Identification of biomarkers to improve the diagnosis and treatment of Multiple Sclerosis

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List of Acronyms	List of Acronyms
AD = Alzheimer's disease	HER2 = Human epidermal growth factor receptor-2
ALS = Amyotrophic lateral sclerosis	IBD = Inflammatory bowel disease
ALK = Anaplastic lymphoma kinase	IFN = Interferon
APC = Antigen presenting cell	IHC = Immunohistochemistry
AQP4 = Acquaporin-4	IRE1α = Inositol requiring enzyme one alpha
ASD = Autism spectrum disorder	JCV = John Cunningham virus
ASCO = American society of clinical oncology	LFA-1 = Lymphocyte function-associated antigen 1
ATF6 = Activating transcription factor 6	MAG = Myelin associated glycoprotein
BB = Breath biopsy	MET = Hepatocyte growth factor receptor
BD = Bipolar disorder	MHC = Major histocompatibility complex
BG = Basal ganglia	miRNA = Micro RNA
BMI = Body mass index	MRI = Magnetic resonance imaging
CAP = College of American pathologists	MS = Multiple Sclerosis
CIS = Clinical isolated syndrome	NAGM = Normal appearing grey matter
CNS = Central nervous system	NAWM = Normal appearing white matter
CTRL = Controls	NGS = Next-generation sequencing
DAB = Diaminobenzidine tetrachloride	NIST = National Institute of Standards and Technology
DAPI = 4'6-Diamidino-2-Phenylindole	PBMC = Peripheral blood mononuclear cell
DAWM = Diffusely abnormal WM	PBS = Phospho-buffered saline
DBS = Dynamic background subtraction	PCR = Polymerase chain reaction
EBNA = Epstein Barr virus nuclear antigen	PERK = Pancreatic endoplasmic reticulum kinase
EDSS = Expanded Disability Status Scale	PD-L1 = Programmed death ligand-1
EGFR = Epidermal growth factor receptor	PLP = Proteolipid protein, myelin
ELISA = Enzyme-linked immunosorbent assay	PML = Progressive multifocal leukoencephalopathy
ER = Endoplasmic reticulum	PP = Primary progressive
ERAD = ER associated protein degradation	RR = Relapsing remitting
FC = Frontal cortex	SP = Secondary progressive
FFPE = Formalin-fixed paraffin-embedded	TL = Temporal lobe
FGFR = Fibroblast growth factor receptor	TOF-MS = Time-of-flight Mass spectrometry
FISH = In situ hybridization	UPR = Unfolded protein response
GFAP = Glial fibrillary acidic protein	UV = Ultraviolet
GM = Grey matter	VOCs = Volatile organic compounds
GC-MS = Gas chromatography mass spectrometry	VLA-4 = Very late activation antigen 4
GS = Goat serum	WM = White matter

ABSTRACT

Worldwide, 2.5 million of people are affected by multiple sclerosis, most commonly between 20 and 40 years old, with a prevalence among females. The lack of consensus over the causes of this disease is depicted by the presence in the literature of two antithetic visions about the multiple sclerosis etiopathogenesis. This has dramatic consequences on the clinical approach to the disease, which presently can be effective for a restricted percentage of patients, and on the market, currently ruled by immunomodulator drugs. This variability is one of the key points in the research of biomarkers for multiple sclerosis, and in this thesis, conducted at University of Huddersfield and at the Centre for Biomarker Research, two emerging targets are evaluated. The first one, the *XBP1* gene, involved in endoplasmic reticulum stress response, codes two different isoforms that we evaluated through immunohistochemistry in *post-mortem* human brains of multiple sclerosis patients and healthy controls, in three areas: the frontal cortex, the basal ganglia and the temporal lobe. We found that the expression and the ratio of these isoforms are dysregulated between multiple sclerosis patients and controls. The second biomarker candidate investigated, instead, are the volatile organic compounds, collected via breath samples of multiple sclerosis participants and healthy controls. Unfortunately, due to the novel Coronavirus pandemic, the results of this study cannot be analysed further, but the methodology used in order to perform the breath tests was positively evaluated as non-invasive, providing an alternative for the diagnostic assessment of multiple sclerosis.

INTRODUCTION

1.1 PATHOLOGY OF MS

Multiple Sclerosis (MS) is a chronic immune-mediated demyelinating disease of the central nervous system (CNS), which most commonly affects young adults between 20 and 40 years old (Calabrese et al., 2015). The main target of this disease is the myelin sheath, the membrane that covers neurons and which biological functions are electrical insulation, allowing a faster signal transduction, protection, compaction, maintenance and development of the CNS and the peripheral nervous system (Greenfield et al., 2006). The myelination process happens with the involvement of myelin-specific proteins: the myelinating glial cell establishes its contact with axons by covering it with its glial plasma membrane for a number of times (Han, Myllykoski, Ruskamo, Wang, & Kursula, 2013). When the myelin sheath is damaged or removed, the conduction of impulses along nerve fibres slows down or fails, leading to an impairment of brain functionality; in the case of MS this is clinically related with motor and cognitive impairments (Pronk et al., 2008).

The distinctive features of MS are the lesions where the focal demyelination occurs, accompanied by infiltrations of the immune system and glial activation (Awal et al., 2018). These lesions are disseminated in space and time at the CNS level. In particular, the most studied are those of the white matter (WM) because they are easier to examine and visualize by magnetic resonance imaging (Fig.1.1).

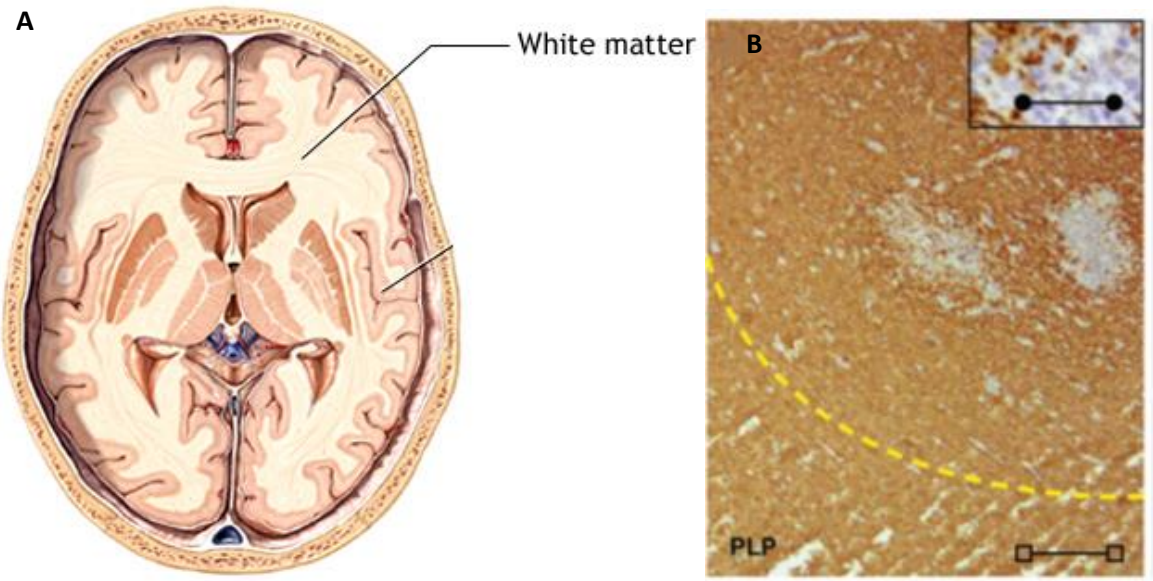


Figure 1.1 Representation of the white matter.

(A) Image of WM in the brain (ADAM Health, 2020). (B) WM portion visible by PLP (proteolipid protein,myelin) staining and WM lesion highlighted by the lack of stained PLP in the area delineated by the yellow dotted lines Image scale bars: 50 μm (Dunham et al., 2017).

The WM is a portion of the brain organized in nerve fibres and myelin (Fields, 2008). The normal appearing white matter (NAWM), instead, is defined as a portion of the brain in which the myelin appears normal and no inflammatory factors are present (Bramwell & Jones, 1941). The grey matter (GM) contains the same connections of the WM but lesser in number, it is mostly characterized by cell bodies, dendrites and axon terminals of neurons and even if lesions are localised there, they are hard to visualize through magnetic resonance imaging, so most of the studies have been performed through neuropathological techniques on autoptic material (Fig.1.2) (Ganepola et al., 2018).

