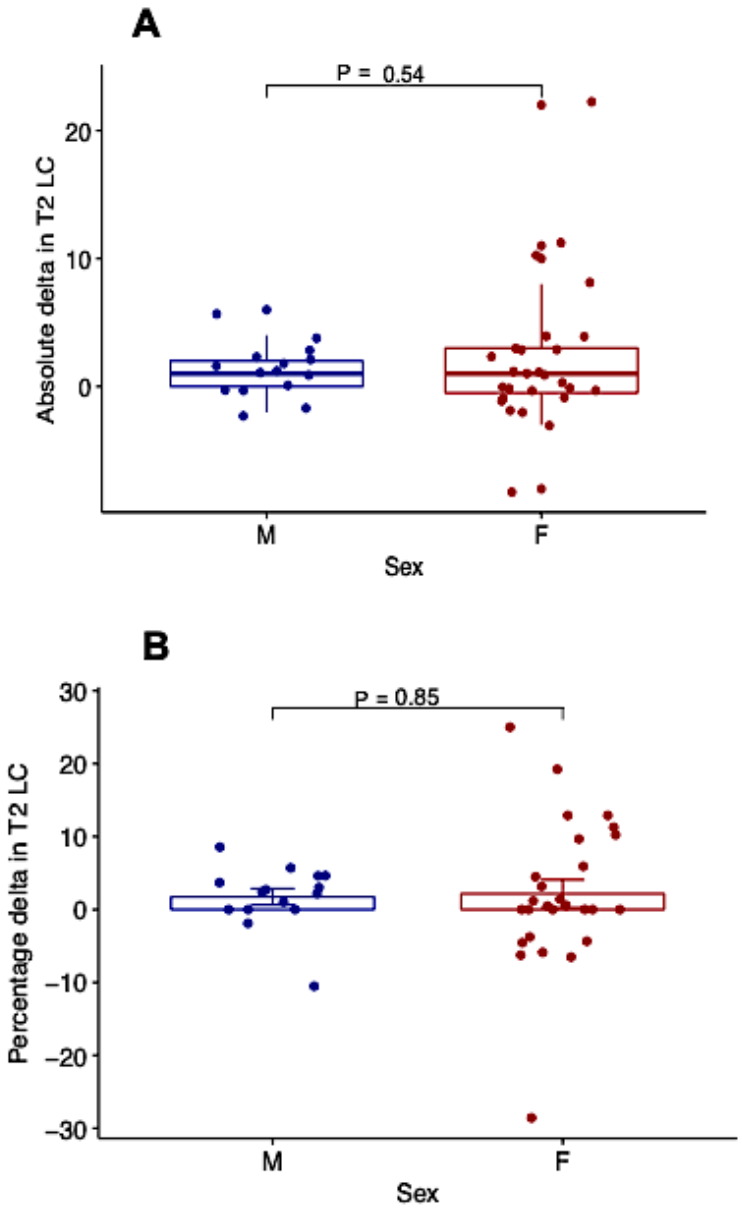

Figure 14 | A) Longitudinal analysis of T2 lesion count change over 18 months. B) Longitudinal analysis of T2 lesion count change over 18 months expressed as percentages.

Figure 14A illustrates the absolute difference in T2 lesion count during the observation time in both sexes, and ***Figure 14B*** depicts this difference expressed as percentages.

4.4. Sex differences in atrophy of subcortical gray matter

We found significant sex differences only in the atrophy of thalamus, which we describe in this section in more detail. Results from the remaining subcortical structures are shown in ***Table 6*** and ***Table 7***. ***Table 5*** describes the demographics of MS patients according to period of disease duration in years.