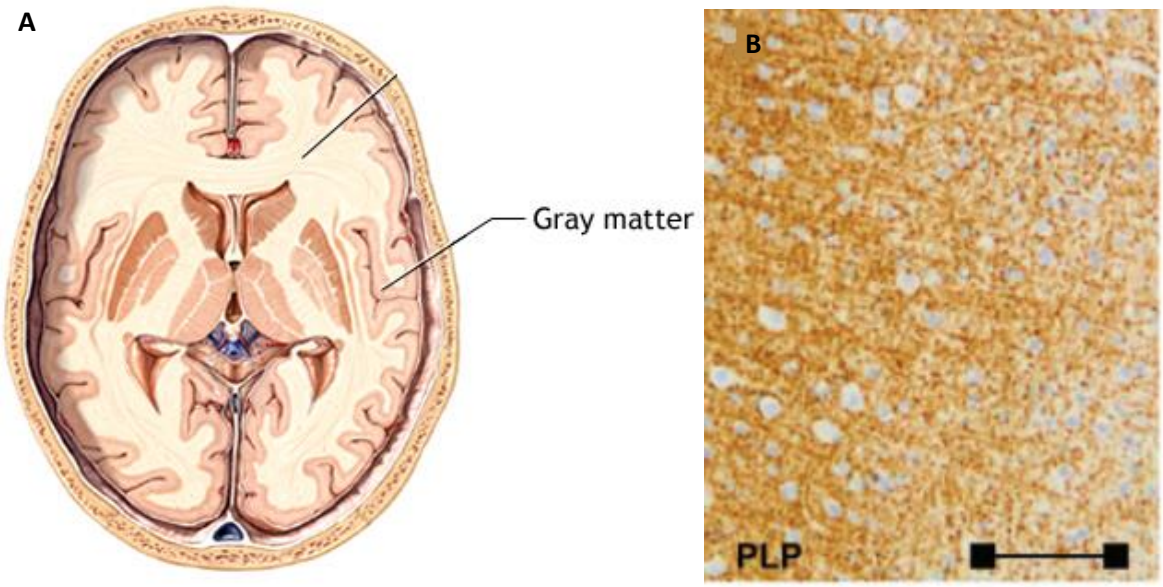


Figure 1.2 Representation of the grey matter.

(A) Image of GM in the brain (ADAM Health Solutions). (B) GM portion visible by PLP staining Image scale bars: 100 µm (Dunham et al., 2017).

The lesions show differences between grey and white matter not only according to the morphology and the position, but also to the degree of lymphocyte infiltration, complement deposition, and blood–brain barrier disruption, which are not present in GM lesions, showing inflammatory features just in the WM (Geurts, Stys, Minagar, Amor, & Zivadinov, 2009). Despite being known for more than a century, at the moment there is no consensus over the causes of this debilitating disorder, and consequently, the pharmacological strategies are simply aiming to reduce the immunological and inflammatory symptoms of the patients (Brotman & Jaffer, 2004).

1.2 CLINICAL FORMS OF MS

There are several classifying criteria developed in order to diagnose the different types of MS forms. The first ones appeared are the Schumacher Criteria (Schumacher et al., 1965) that were based on clinical findings. Then, in 1983, thanks to the presence of diagnostic tests such as evoked potentials, the Poser criteria made their appearance (Milo & Miller, 2014). The most famous diagnostic criteria are undoubtedly the McDonald criteria, established in 2001, that use imaging in order to show lesions in the CNS in space and time (Polman et al., 2011). Since these criteria seem to oversimplify the diagnosis of the disease (Milo & Miller, 2014), they have been reviewed during the years in order to include a variety of symptoms and make use of the state of the art technology . As there is still a lot of debate regarding the criteria that should be used for diagnosis, the Lublin and Reingold classification so far seems to be the only classification that explains the clinical courses of MS, grouping them in the following forms: *Relapsing Remitting* (RR), *Primary Progressive* (PP) and *Secondary Progressive* (SP) MS.

1) RR is characterized by the presence of clearly defined inflammatory recurrences with complete recovery or with after effects and deficit residues in the recovery. Periods of recurrence are characterized by absence of disease progression (Fig.1.3). This form represents the most frequent course of the disease, in about 85% of cases (Lublin & Reingold, 1996).

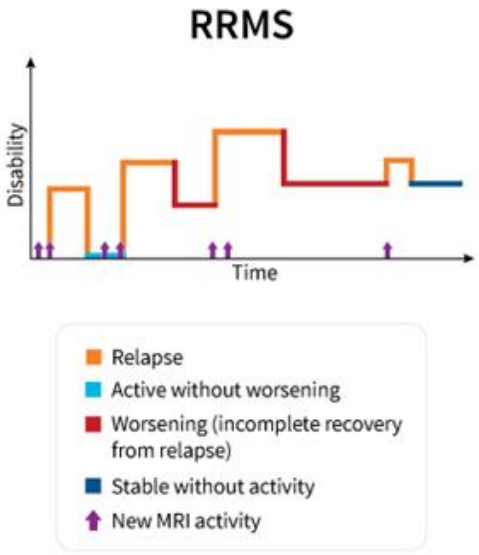


Figure 1.3 Clinical course of Relapsing-Remitting multiple sclerosis.
There could be present acute attacks with full recovery or residual deficits after recovery (Lublin et al., 2014).

2) The essential element in PP is a gradual and continuous worsening of the clinical condition with no distinct relapse but with occasional *plateaus* (Fig.1.4). This form is the rarest course of the disease, around 15% of cases (Polman et al., 2011).

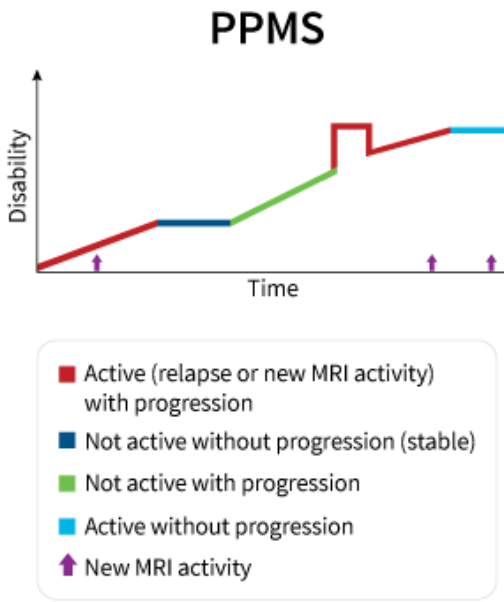


Figure 1.4 Clinical course of Primary-Progressive multiple sclerosis.

There could be progression of disability from onset, without plateaus or remissions; or progression of disability from onset, with occasional plateaus (Lublin et al., 2014).

3) SP is characterized by an initial course of the disease such as the RR form followed by progression with or without occasional recurrences, minor remissions, and *plateaus* (Fig.1.5). About 80% of RR forms progress to SP in 10-15 years from the onset (Polman et al., 2011).

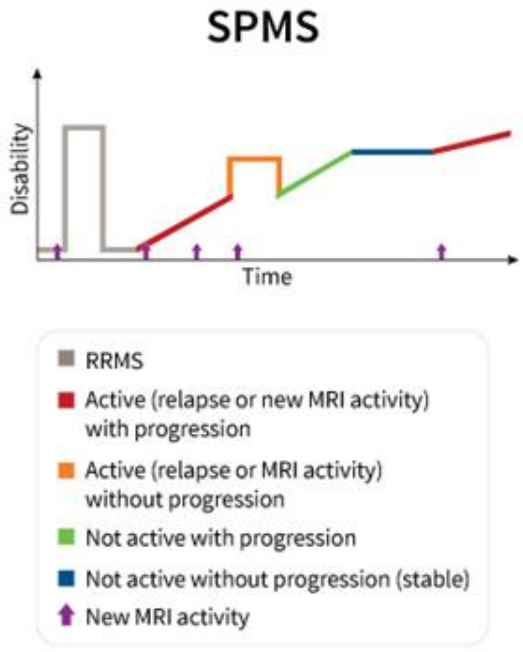


Figure 1.5 Clinical course of Secondary-Progressive multiple sclerosis.

There is an initial RR course, followed by a variable progression or by occasional relapses and remissions (Cook et al., 2010; Lublin et al., 2014).

1.3 AETIOLOGY OF MS

As previously mentioned, the pathological mechanisms that underlie the aetiology of MS are not yet fully understood. The lack of consensus over the causes for MS is depicted by the presence in the literature of two antithetic concepts about the MS aetiopathogenesis. The first and the most prevalent in the literature states that a faulty immunological attack, driven by CD4+ T lymphocytes, leads to myelin destruction and consequently cytodegeneration (Holmoy & Hestvik, 2008). This identifies the autoimmune response as the cause for MS and was named "*outside-in"* theory. In contrast, an alternative hypothesis explaining the aetiology of MS, was named then "*inside-out"* theory. This theory suggests that cytodegeneration leads to autoimmunity, supporting the idea that the immune system is a secondary player that responds to a disrupted brain function (Fig.1.6) (Stys, Zamponi, van Minnen, & Geurts, 2012).

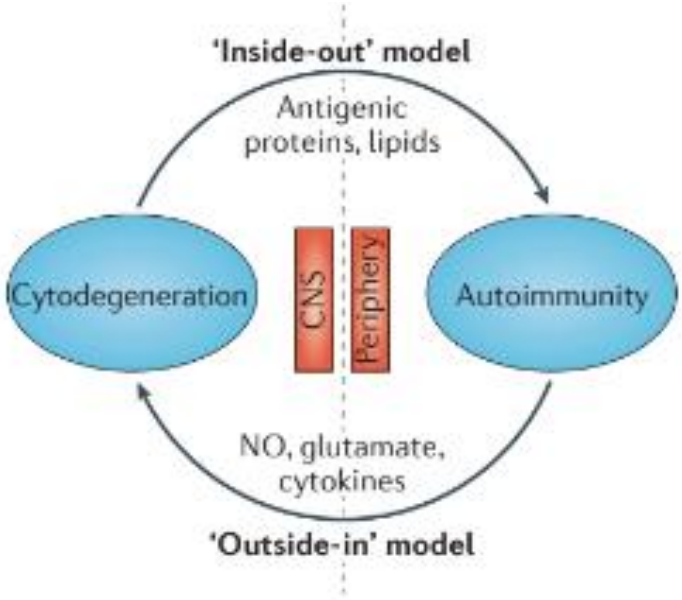


Figure 1.1 Scheme of "*inside-out/outside-in*" models.
According to the "*outside-in"* model, MS is an autoimmune disease that causes demyelination and tissue injury in CNS. The "*inside-out"* one instead, states that the early dysregulation occurs in the CNS, probably on the oligodendrocyte–myelin complex, and by releasing antigenic proteins, drives a second phase in which the autoimmune response is triggered in the predisposed subject (Stys et al., 2012).