Variable	***< 5 years of disease duration***			***5 – 10 years of disease duration***			***> 10 years of disease duration***		
	Female	*Male*	*p*	*Female*	*Male*	*p*	*Female*	*Male*	*p*
n (%)	13 (72)	5 (28)	NA	11 (64)	6 (36)	NA	14 (70)	6 (30)	NA
Age (years), Mean ± SD	36 ± 10.8	41 ± 14.8	0.373	44 ± 10.7	45 ± 6.9	0.765	47 ± 5.7	44 ± 10.5	0.170
Age at diagnosis (years), Mean ± SD	33 ± 10.2	40 ± 13.8	0.176	37 ± 9.4	40 ± 7.1	0.659	35 ± 7.3	33 ± 12	0.366
Disease duration (years), mean ± SD	3 ± 1.6	1.5 ± 1	0.552	8.5 ± 1.4	8 ± 1.5	0.624	16.8 ± 4.8	14.8 ± 3.5	0.711
EDSS (Score), Median (range)	1.5 (0 – 2.5)	2.5 (1 – 2.5)	0.087	2 (1 – 6)	3 (3 – 5)	0.306	2.5 (1 – 4.5)	2 (0 – 3)	0.052

Table 5 | Demographic characteristics of sample according to periods of disease duration. P corresponds to p value.

Our results demonstrated that the volume of thalamus correlated negatively with disease duration (p-value = <0.001). ***Figure 15*** illustrates this correlation, where we can also observe that the volume of thalamus in males is smaller than the one of females over the disease course.

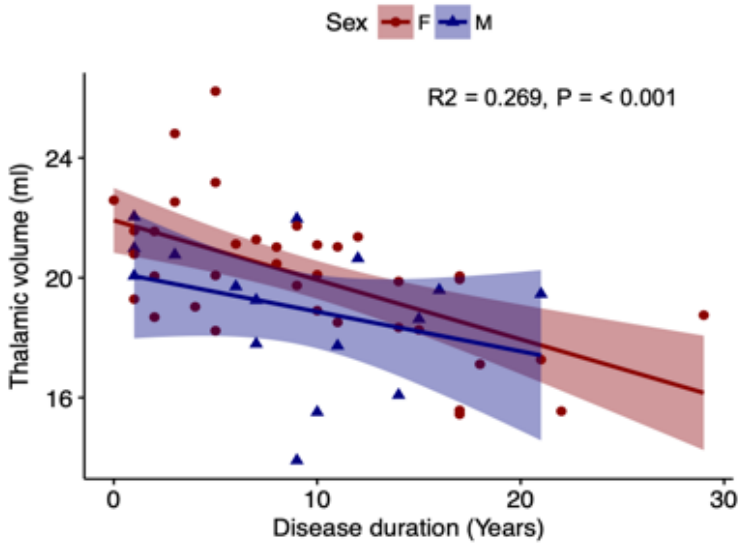

Figure 15 | Correlation between thalamic volume and disease duration in both sexes.

This negative correlation of disease duration with subcortical deep grey matter volume in both sexes was also observed in the case of putamen, pallidum, hippocampus and accumbens. ***Table 6*** describes this association of SDGM volumes with years of disease duration, sex and T2 lesion volume and count. We demonstrated that sex was not a strong predictor in the multivariate linear regression model, but T2 lesion volume contributes strongly to the volume loss in thalamus, putamen and pallidum. T2 lesion count only showed an association with the volume of the pallidum.

Variable	***Thalamus***	***Caudate***	***Putamen***	***Pallidum***	***Hippocampus***	***Amygdala***	***Accumbens***
Disease Duration	*t = -4.12* **p = <0.001**	*t = -0.08* *p = 0.934*	*t = -2.16* **p = 0.035**	*t = -2.27* **p = 0.027**	*t = - 2.25* **p = 0.028**	*t = -1.22* *p = 0.226*	*t = -2.56* **p = 0.013**
Sex (M)	*t = -1.38* *p = 0.172*	*t = -0.97* *p = 0.334*	*t = -0.05* *p = 0.960*	*t = -1.62* *p = 0.111*	*t = -1.48* *p = 0.144*	*t = -0.59* *p = 0.552*	*t = -0.19* *p = 0.850*
T2 Lesion Volume	*t = -2.42* **p = 0.019**	*t = -1.87* *p = 0.062*	*t = -3.39* **p = 0.001**	*t = -2.22* **p = 0.030**	*t = -0.67* *p = 0.500*	*t = -0.36* *p = 0.719*	*t = -0.56* *p = 0.576*
T2 Lesion Count	*t = 0.23* *p = 0.812*	*t = 0.16* *p = 0.868*	*t = 1.08* *p = 0.281*	*t = 2.30* **p = 0.025**	*t = -1.14* *p = 0.257*	*t = -1.27* *p = 0.208*	*t = -0.42* *p = 0.671*
F statistic	*8.067*	*4.210*	*5.183*	*4.355*	*2.108*	*1.429*	*2.147*
Adjusted R-squared	*0.352*	*0.198*	*0.243*	*0.205*	*0.096*	*0.031*	*0.081*
p-value of multivariate linear model	***<0.001***	***0.009***	***0.001***	***0.004***	*0.080*	*0.238*	*0.089*