OUTSIDE-IN THEORY

In the "*outside-in"* theory, the triggering event for the onset of MS occurs when autoreactive CD4+ T cells are activated in the periphery, due to a series of genetic and environmental factors. These cells enhance the immune response through the recruitment of additional pro-inflammatory immune cells (including cytotoxic T cells, B cells, granulocytes, monocytes, dendritic cells and mast cells), which adhere to the endothelium forming part of the blood-brain barrier, and traverse it. After entrance into the CNS, autoreactive CD4+ T cells are reactivated by antigen-presenting cells and release pro-inflammatory cytokines (IFN-gamma, Tumour Necrosis Factor-alpha) and a variety of chemokines. The release of these factors can amplify local inflammation by activating microglia and astrocytes, which can stimulate a myelin attack by macrophages with subsequent demyelination and axonal damage. The activation of B cells, the production of autoantibodies by the plasma cells, and the involvement of complement factors entering the CNS once the inflammation process has been triggered, produce a variety of responses that cause further damage to the CNS, related to myelin destruction and axonal damage (Fig.1.7) (Holmoy & Hestvik, 2008).

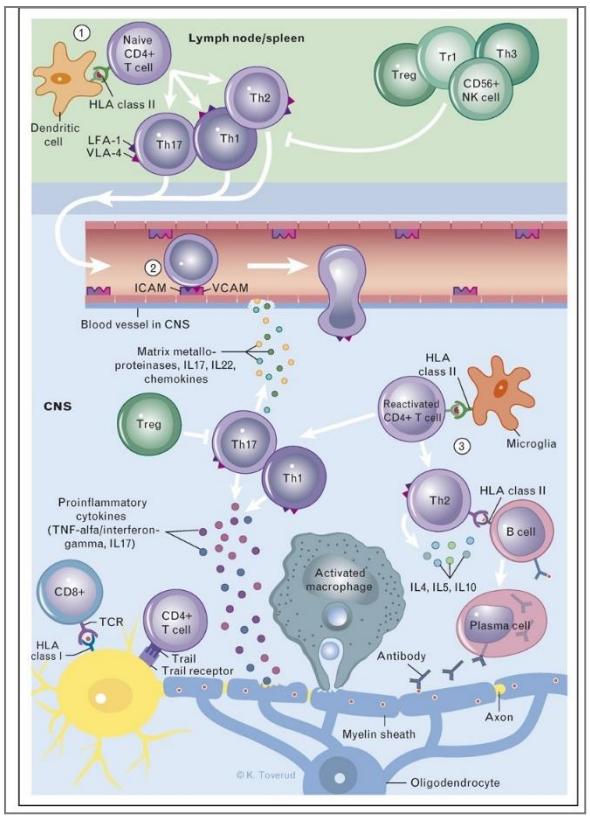


Figure 1.2 Pathogenic mechanisms that intervene in multiple sclerosis.
(1) According to the cytokine environment, the activated CD4+ T cells mature into subtypes of T helper (Th) cells. (2) Activated T cells express the very late activation antigen 4 (VLA-4) and lymphocyte function-associated antigen 1 (LFA-1), in order to cross the blood–brain barrier (Holmoy & Hestvik, 2008).

INSIDE-OUT THEORY

The "*inside-out"* theory rose up in order to explain some phenomena that occur in the disease that couldn't be explained by the "*outside-in"* model.

The first discrepancy occurs in the early stage of the disease: as previously described, myelin sheath and myelin-specific proteins play a key role in MS and, as pathological and immunohistochemical examinations confirm, the disruption occurs in the inner part of axons that are still myelinated. In this process the internal lamellae swell, with the loss of myelin-associated glycoprotein (MAG) and adhesion molecules, while the external part remains intact (Fig.1.8).

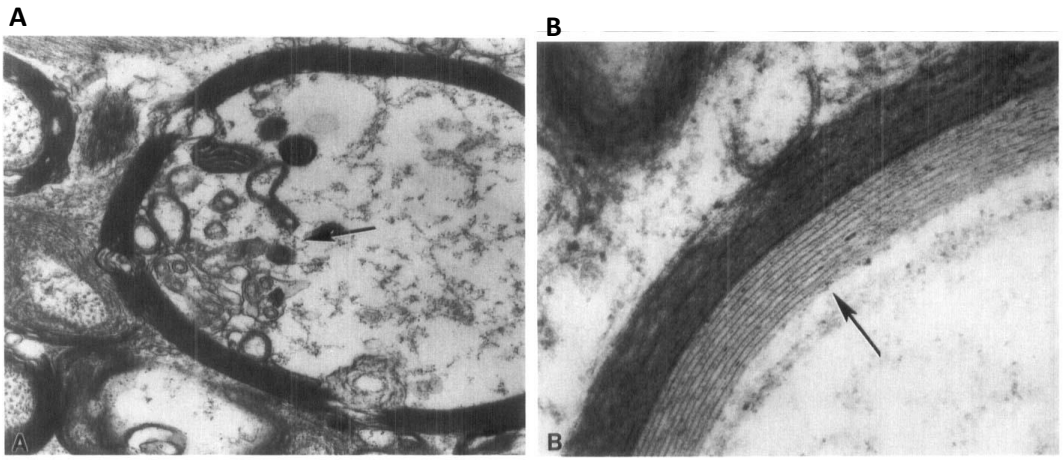


Figure 1.3 Electron microscopy image of an acute demyelinating lesion of multiple sclerosis. (A) The arrow indicates an abnormal oligodendrocyte cell enveloped by an intact myelin sheath. (B) The arrow indicates intact inner myelin lamellae (Rodriguez & Scheithauer, 1994).

How could the first event be the immunological response, as affirmed by the "*outside-in"* hypothesis, if the disruption is in the inner part of myelin sheath? Also, through autopsy material (*post-mortem* brain tissue) from patients in early active stages of MS, it has been discovered the sole presence of macrophages and microglia in areas of demyelination and oligodendrocyte loss, with scarce T and B cell infiltration, meaning that only the innate immune response occurred (Fig.1.9) (Rodriguez & Scheithauer, 1994).

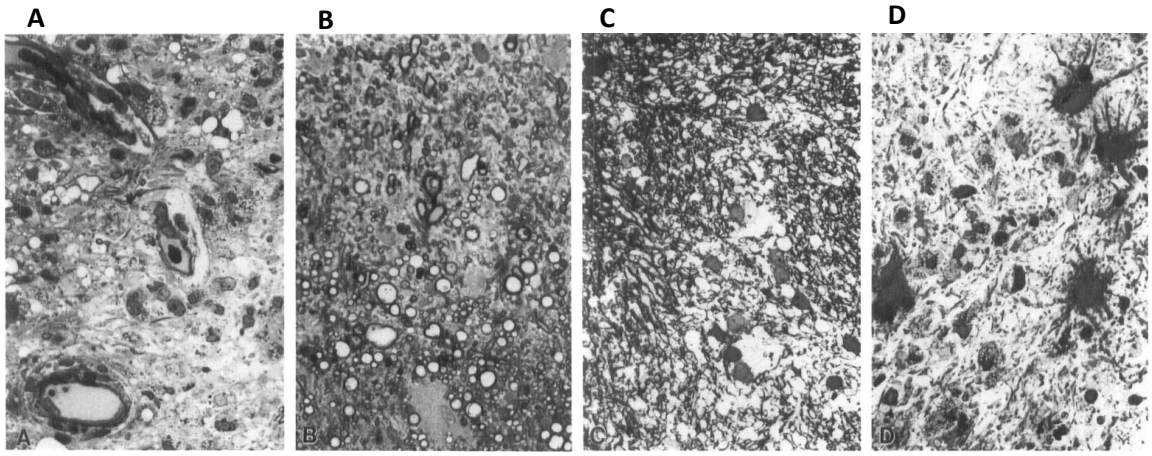


Figure 1.4 Autopsy material from patients in early active stages of multiple sclerosis.
(A) Images of active demyelination with perivascular inflammatory cells, macrophages, myelin debris and demyelinated axons. (B) Area of partial demyelination with demyelinated axons and absence of inflammatory infiltrates. (C) Oligodendrocytes in area of remyelination. (D) Demyelinated lesions with astrocytes and macrophages (Rodriguez & Scheithauer, 1994).

Moreover, in the active cortical MS lesions, a consistent amount of neuritic injury was found to have less inflammatory infiltration relative to the rest of the white matter lesions (Peterson, Bo, Mork, Chang, & Trapp, 2001), which means that the progression of the degenerative process may occur in a different way in the white and grey matter (Fig.1.10).

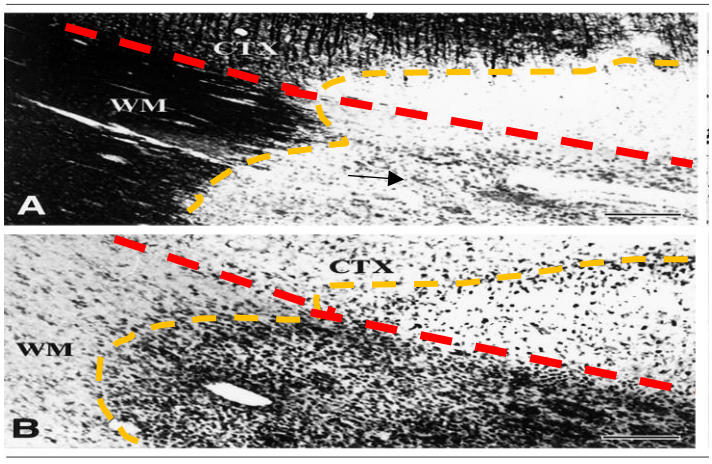


Figure 1.5 Cortical lesions contain less inflammatory cells than white matter lesions.
(A) Myelin staining has been performed in order to identify the WM area and the cortical area (divided by a red dotted line) and the lesion area (delimited area with yellow dots and arrow). (B) Major Histocompatibility Complex class II staining has been performed and shows a higher presence of inflammatory process in the WM lesions rather than the cortical lesions (Peterson et al., 2001).

The second discrepancy is the reduced presence of immune infiltration in the NAWM where there are myelin and axonal losses (Fig.1.11), which shouldn't be the case according to the "*outside-in"* model.(Seewann et al., 2009).

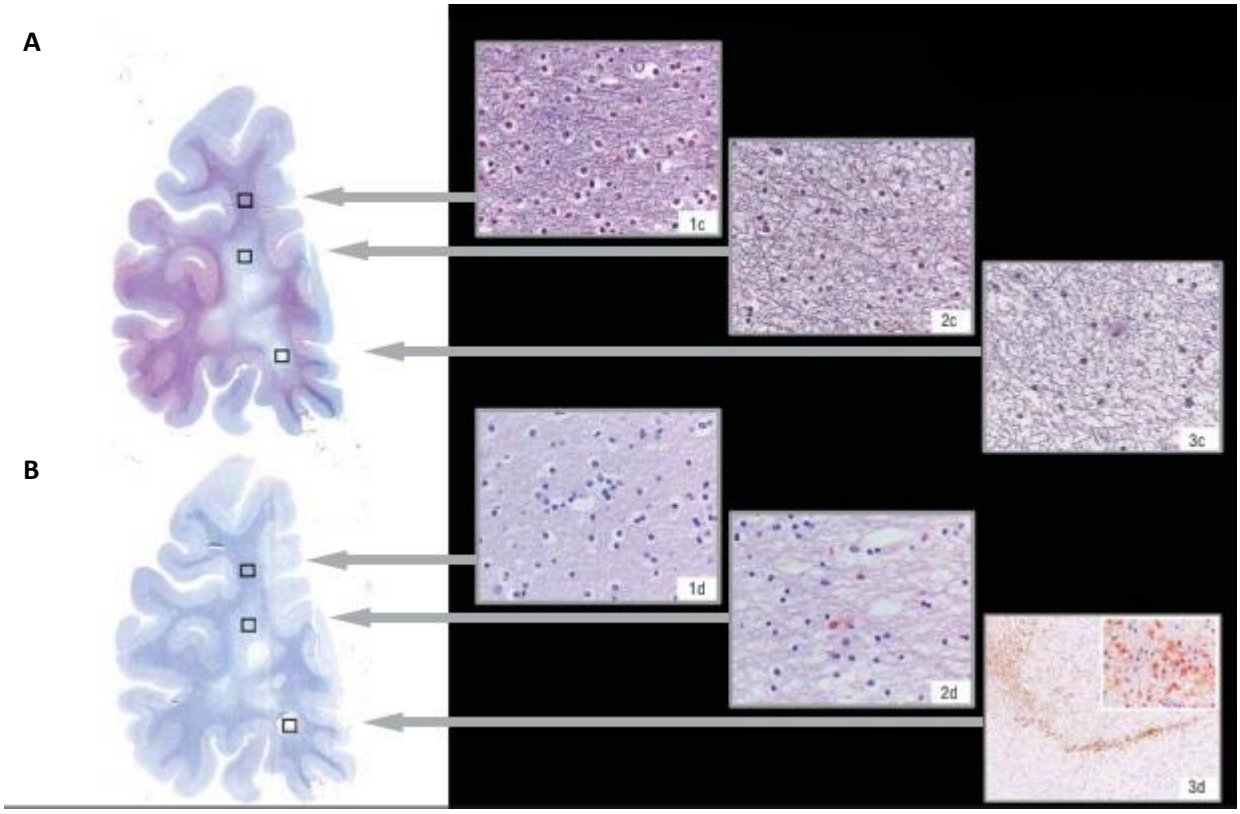


Figure 1.6 Normal appearing white matter of a patient showing myelin and axonal loss but reduced immune infiltration.
(A) Bodian silver staining has been performed in order to show nerve endings and nerve fibre tissue on a MS brain section. (B) HLA-DR (an MHC class II cell surface receptor) immunostaining of the same brain section has been performed in order to highlight the inflammatory process. (1c) NAWM, (2c) diffusely abnormal WM (DAWM), (3c) WM lesions. As can be seen through the bodian silver staining, there is higher axonal loss in 3c and 2c than 1c. (1d) No activated Antigen presenting cells (APC) found in NAWM. (2d) Some activated APCs found in DAWM. (3d) Chronic active lesion with a bulk of activated APCs. Considering both the bodian silver staining and the HLA-DR staining, there is a higher presence of immune infiltration in the NAWM area where there is axonal loss (Seewann et al., 2009).

The third discrepancy is more clinically related. It has been shown that the immune modulator drugs that are administered to RRMS patients are highly effective at reducing either relapses or inflammation, but the same compounds are ineffective in PPMS, since, as previously described, the onset of the disease is not inflammatory-related (Hawker, 2011). Another clinical approach is the autologous hematopoietic stem cells transplantation, this has been found to be very effective in reducing the inflammatory activity in the CNS, but it does not stop the progression of the demyelination and axonal degeneration (Lu et al., 2010). Why do these treatments fail in blocking the degeneration if the "*outside-in"* theory is correct? There are

arguments stating that the degeneration, at a certain stage of the disease, becomes independent from the "primary" inflammation (Brotman, Jaffer, Hurbanek, & Morra, 2004). In support to the latter, the patients that are treated with the drug Alemtuzumab (a monoclonal antibody directed against CD52, which is expressed on lymphocytes) that show a higher inflammatory profile at the beginning of the disease, continue to accumulate disability more rapidly despite the treatment therapy. However, the immunosuppressing activity of this drug can fail to remove pathogenic CNS-resident immune cells, leading to a continuous degeneration process (Coles et al., 1999). As such, the observations can also be explained with the "*inside-out"* theory, in which a highly aggressive primary degenerative process causes the release of high levels of autoantigens, which cause a secondary inflammatory reaction. In this way, even if there is the suppression of the inflammatory reaction, the degeneration will still be present. Another clinical observation is the difference in the progression of the MS disease, in which the relapsing inflammatory activity is separated from the primary progressive course (Scalfari et al., 2010), which means that in these cases the inflammation cannot lead to the degeneration.

The last discrepancy relates to the genetic profile of the disease. The largest genome-wide association study on the subject affirms that the *major histocompatibility complex II* is the key player to develop MS in addition to other genes that are involved in T-cell functions. Going deeper in this study it was found that 90% of the patients studied were RRMS, which is consistent with the strong involvement of immune related genes. The interesting finding is that the remaining 10%, composed of PPMS, shows robust associations only with four genes, all of them unrelated to the immune system (International Multiple Sclerosis Genetics et al., 2011). This study confirms the relation between the disease and the immune reactions but, obviously, it does not discern which is the primary mechanism.

1.4 DIAGNOSIS OF MS

Currently there are no diagnostic laboratory tests to assess MS (Venkateswaran et al., 2010). The current procedure involves evaluating patients through neurological examination, blood test, MRI (Magnetic Resonance Imaging) scan, lumbar puncture and evoked potential tests according to MS diagnostic criteria: the McDonald criteria (McDonald et al., 2001). During the neurological examination physical changes, weakness, vision and motor coordination, reflexes, balance and speech are tested in order to see whether there are nerve damages (NationalMSsociety, 2020). The blood test is performed to exclude other diseases that share similar symptoms with MS such as neuromyelitis optica, characterised by the presence of aquaporin-4 (AQP4) antibodies. The MRI scan is required in order to see whether lesions are present in the brain or in the spinal cord and can also be used to confirm if a lesion has occurred at two different places during the time (Broza et al., 2017). The most invasive procedure undertaken in order to confirm MS diagnosis is the lumbar puncture. The cerebrospinal fluid is taken from spinal canal in order to see the production of antibodies and the presence of oligoclonal bands (Diplock, Sullivan, Jaffer, & Opps, 2004). An emerging examination is the evoked potential tests, in which the recording of electrical signals, produced by the nervous system in response to stimuli (that can be visual or electrical), allows to assess the speed of the nerve signalling between neurons. This information is an indirect one of myelin status, the membrane responsible for saltatory conduction of impulse, which is disrupted in MS disease (Mayoclinic, 2020). The diagnosis of MS is assessed from the evidence of lesions in at least two separate areas of the brain or spinal cord, and disseminated in time, together with the exclusion of other possible disease that could share MS patterns (Omerhoca, Akkas, & Icen, 2018). The assessment of MS types (RRMS, PPMS, SPMS) is based on symptoms and the presences of relapses, remissions, clinical degeneration (NHS, 2020) and, according to the McDonald criteria, on the dissemination of lesions in space and time visible through MRI scan. The exact selection of MS type, unfortunately, can be assessed only during time due to the variability of symptoms and to the progression of the disease.