Table 6 | Multivariate regression model of SDGM volumes. Statistically significant values are shown in bold.

Additionally, in the cross-sectional analysis during different periods of disease duration, we observed that the thalamic volume in male patients between 5 and 10 years of disease duration was significantly reduced (2.9 ml less volume) when compared to females (p-value = 0.03; total volume in males was 18 ml and in females 21 ml). After post-hoc correction, this value was not significant anymore (p-value = 0.46). Before 5 years and after 10 years of disease evolution no significant differences were found.

Table 7 describes the rest of the SDGM structures according to period of disease duration. In the rest of the SDGM structures, we found that males had less caudate volume than females after 10 years of disease duration. Pallidum volume was smaller in males than females at 5 to 10 years of disease duration, and the amygdala volume in men was also reduced when compared to women at less than 5 years of disease evolution. After Bonferroni correction, no significant values were found.

SDGM	***< 5 years of disease duration***			***5 – 10 years of disease duration***			***> 10 years of disease duration***		
	Female	*Male*	*p-value*	*Female*	*Male*	*p-value*	*Female*	*Male*	*p-value*
Caudate	*9.49*	*8.52*	*0.087*	*9.04*	*8.06*	*0.087*	*8.97*	*8.08*	***0.021***
Putamen	*12.84*	*12.47*	*0.733*	*12.36*	*11.11*	*0.322*	*11.32*	*11.59*	*0.624*
Pallidum	*4.82*	*4.68*	*0.453*	*5.06*	*4.14*	***0.012***	*4.42*	*4.37*	*0.970*
Hippocampus	*10.27*	*8.88*	*0.075*	*10.35*	*9.36*	*0.638*	*9.23*	*9.43*	*0.922*
Amygdala	*3.52*	*2.20*	***0.003***	*3.68*	*3.33*	*0.21*	*3.51*	*3.24*	*0.249*
Accumbens	*1.15*	*1.27*	*0.303*	*1.06*	*0.93*	*0.415*	*0.96*	*0.99*	*0.819*

Table 7 | Cross-sectional analysis of SDGM volume at different time points of disease duration. Statistically significant values are shown in bold.

In the longitudinal analysis, our results demonstrated that males had larger thalamic atrophy than females. Men lost 0.41 ml over 18 months, whereas women lost only 0.18 ml (p-value = 0.017). This corresponds to a 2% and 0.8% decrease in thalamic volume, respectively (p-value = 0.014). ***Figure 16*** illustrates these longitudinal results of the thalamus. In ***Figure 16A*** we can observe the absolute difference in thalamic volume during the observation time in both sexes, and in ***Figure 16B*** this difference expressed as percentages.

In the longitudinal analysis, we did not find any sex effect for any SDGM structure other than the thalamus. ***Table 8*** describes these results regarding the volume change for each SDGM structure, expressed in absolute values and percentages.

SDGM	*Absolute volume change (ml)*			*Percentage volume change (%)*		
	Female	*Male*	*p-value*	*Female*	*Male*	*p-value*
Caudate	*-0.11*	*-0.09*	*0.55*	*-1.20*	*-1.19*	*0.62*
Putamen	*-0.15*	*0.04*	*0.16*	*-1.25*	*0.27*	*0.13*
Pallidum	*-0.05*	*-0.06*	*0.51*	*-1.30*	*-2.27*	*0.55*
Hippocampus	*-0.06*	*-0.19*	*0.06*	*-0.49*	*-2.06*	*0.06*
Amygdala	*0.02*	*0.20*	*0.28*	*0.73*	*5.59*	*0.22*
Accumbens	*-0.008*	*-0.041*	*0.75*	*-1.03*	*-5.68*	*0.73*

Table 8 | Longitudinal analysis of SDGM volume change over 18 months expressed as percentages and absolute values. Statistically significant values are shown in bold.

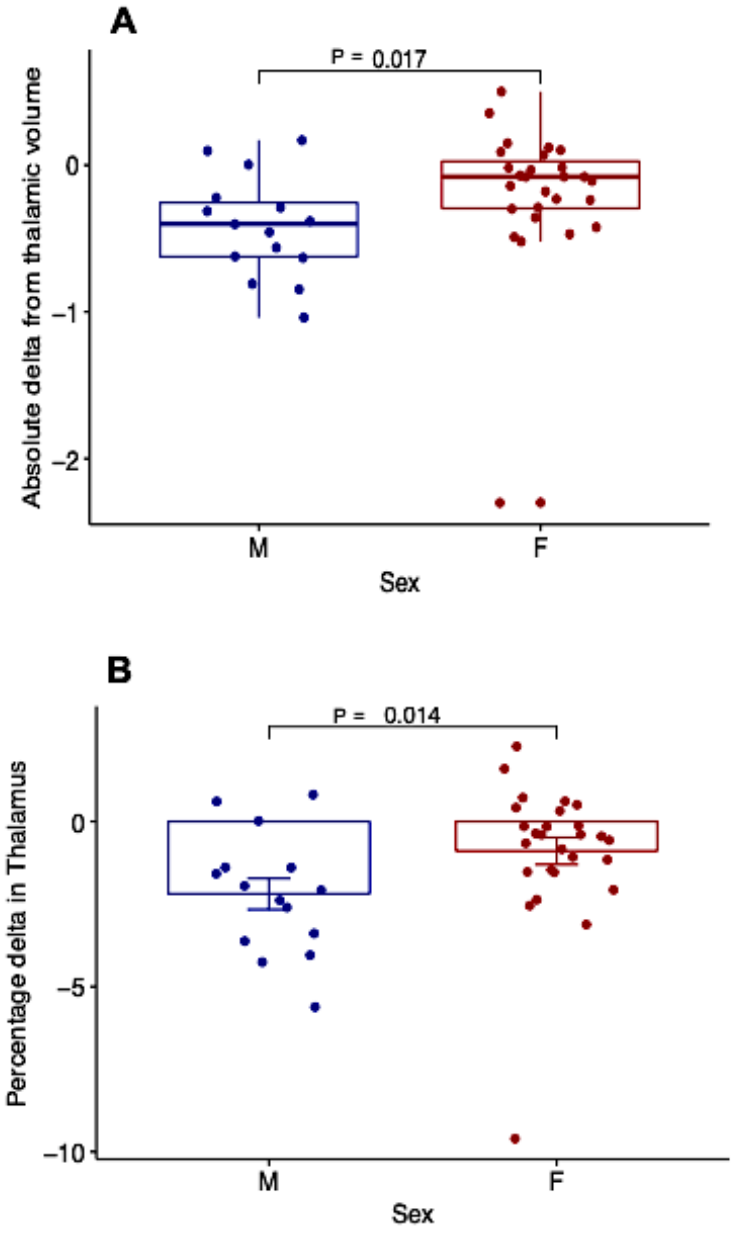

Figure 16 | A) Longitudinal analysis of thalamic volume change over 18 months. B) Longitudinal analysis of thalamic volume change over 18 months expressed as percentages.

5.- DISCUSSION

In this study we aimed to investigate sex related differences in the progression of disability, T2 lesion burden and atrophy of subcortical deep gray matter in patients with multiple sclerosis. Major findings are discussed in the following sections.