1.5 CLASSIFICATION OF MS BIOMARKERS

According to the Collins dictionary, the definition of biological biomarker is “a substance, physiological characteristic, gene, etc that indicates, or may indicate, the presence of disease, a physiological abnormality or a psychological condition” (CollinsEnglishDictionary, 2020). The entity of a biomarker, which in laboratory routine is commonly identified as a molecule, whether this could be a protein, a vesicle, or a compound, is an element of distinction between a healthy person and a sick subject. Another even broader definition considers also what affects a disease, so not only the presence of the difference, but also the elements that contribute to the onset of the disease, such as chemicals or nutrients (Strimbu & Tavel, 2010).

For MS it is important to have: predictive biomarkers that help to determine the onset of the disease before it occurs, or at an early stage, and help with the planning of future treatments; diagnostic biomarkers to assess MS diagnosis; disease activity biomarkers that can distinguish the different forms of MS disease; treatment-response biomarkers to help determine the efficiency of the response to drug therapies (Paul, Comabella, & Gandhi, 2019).

PREDICTIVE BIOMARKERS

Among the predictive biomarkers there are the genetics biomarkers and the potentially risk genes. On the first category (the genetics biomarkers) there is no evidence for a specific gene that causes MS, but the presence of antibodies against Epstein–Barr virus nuclear antigens (anti-EBNA) in blood seems to correlate with MS disease (Sundstrom et al., 2004). Nevertheless, the probability of developing MS with a family history of the disease is about: 1.5% (1 in 67) if one parent has MS; 2.7% (1 in 37), if a sibling has MS; 20% (1 in 5), if an identical twin as MS (Sadovnick, Ebers, Dyment, & Risch, 1996). In the second category (the potentially risk genes) there are some genes that make a subject more likely to develop it, such as the ones that have a role in the immune system. Some of these genes are related to other autoimmune diseases such as Crohn’s disease and rheumatoid arthritis (Zuvich, McCauley, Pericak-Vance, & Haines, 2009), while other genes have a connection with the lower levels of vitamin D. In 2016, the correlation between body mass index (BMI) and MS emerged, identifying that people who are genetically predisposed to have a higher BMI are more likely to develop MS (Gianfrancesco et al., 2017).

DIAGNOSTIC BIOMARKERS

The diagnostic biomarkers, or exploratory biomarkers, are used to assess MS disease. In this field, the biomarkers able to determine Clinical isolated syndrome (CIS) are investigated, since CIS is a form of MS considered to be the first step into the disease, due to the presence of a single lesion (instead of the minimum two lesions required to confirm MS according to the McDonald criteria). The majority of the studies are focused on diagnostic biomarkers, as can be seen in Fig.1.12, and can be divided in three sub categories: the exploratory biomarkers, in which there is still a base research approach, the validated ones, that are confirmed by a large majority of laboratories worldwide, and the clinically used (Paul et al., 2019).

Exploratory biomarkers	Validated biomarkers	Clinically useful biomarkers
Cytokines (D, DA, IFN-β-R, GA-R)	Anti-EBNA (P, D, DA)	Anti-NZ (NZ-R)
Adhesion molecules (D, DA, IFN-β-R, NZ-R)	KFLC (D)	NAbs (IFN-β-R)
Chemokines and receptors (D, DA, IFN-β-R)	IGM OB (D, DA, IFN-β-R, NZ-R)	IgG OB (D)
MMPs and inhibitors (D, DA, IFN-β-R)	NCAM1 (D, DA)	IgG index (D)
Proteomics (D, DA, IFN-β-R)	NO metabolites (D, DA)	Anti-AQP4 (D)
Cystatin C (D)	MMP9 (D, DA, IFN-β-R)	Anti-JC virus (NZ-R)[a]
microRNA (D, DA, GA-R)	MBP (D, DA)	Anti-VZV (F-R)
C31/C4b (D, DA)	MMP9 (D, DA, IFN-β-R)	
sCD146 (DA)	MBP (D, DA)	
sCD14 (D, DA)	SPP1 (D, DA)	
sHLA I and sHLA II (D, DA, IFN-β-R)	CXCL13 (D, DA)	
sHLA-G (D)	GFAP (D, DA)	
sNogo-A (D, DA)	BDNF (D, DA, IFN-β-R, GA-R)	
Anti-Nogo-A (D, DA)	KCNJ10 (D)	
Anti-MBP (D, DA)	MRZ reaction (D, DA)	
Anti-MOG (D, DA)	CHI3L1 (D, DA, NZ-R)	
Anti-HHV6 (DA)	Complement factor H (DA)	
Anti-proteasome (D)	Type I IFNs (DA, IFN-β-R)	
Anti-CD46 and anti-CD59 (DA)	GPC5 (IFN-β-R)	
Lipocalin 2 (DA)	HLA-DRB1*04:01, HLA-DRB*04:08 (IFN-β-R)	
VEGFA (DA)	IL-17 (D, DA)	
AMCase and Chit (D,DA)	BAFF (D, DA, IFN-β-R)	
Fetuin-A (D, DA, NZ-R)	TNF, IL-12, IL-23 (D, DA)	
APRIL (DA)	GWAS genes (P, D)	
CSF cells (D, DA)	NEFH (DA)	
S/GPL (P, D)	NEFL (D, DA, NZ-R)	
HMGB1 (D)	25(OH) vit D (P, D, DA, IFN-β-R)	
TOB1 (D)	$CD56^{bright}$ NK cells (DC-R, IFN-β-R)	
S100B and ferritin (D, DA)		
Isoprostanes (P, D, DA)		
Oxysterols (D, DA)		
Pentosidine (D, DA)		
Tau (D, DA)		
14-3-3 (D, DA)		
NAA and NSE (D, DA)		
Anti-TUb and b-TUb (D, DA)		
Anti-NEFL (DA)		
Neurotrophic factor (D, DA)		
Tregs (DA)		
KCNK5 (D, DA)		
FGF2 and PDGF-AA (DA)		
gMS classifier 1 (D, DA)		
Myeloid MVs (D, DA)		
sAPP, Aβ peptides (D, DA)		
Apoptosis-related molecules (D, DA, IFN-β-R)		
Cosignaling molecules (DA, IFN-β-R)		
GWAS genes (IFN-β-R)		
Candidate genes (IFN-β-R, GA-R)		

Figure 1.12 Diagnostic biomarkers currently used (Paul et al., 2019).
P, Predictive biomarker; D, diagnostic biomarker; DA, disease activity biomarker; AL-R, alemtuzumab-response biomarker; DC-R, daclizumab-response biomarker; F-R, fingolimod-response biomarker; GA-R, glatiramer-acetate-response biomarker; IFN-β-R, interferon β response biomarker; MT-R, mitoxantrone-response biomarker; NZ-R, natalizumab-response biomarker; MMP, matrix metalloprotease; C3/C4b, complement components C3 and C4b; sCD146, soluble CD146; sCD14, soluble CD14; sHLA, soluble human leukocyte antigen; sHLA-G, soluble HLA-G; sNogo-A, soluble Nogo-A; anti-Nogo-A; anti-Nogo-A antibodies; anti-MBP, anti-myelin basic protein; anti-MOG, anti-myelin oligodendrocyte protein; anti-HHV-6, anti-human herpesvirus 6; VEGFA, vascular endothelial growth factor A; AMCase, acid mammalian chitinase; Chit, chitinase 1 (chitotriosidase); TNFSF13 (also known as APRIL), tumor necrosis factor (ligand) superfamily, member 13; S/GPL, sulphatide and glycosphingolipid antibody titers; HMGB1, high mobility group box 1; TOB1, transducer of ERRB2 1; NAA, N-acetyl aspartic acid; NSE, neuron-specific enolase; anti-TUb, anti-tubulin antibodies; β-TUb, β-tubulin isoforms II and III; anti-NEFL, anti-neurofilament light chain antibodies; Tregs, T-regulatory cells; KCNK5, potassium channel subfamily K member 5; FGF2, fibroblast growth factor 2; PDGF-AA, platelet-derived growth factor-AA; gMS classifier 1, anti-Glc(αl,4) Glc(α) IgM antibodies; myeloid MVs, myeloid microvesicles; sAPP, soluble amyloid precursor protein; Aβ peptide, amyloid β peptide; GWAS, genome-wide association study; CIITA, class II transactivator; APLA, antiphospholipid antibodies; ABCB1, ATP-binding cassette, subfamily B, member 1; ABCG2, ATP-binding cassette, subfamily G, member 2; anti-EBNA, antibodies against Epstein–Barr virus nuclear antigens; KFLC, κ-free light chain; OB, oligoclonal bands; NCAM1, neural cell adhesion molecule 1; NO metabolites, nitric oxide and its metabolites, nitrates, and nitrites; SPP1 (also known as osteopontin), secreted phosphoprotein 1; CXCL, CXC ligand 1; GFAP, glial fibrillary acidic protein; BDNF, brain-derived neurotrophic factor; KCNJ10 (also known as KIR4.1), potassium inwardly rectifying channel, subfamily J, member 10; MRZ reaction, intrathecal humoral immune response against measles, rubella, and varicella zoster virus; CHI3L1, chitinase-3-like protein 1; GPC5, glypican-5; BAFF, B-cell activating factor; TNF, tumor necrosis factor; NEFH, human neurofi lament heavy chain; NEFL, human neurofilament light chain; 25(OH) vit D, 25-hydroxyvitamin D; CD56bright NK cells, CD56bright natural killer cells; anti-NZ, anti-natalizumab antibodies; NAbs, neutralizing antibodies; anti-AQP4, anti-aquaporin 4 antibody; anti-JC virus, antibodies against JC virus; anti-VZV, anti-varicella zoster virus antibodies (Paul et al., 2019).