5.1. Sex differences in disability

We were able to replicate the findings from previous studies regarding disability progression in MS (Confavreux et al., 2003; Leray et al., 2010; Ribbons et al., 2015). Our results demonstrated that males showed more clinical disability expressed by a higher EDSS score than females at early stages of the disease, and this disability remained stable, whereas in women it kept progressing. Based on this finding, we assume that males reach higher EDSS scores in a shorter time than females. This may not be necessarily due to faster progression, but because they start out with a higher disability score. This was described by Leray et al. in 2010, when they reported that men took around 9 years to reach a moderate disability (DSS 3), whereas females took around 10 years. Contrarily, in our longitudinal analysis we observed a larger increase of disability in females when compared to males. However, our sample size for each sex was different in proportions and the EDSS rating is subject to inter- and intra-rater variability. Therefore, we cannot exclude an interaction of these two factors as a main influence on our observations.

Leray et al., (2010) and Ribbons et al., (2015) proposed that MS is a two-stage disease. In the first stage, the focal inflammatory lesions have a strong impact on disability, and in the second stage, disability is independent of focal inflammatory markers. This may explain why the influence of sex on disability progression can mainly be identified during the first stage of the disease, due to disparities in the inflammatory response. The reasons that could explain a higher disability in males at initial stages are that the neuronal damage existed already for some time in a subclinical way, or that the current therapies are not effectively targeting the stronger immune response during the first and crucial years of disease specifically in males.

5.2. Sex differences in T2 lesion burden

We were able to identify sex differences in T2 lesion volume but not in lesion count at early stages of MS evolution, whereas no difference in longitudinal change throughout

our observation time was found. Our results demonstrated that at early stages of the disease, men showed a significant higher T2 lesion volume than females.

Previous studies focused on sex differences in T2 lesion load and found that there were no significant differences (Antulov et al., 2009; Tedeschi et al., 2005). A reason that could explain the contradictory findings may be that they evaluated the T2 lesion load in a cohort of patients with a mean of 13.3 and 9.7 years of disease duration, respectively. This corresponds to a later disease stage, and according to our results, differences in T2 lesion load were not significant anymore at this time point. On the other hand, our results corroborate Li et al., (2006), who reported a larger T2 lesion burden for men than women in a cohort of 463 patients with a mean disease duration of 6.8 years.

A larger T2 lesion load was reported to increase the risk of disability progression in patients with RRMS (Mostert et al., 2010). Therefore, our results could explain why in our cohort men had higher disability than females during the first years of disease evolution, since it correlated with a larger T2 lesion load. The larger T2 lesion volume in males may not only explain a cross-sectional higher clinical impairment expressed by worse EDSS scores, but also predicts higher levels of disability at follow up (Fisniku et al., 2008; Mostert et al., 2007; Rudick et al., 2006; Tintoré et al., 2006). Furthermore, our results illustrated that during the disease course, females accumulated a larger T2 lesion load, to a degree where they exceeded the load of males after 15 years. However, this may not translate into greater disability. According to the literature, this is most likely explained through the fact that when the disease is already advanced, disability is independent of focal inflammatory markers, and several other neurodegenerative mechanisms are more influential (Leray et al., 2010; Li et al., 2006). In our longitudinal analysis over a period of 15-18 months, we found a 0.28 ml T2 lesion volume increase in males, and only an increase of 0.04 ml in females (p-value 0.43). This is in line with our cross-sectional results, where at 10 years of disease duration men still had more T2 lesion volume than females, but the differences were not significant anymore (13.823 ml in men and 6.307 ml in females; p-value = 0.059). The absence of significance in our results could be related to the differences in sample size. Also our results showed a much slower accumulation of T2 lesion load, namely 80% lower for males and 98% lower for females when compared to the study of Minneboo et al., (2009), who described a median rate of 1.4 ml/year, but did not specify sex differences. On the other hand, our findings are in line with Rojas et al., (2013), who described a larger increase of lesion load in males than in

females (2 ml and 1 ml, respectively) at 6 years of follow-up after onset of the disease (p-value 0.01).