CLINICAL BIOMARKERS

The lower number of biomarkers clinically used is due to the process required to allow a biomarker validated in research studies to become a clinical one. The three characteristics that a clinical biomarker should have are 1) reproducibility, meaning that it can be assessed worldwide, different laboratories have to give the same result on the same person; 2) high sensitivity and specificity, it can be detected with a 100% of confidence and can detect only this specific disease; 3) cost attractive (Katsavos & Anagnostouli, 2013).

DISEASE ACTIVITY BIOMARKERS

To this category belong the biomarkers able to distinguish between the different forms of MS disease. The presence of oxidative stress and inflammation are a peculiarity of the RRMS, while the demyelination and recovery process are characteristics of the PPMS (Lassmann et al. 2007). In clinical practice, the distinction between the two forms is made according to the symptoms, the progression of the disease and MRI scans. An earlier identification of the forms of the disease could allow a better treatment plan, psychological and physical support.

TREATMENT-RESPONSE BIOMARKERS

The personalised treatment in clinical practice is made possible by pharmacokinetics and pharmacodynamics biomarkers. The role of these biomarkers is to predict the failure of the treatment, the dose required, the poor or extensive response to the drugs, and the propensity in developing side effects. One example is the detection of antibodies against John Cunningham virus (JCV) in MS patients that are taking the Natalizumab drug. The presence of these antibodies, in fact, can advise about the degeneration in progressive multifocal leukoencephalopathy (PML), so the need to stop the treatment (Antoniol & Stankoff, 2014).

1.6 TECHNIQUES FOR INVESTIGATION OF BIOMARKERS

As previously mentioned, the main characteristics in order to validate a biomarker in clinical practice are the reproducibility and the specificity. In order to overcome the problems related to the traditional methods for testing biomarkers, the automated assays have been introduced, reducing the variability of the results (Engel, Jaffer, Adkins, Riddle, & Gibson, 2004). However, despite the implementation of these new techniques, the validation of biomarker candidates remains elusive, encountering several issues such as the variation among which the population studied, the eligibility criteria, the timing of sample collection and the variation among the techniques used which include molecular and cellular techniques (Finer, Jaffer, & Santerre, 2004). The reasons for one technique being a preferred choice over another remains to be clarified for several biomarkers (Teixido, Karachaliou, Gonzalez-Cao, Morales-Espinosa, & Rosell, 2015). Among the most used there are PCR (polymerase chain reaction), NGS (next-generation sequencing), FISH (fluorescent *in situ* hybridization), IHC (immunohistochemistry), Western blot, ELISA (enzyme-linked immunosorbent assay), gel electrophoresis. The detection of anaplastic lymphoma kinase (ALK) in a sample is a perfect example to better explain the limitation of these techniques: with the PCR it is possible to detect the ALK rearrangements, but only with known fusion partners and when high-quality RNA is provided, excluding the possibility to extract it from a formalin-fixed paraffin-embedded (FFPE) samples (Ying et al., 2013). NGS is able to detect the ALK rearrangements even in FFPE samples, but the sensitivity varies among the platforms, some can detect genomic breakpoints located in introns, some other can detect the rearrangements only when their breakpoints are adequately covered (Soda et al., 2007). Plus, these advanced techniques are not available in all the laboratories and the results may require weeks in order to be produced, which in a clinical laboratory is a long time for lung cancer patients. FISH is a technique which requires short time to process, but the close proximity of *ALK* gene with other genes may produce false positive results and this in turn leads to incremented costs (Travis et al., 2015). IHC instead is a cost-efficient methodology, but it requires a uniform standard set of guidelines in order to standardize the testing process (Mino-Kenudson, 2017). Efforts in evaluating the strength and weakness of these techniques, together with a standardization of protocols for the performance and the analysis, will widen the possibilities for the clinical biomarkers to be used in clinical practice (Ford et al., 2009). A testing methodology that will explore biomarkers in a non-invasive manner while being specific, sensitive and reliable would be a great advantage.

1.7 XBP1 GENE

The endoplasmic reticulum (ER) is a structure present in the cell whose prevalent function is protein synthesis and transport (Schwarz & Blower, 2016). The malfunction of the ER is strictly related to the neurodegenerative disease's pathways, such as MS, making it worth of attention due to its function. The ER is formed by two parts: the rough ER, called this way due to the presence of ribosomes attached on the surface; and the smooth ER characterized by the absence of the ribosomes (Cubillos-Ruiz et al., 2015). After the mRNA translation into protein, a process that occurs in the rough ER, all the sub sequential post-translational modifications that activate the proteins take place in the same organelle (Back, Schroder, Lee, Zhang, & Kaufman, 2005). Upon accumulation of unfolded proteins, the ER activates a transcriptional pathway called unfolded protein response (UPR). This response has two main effects: on one side it increases the protein folding and on the other it reduces the protein influx as a safety mechanism to reduce ER stress (Bartoszewski et al., 2011). The pathway triggered by UPR cascade involves genes of protein transfer, folding, glycation, proteolysis, lipid biosynthesis, vesicle transport, and oxidation-reduction metabolism (Shanmuganathan et al., 2019). In mammals, this signalling system can be mediated by inositol requiring enzyme one alpha (*IRE1*α), activating transcription factor 6 (ATF6) and pancreatic endoplasmic reticulum kinase (PERK) (Walter & Ron, 2011). All of these genes are sensors present in the ER, but the one which is evolutionarily conserved is *IRE1* (Mori, 2009). In fact, in yeast the activation occurs with direct binding of unfolded proteins to the luminal core regions of *IRE1* (Gardner and Walter, 2011; Kimata et al., 2007), but the same mechanism has not been proved yet in mammal cells (Kohno, 2010). *IRE1* is a Ser/Thr protein kinase that autophosphorylates after unfolded proteins accumulation, and brings to an unconventional splicing in the cytoplasm, activating *XBP1* gene. *XBP1* is a gene that encodes a transcription factor that regulates *MHC* (major histocompatibility complex) class II genes by binding to a promoter element referred to as an X box. The splicing is defined as "unconventional", since the removal of a 26-nt intron from *XBP1u* pre-mRNA, causes a translational frame-shift with a sub sequential replacement of the C-terminal into a new one generating *XBP1s*, which is the functionally active transcription factor (Calfon et al., 2002). In the nucleus, *XBP1s* activates genes encoding ER-resident chaperone molecules that increase the folding of other proteins, such as transcription factors with the bZIP domain, enhances ER associated protein degradation (ERAD) components, responsible to degrade the unfolded proteins, and promotes the differentiation of secretory cells (Sriburi, Jackowski, Mori, & Brewer, 2004).

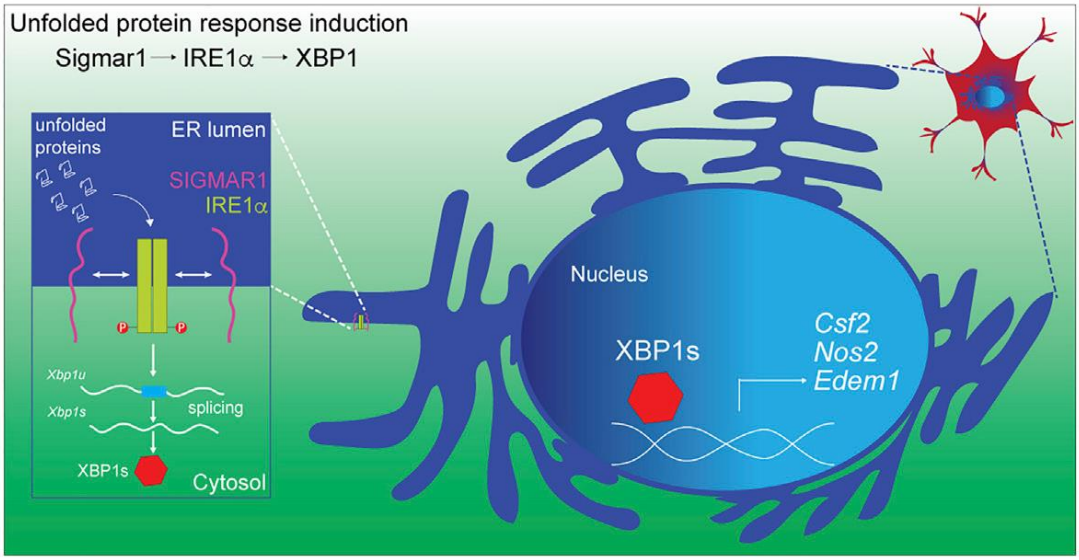


Figure 1.13 XBP1 pathway (Wheeler et al., 2019).
On the left side of the figure, the unfolded proteins response pathway is shown, including how it affects *XBP1* gene regulation, while on the right side an enhanced image of the structure in which the event occurs.

ER stress influences the secretory pathways, the lipid metabolism and the immune response. These changes are a particular hallmark that can be used to explore the causes of neurodegenerative diseases (Yoon et al., 2019). Among these diseases, a recent work has highlighted the correlation between gene mutation in autism spectrum disorders (ASD) and ER stress. The ER-stressed genes expression has been analysed in *post-mortem* middle frontal gyrus of ASD and found an increase in *XBP1* and *IRE* levels. Apart from that, it was found that the higher levels correlate with the diagnostic score for stereotyped ASD behaviour (Crider, Ahmed, & Pillai, 2017). Another neuro-disorder involved in ER-stress is schizophrenia: among the proteins involved in UPR cascade, in dorsolateral prefrontal cortex, there is an increase in *XBP1*s expression, together with the higher ratio of *XBP1*s/u transcripts. Altogether, these results suggest an increase in *IRE1*α splicing in schizophrenic patients, which means an unbalance in cellular stress response (Kim, Scott, & Meador-Woodruff, 2019). Other neurodegenerative diseases correlated with ER-stress are amyotrophic lateral sclerosis (ALS) and Alzheimer's disease (AD), in which the *IRE1α-XBP1* pathway is highly activated, but, particularly in ALS, *XBP1* activation is sustained by an increase of co-chaperone activity and *ERAD* genes (Montibeller & de Belleroche, 2018). Regarding MS, the *XBP1* protein expression was found to be increased both in lesions and NAWM. Furthermore, there is an *XBP1*s incremental expression in NAWM and normal-appearing grey matter (NAGM) in MS astrocytes (Wheeler 2019). Interestingly, in bipolar disorder (BD) the total *XBP1* and XBP1u levels were decreased in peripheral blood. Thus, the decrease could open the possibility that the lower *XBP1u* expression does not allow the restoration of ER stress level, instead it allows

an increase of *XBP1s* levels. Controversially, the XBP1s/u ratio did not differ between BD and controls (Bengesser et al., 2018). However, this does not exclude the possibility to consider *XBP1* gene as predictive or diagnostic biomarker.

1.8 VOLATILE ORGANIC COMPOUNDS

Volatile organic compounds (VOCs) are gaseous molecules that are becoming promising in the clinical biomarkers field as they can be collected in a fast and non-invasive way.

They have been described for the first time in 1971 by Linus Pauling, who identified 250 substances from the breath (Pauling, Robinson, Teranishi, & Cary, 1971). By definition, VOCs include all the organic compounds based on carbon molecules, having a vapour pressure of 0,01 kPa at 293,15 K and a low molecular weight (<1 kDa) (Cicolella, 2008). They are part of the exhaled breath and represent the volatile fraction of metabolites.

They are produced by the human metabolism, and represent the endpoint of gene transcription and protein expression. They can originate either from within the body (endogenous VOCs) or from external sources such as diet, prescription drugs and environmental exposure (exogenous VOCs) (OwlstoneMedical, 2019).

There are three different types of VOCs that can be detected during a breath test: first, the VOCs that arise from metabolic activity in local airways tissue; they are modulated by gene activity as they are directly linked with them, therefore any changes in the genomic pathway will consequentially lead to a different pattern of molecules (Hurbanek, Jaffer, Morra, Karafa, & Brotman, 2004). Second, the VOCs that come from the systemic circulation, as in the lungs there is a direct exchange of molecules between the pulmonary system and the circulatory system. In 1 minute, the VOCs originated from the body periphery reach the lungs and pass from the blood into exhaled breath (Fig. 1.14).

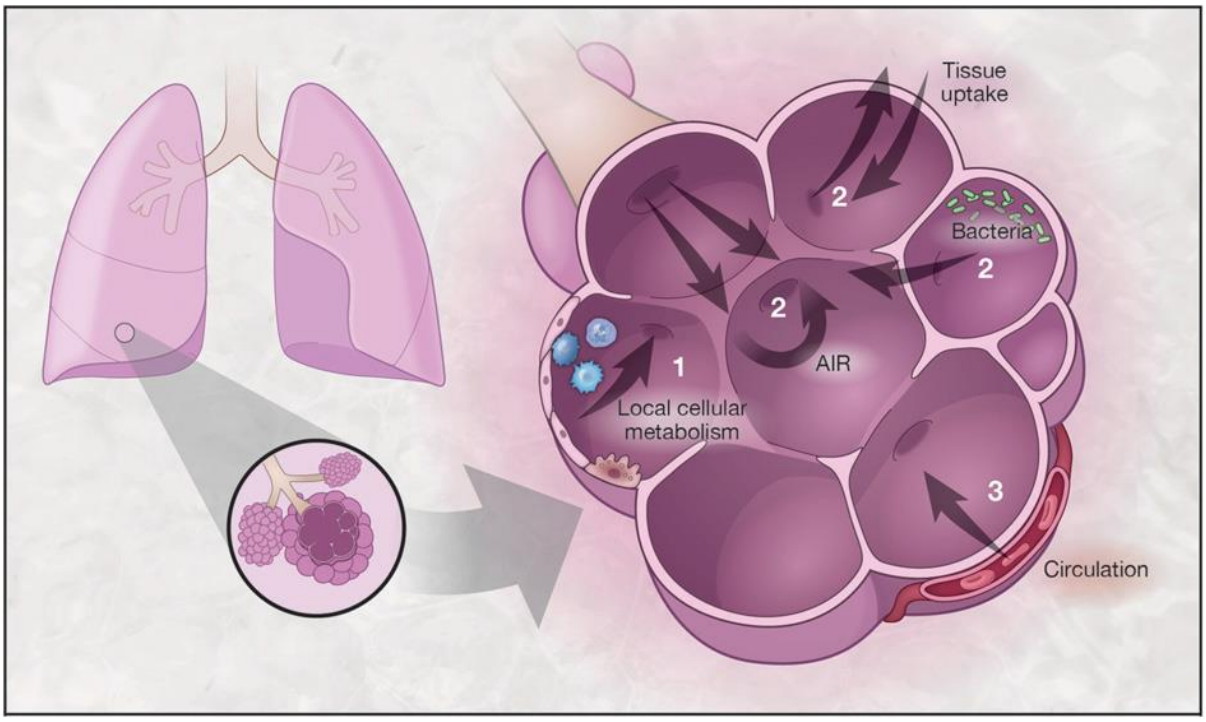


Figure 1.14 Exchange from blood to airways (van der Schee et al., 2015).
In the lungs, the exchange of volatile organic compounds occurs between blood and alveolar air and on into exhaled breath. In the alveolus the sources of the volatile organic compounds are blood, tissue uptake, local metabolism and bacteria.

Third, the VOCs arising from the microbiome or introduced from the environment, diet or prescription drugs metabolites (Boyle & Murphy, 2016). The high specificity rate of VOCs and the rapid and pain-free characteristics of the breath biopsy make them a useful tool for the investigation of diseases, whose need of biomarkers is urgent. One of the first diseases investigated was cancer: the metabolites released in the early stages of the disease, with a particular focus on lung cancer, were used as predictive biomarkers, as they are hallmarks able to distinguish cancer patients from healthy ones (Krilavicbutesiute et al., 2015). Among the inflammatory diseases, the breath biopsy has been used on inflammatory bowel disease (IBD), and it was successful not only in distinguishing IBD patients from healthy controls, but also in differing between the two forms of this disease (Arasaradnam et al., 2016). This powerful tool has also been used in neurodegenerative diseases, such as AD, with the same discrimination capacity between AD patients and healthy controls (Mazzatenta, Pokorski, Sartucci, Domenici, & Di Giulio, 2015). Regarding the MS field, three breath biopsy studies (Ionescu et al., 2011) (Broza et al., 2017) with the usage of gas chromatography mass spectrometry (GC-MS), have proven the different variety of VOCs among MS patients and healthy controls. In the first study, they found that only hexanal and 5-methylundecane levels are higher in MS patients than healthy control. The possible explanation is that these compounds are related to lipid peroxidation, an event that contributes to expand the MS

cascade. They further validated the results via a blind approach, testing all the participants and then assessing them as MS disease patients or as controls thanks to the higher level of these two compounds, showing statistically relevant results (Ionescu et al., 2011). In the second study instead, the number of compounds that were found higher in MS participants amounts to 4: heptadecane, nonanal, decanal and sulphur dioxide. The first three compounds participate in the process of lipid peroxidation, while the latter is involved in the oxidative stress process. Interestingly, only one compound was found more present in healthy controls respect to MS and it's acetophenone, still involved in lipid peroxidation. Again, the predictive model, able to assess the presence of the disease according to the VOCs pattern, generated statistically significant results (Broza et al., 2017). The last study is a Master thesis from University of Huddersfield conducted by Phoebe Tate and Dr. Patrick McHugh at the Centre for Biomarker Research. In this study acetic acid, d-limonene, alpha-pinene and toluene levels were found higher in MS patients compared to controls. With the exception of acetic acid, which is involved in the fermentation process, the other compounds all have exogenous origin. Acetone and methylene chloride levels instead, were reduced in MS participants. Taken together all these results reinforce the idea of employing VOCs and breath biopsy as promising tools, as the procedure is less invasive than lumbar puncture and quicker since it takes 10 minutes to perform it, mainly for the clinical related biomarkers, but with an open possibility on predictive and treatment-response biomarkers.

1.9 PURPOSE OF THE THESIS

Multiple sclerosis is a complex disease, and its etiopathogenesis is still a matter of debate. This has dramatic consequences on the clinical approach to the disease, which presently can be effective for a restricted percentage of patients, and on the market, currently ruled by immunomodulator drugs (Irshad, Veillard, Roux, & Racoceanu, 2014). For the progressive forms of the disease, the most common drugs can help to manage the symptoms, but do not eradicate the disease or prevent the relapses. The need of therapeutic targets for the progressive disease forms is intertwined to the investments in research to understand not only the succession of events that leads to this disease, but also the causes leading to a variability among patients (Karussis, 2014).