Regarding lesion count, we found that men had fewer numbers of lesions than females at early stages of the disease, whereas at late stages this relationship inverted without reaching significance. Our findings regarding the increase in T2 lesion count for males is in line with previously described sex differences, as the increase of T2 count was associated with higher risk of developing secondary progressive MS (Mostert et al., 2007), and men seem more prone to develop progressive type disease (Ribbons et al., 2015).

5.3. Sex differences in atrophy of subcortical gray matter volume

Our analysis of sex effect in the atrophy of SDGM showed that: 1) No sex differences were found in the longitudinal analysis for any subcortical gray matter structure other than thalamus; 2) During our observation period the atrophy of the thalamus was more pronounced in men than women; 3) The volume reduction in the thalamus, putamen, pallidum, hippocampus and accumbens was proportional to disease duration for both sexes; 4) The reduction of volume in the thalamus, putamen and pallidum correlated with higher T2 Lesion volume and in the case of the pallidum also with an increase of T2 lesion count; 5) No sex differences were found in the cross-sectional analysis for any subcortical deep gray matter structure but males had a non-significant larger thalamic atrophy at 5 to 10 years of disease duration (2.9 ml volume reduction in males compared to females); 6) The volume of caudate, pallidum and amygdala showed a larger reduction in males than in females at different disease duration time points.

In our longitudinal analysis, the thalamus was the only subcortical gray matter structure that showed a stronger atrophy in males when compared to females during our observation time. These findings may be related to the rich reciprocal connectivity of thalamus with several brain areas, which makes it particularly susceptible in comparison with other subcortical gray matter structures to hypometabolism and Wallerian degeneration from remote connected brain regions (Cifelli et al., 2002; Eshaghi et al., 2018; Houtchens et al., 2007). Similarly, previous studies identified the thalamus as the subcortical gray matter structure with largest atrophy (up to 22 % less volume compared to healthy controls) and earliest affection (from 3.9 years of disease duration) in patients with MS (Bermel and Bakshi, 2006; Chu et al., 2018; Datta et al., 2015; Eshaghi et al., 2014; Houtchens et al., 2007; van de Pavert et al., 2016; Vercellino et al., 2009). The

reasons that could explain a preferential neurodegeneration in males may be related to the modulation of the immune response through hormonal regulation (See Chapter 2). A predominance of Th1 response in males, combined with a higher vulnerability of oligodendrocytes to excitotoxic death driven by testosterone, leads to a faster neurodegeneration in susceptible areas such as the thalamus, preferentially in males (Caruso et al., 2004; Gold et al., 2019). We found a proportional volume decrease of the thalamus, putamen, pallidum, hippocampus and accumbens with disease duration. This is in line with Chu et al., (2018), who also reported disease duration to be a strong predictor for atrophy in the thalamus, caudate and pallidum after 5 years of disease evolution in comparison to healthy peers. Eshaghi et al., (2018), similarly found a significant association between the rate of increase in atrophy of gray matter areas (including SDGM) and disease duration in all patients with multiple sclerosis. One of the mechanisms by which disease duration contributes to neurodegeneration is driven by a persistent state of demyelination. During this state, neurons consume more energy to survive, which creates a micro-environment that is similar to a state of hypoxia, i.e. decreased nutrients and oxygen, facilitating neurons to be more vulnerable to neurodegeneration (Zhang and Raichle, 2010). A reason why we did not observe a proportional volume decrease with disease duration in all investigated subcortical gray matter structures may be linked to the small sample in our study.

Our results showed a significant correlation between atrophy of the thalamus, putamen and pallidum with T2 lesion volume. These findings were previously described by Pontillo et al., (2019), in a cohort of 52 RRMS patients and by Datta et al., (2015), in a cohort of 924 RRMS patients. Our findings support the suggested explanation that subcortical gray matter atrophy is a consequence of microstructural damage, due to demyelination and cell injury as expressed by T2 lesions. Lesions in WM were described as the most likely origin of this atrophy, as an axonal transection that leads to disconnection and subsequent degeneration along axonal projections was widely proposed (Bergsland et al., 2012; Cifelli et al., 2002; Pontillo et al., 2019; van de Pavert et al., 2016). Since we did not categorize lesions according to the area where they were found, i.e. white or gray matter lesions, we cannot corroborate that the atrophy of SDGM is mostly driven by WM lesion load. Nevertheless, we similarly demonstrated a strong association between atrophy and lesion volume.

Studies focusing on sex differences and SDGM atrophy showed several discrepancies. Dolezal et al., (2013), found larger thalamic volume in males than females at 5.2 and 5.7

years of disease duration, respectively. However, no sex differences were identified at 5 years follow-up. Nevertheless, Schoonheim et al., (2012), found a larger thalamic volume in females than in males at 7.5 and 7.6 years of disease duration, respectively, as well as Houtchens et al., (2007), at a mean of 9.7 years of disease duration.

Even though we did not find sex differences in thalamic atrophy after post-hoc analysis, our results are in agreement with the susceptibility of males to develop a stronger thalamic atrophy than females at 5 to 10 years of disease duration (Wilcoxon-Mann-Whitney test: p-value = 0.03). A concept that may explain this atrophy during this specific period of time would be the suggested two-step model of MS pathology. At early stages of the disease demyelination and inflammation have no effect on volume reduction, but chronically demyelinated axons undergo degeneration due to the lack of trophic support, so that atrophy can be identified after a longer disease duration (Abdurasulova and Klimenko, 2011). The specific time point when this shift occurs may be when the compensatory resources are exhausted, which vary according to patients' susceptibilities. Still, it correlates with progressive irreversible functional impairment, which is reflected at 6 years of disease duration in males, and 8 years of disease duration in females (Leray et al., 2010). In our cohort, the mean years of disease duration for men was 9 (median 9), and for women 9.4 (median 9; p-value = 0.899) with a mean follow-up of 18.6 $\pm$ 3.9 months. During this time, and according to the literature, neurodegenerative mechanisms take place (Leray et al., 2010). This could be the reason why we observed a sex effect in thalamic atrophy during this period of time.

Our results indicated a stronger atrophy of caudate at more than 10 years of disease duration, of pallidum at 5 to 10 years of disease duration, and of amygdala at less than 5 years of disease duration in males when compared to females (although results were not significant after Bonferroni correction). These results agree with Schoonheim et al., (2012), who found a larger volume reduction in caudate, pallidum and thalamus in male MS patients compared to females in a RRMS cohort with 7.5 and 7.6 years of disease duration, respectively. Bermel et al., (2003), also found a significantly lower caudate volume in an MS cohort with mean of 11.5 years of disease duration, but they did not study sex differences. Although we did not find a sex effect concerning the atrophy of putamen in our cohort, a larger atrophy of basal ganglia in males than females is suspected at later stages of the disease, as a consequence of the ongoing stronger neurodegeneration in men during the second phase of disease progression. The mechanisms that have been described so far as the cause of larger deep gray matter

atrophy and cognitive dysfunction in males compared to females were strongly linked to the neurodegenerative effect of low testosterone and the protective effect of estrogen (Gosselin and Rivest, 2011; Voskuhl and Gold, 2012). Finally, we found a larger amygdala atrophy in males compared to females at less than 5 years of disease duration. This early atrophy of amygdala has only been described by Bergsland et al., (2012), in a cohort of RRMS patients with 3.9 years of disease duration. Although sex differences were not investigated, the atrophy of the amygdala was the only structure that correlated with disease duration. The early atrophy of amygdala was also explained by the two-step model, where a subclinical inflammation of the amygdala prevails, and at the time of MS diagnosis it is already going through the neurodegenerative phase. The role of the amygdala, according to studies, is important in processing information concerning the eye regions of stimuli with faces, and particularly in making judgments about the direction of gaze from eyes (Adolphs and Tranel, 2003; Gamer and Buchel, 2009). Therefore, it plays a major role in social cognition through decoding stimuli and associating them with their emotional and social significance (Batista et al., 2017). MS male patients have proven to have worse social functioning and emotional well-being than females (Casetta et al., 2009). The early atrophy of the amygdala could be responsible for these results.

6.- LIMITATIONS

Some limitations in our study should be acknowledged. The main one is the small sample size which did not allow for a very high statistical power. However, several sex differences were still detected. Another important limitation was the relatively short observation time for the longitudinal analysis, which did not allow us to detect possible significant differences between sexes in the EDSS score, T2 lesion burden and some SDGM. On the other hand, it is important to investigate sex differences in a short time span, as the detection of early and rapid sex-driven changes that impact disease course and prognosis should be prioritized to identify the susceptible population. Furthermore, due to the rather small cohort, we did not consider other aspects such as age at disease onset, treatment variations and relapsing rates, that may also play an important role in the measured outcomes. Currently, more MRI and clinical data are being collected and analyzed to address this issue. Another limitation is that the EDSS scoring has a relatively high inter- and intra-rater variability. In our sample, the EDSS score from patients was not calculated by the same physician; however, all examiners were experienced and worked at the same research center. This allows for certain standardization in the process of EDSS score assessment. Finally, we did not subclassify the localization of T2 lesions in the brain. Therefore, we cannot discuss whether the inflammation of gray matter or white matter lesions drive SDGM atrophy. Similarly, we did not assess lesions in the spinal cord, which also play a major role concerning clinical disability. In our analyses, the incorporation of spinal cord lesions could reveal a stronger sex-related effect, especially in our longitudinal study section. This is being currently addressed at our research center, to provide a wider scope of sex differences in MRI data from patients with MS.

7.- CONCLUSION

Our results demonstrated that male sex was associated with more clinical disability and more T2 lesion volume at early stages of MS when compared to female sex. Men had more atrophy in some subcortical deep gray matter structures in comparison to women.

Our clinical and MRI findings support the hypothesis that men have a much more aggressive and destructive inflammatory response at early stages of the disease, which leads to stronger clinical impairment and larger lesion burden. Furthermore, males showed more atrophy than females in the thalamus, caudate nucleus, pallidum and amygdala at different time points of MS disease duration. For the thalamus, this atrophy could be identified longitudinally even in a relatively short time span of about 1.5 years. Moreover, we found a significant association between T2 lesion volume and atrophy of SDGM structures.

Our results encourage further investigations of sex effect on MS to dissect the complex underlying immune response at different stages of the disease. If it is true that men start by having a much more aggressive and destructive inflammatory response, a main concern may be to reduce it in a more effective way early on. A strategy to achieve this could be the use of recombinant monoclonal antibodies advised for active-relapsing or progressing types as a first line treatment in men, as they have proven to reduce the annualized relapse rate and the probability of disability progression (Alroughani et al., 2019). Studies that evaluate unwanted effects, safety and effectiveness are essential for these treatment modifications. Additionally, more information about the source, physiopathology and prevention of male susceptibility to SDGM atrophy is required. The development of neuroprotective therapies to counteract the intrinsic mechanisms of atrophy, especially in males at early stages of the disease should be considered.

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9.- APPENDIX

Statutory Declaration

"I, Brenda Carolina Nájera Chávez, by personally signing this document in lieu of an oath, hereby affirm that I prepared the submitted dissertation on the topic:

Sex related differences in clinical disability, MRI lesion load and atrophy of subcortical deep grey matter in patients with multiple sclerosis

Geschlechterspezifische Unterschiede in klinischer Beeinträchtigung, MRT-Läsionslast und Atrophie von subkortikaler grauer Hirnsubstanz bei Patienten mit Multipler Sklerose

independently and without the support of third parties, and that I used no other sources and aids than those stated.
All parts which are based on the publications or presentations of other authors, either in letter or in spirit, are specified as such in accordance with the citing guidelines. The sections on methodology (in particular regarding practical work, laboratory regulations, statistical processing) and results (in particular regarding figures, charts and tables) are exclusively my responsibility.

I declare that I have not yet submitted this dissertation in identical or similar form to another Faculty.

The significance of this statutory declaration and the consequences of a false statutory declaration under criminal law (Sections 156, 161 of the German Criminal Code) are known to me."

Date: 29.10.2019

Lebenslauf

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

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