This variability is one of the key points in the research of biomarkers for MS, as predictive biomarkers can be used to distinguish the various forms of the disease and help with the planning of future treatments. Taking into consideration the literature behind, in this thesis the *XBP1* gene products will be investigated. The first hypothesis to be tested will be the expression of both isoforms of XBP1 in both MS patients and healthy controls (CTRL) in *post-mortem* human brains in the normal appearing white matter area, in order to see whether there will be an altered ratio between the two XBP1 isoforms and if their presence is peculiar to either disease form. Also, the expression of these proteins will be evaluated in three areas: the frontal cortex (FC), the basal ganglia (BG) and the temporal lobe (TL) in order to explore the propensity of accumulation of both the isoforms. To test this hypothesis, immunohistochemistry stainings for XBP1u and XBP1s will be performed on 8 MS and 6 controls *post-mortem* human brains, and a quantification analysis will evaluate the expression of the proteins.

Another biomarker that will be investigated is a diagnostic biomarker, since the lumbar puncture is a painful procedure to assess the MS disease. As the VOCs are becoming a promising tool, the second hypothesis will be to evaluate the presence of particular compounds only on the MS population, using it as a distinction marker from the healthy controls. In order to test this hypothesis, we will perform a breath sampling among MS participants and healthy controls with RECIVA breath technology. This technology is non-invasive and will allow an identification of breath biomarkers relevant for disease occurring throughout the body, thanks to the collection of both upper and lower airflows.

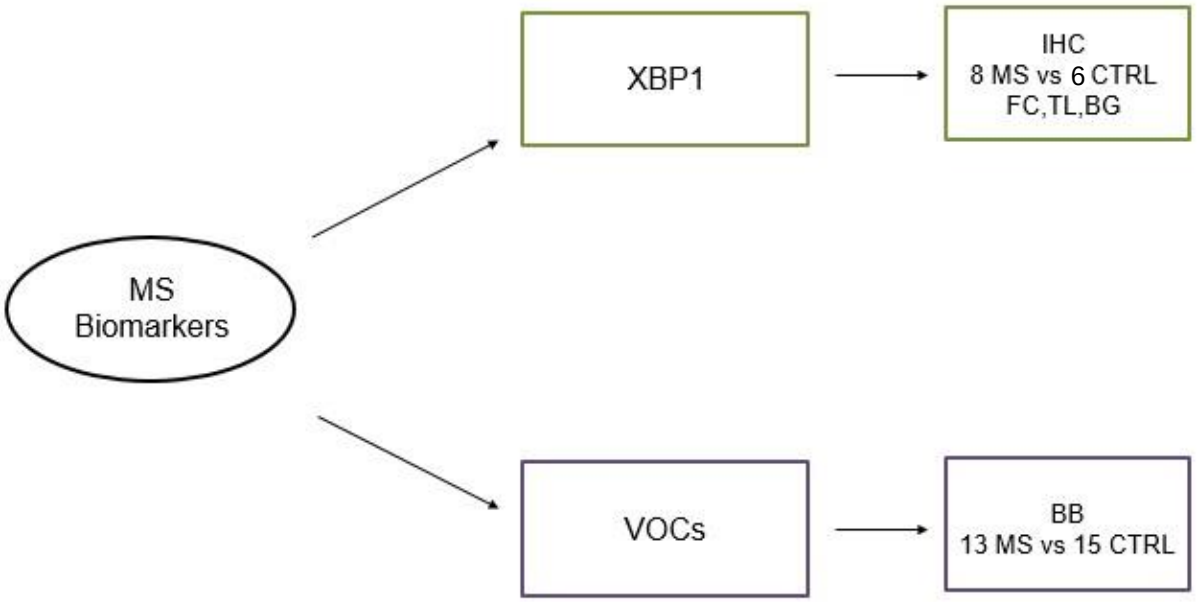


Figure 1.15 Aims and objectives.
The core of this thesis are the multiple sclerosis biomarkers. Among these ones, the focus will be on *XBP1* gene products and volatile organic compounds. The hypothesis will be tested through IHC = immunohistochemistry and BB = breath biopsy.

MATERIALS AND METHODS

2.1 HUMAN BRAIN TISSUE

Formalin-fixed *post-mortem* human brain tissues were used for this thesis. This study was reviewed and approved by the institutional ethics review committee. The MS cases and the controls were selected from the MS Society tissue bank (Imperial College, London), with a collection of 8 MS cases with a higher percentage of NAWM and a lower percentage of lesions. The patients were between 42 and 88 years (Fig.2.1a) old, with a prevalence of females (Fig.2.1b). The controls instead, were a total of 6 cases, ensuring that no patients deceased due to neurological disorders were selected (Fig.2.1c). The cases were between 63 and 84 years old (Fig.2.1a), with equal number of females and males (Fig.2.1b). For both sample pools only the FC, BG and TL of WM areas were selected, since they represent three hallmark spots for MS disease. Both MS patients and controls brain material was fixed in 4% formalin for 1 year, then put in 24% sucrose solution and preserved at -30 °C. Then, slices of 10 µm of thickness were cut with a cryostat.

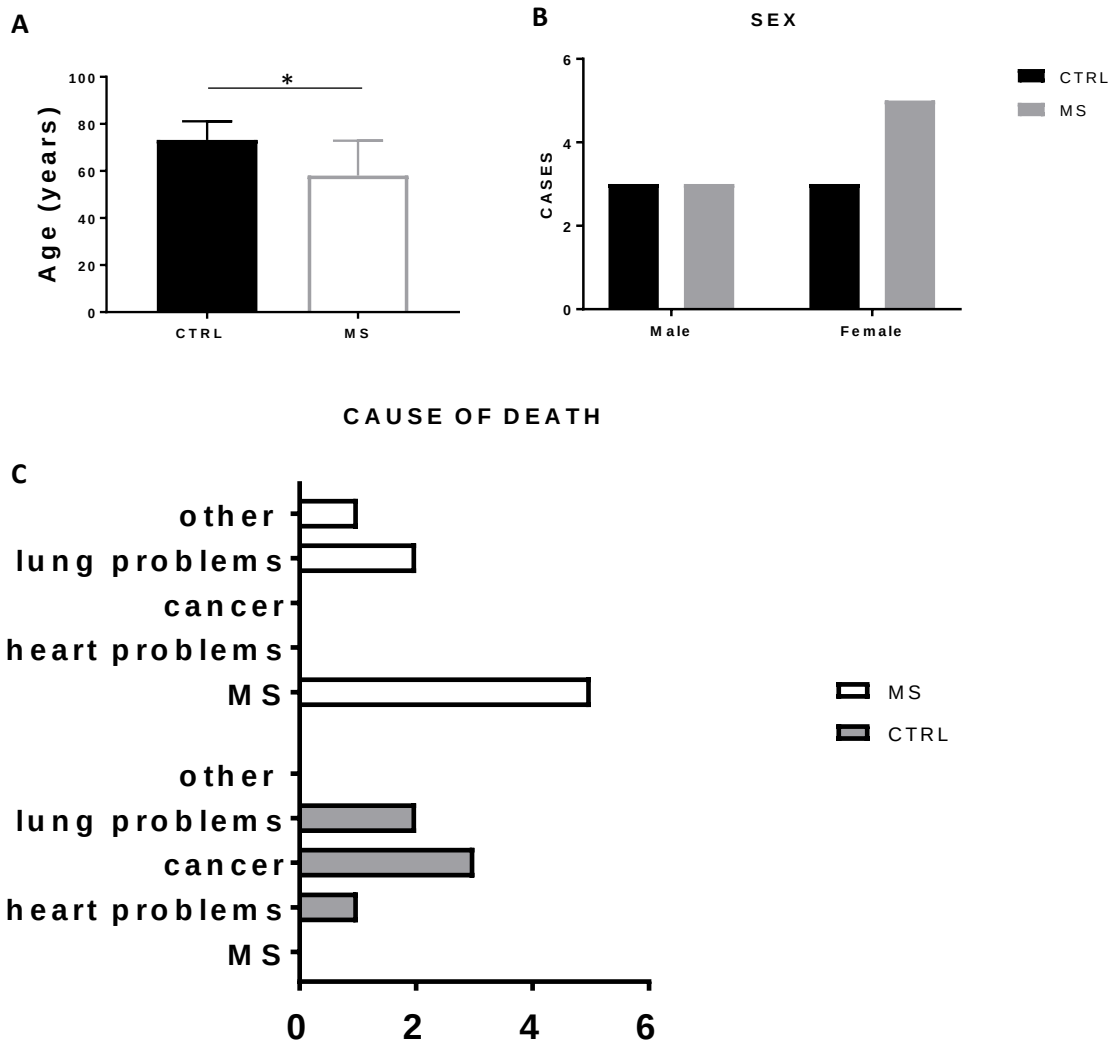


Figure 2.1 Immunohistochemistry cases details.
(A) Age at which the cases died. Mann-Whitney two tailed test p = 0.0413 (B) Representation of the sex of the cases, comprehending 3 male controls and 3 female controls; 3 MS males and 5 MS females. Fisher's exact test p > 0.999 (C) Causes of death, none of them included neurological disorders. Chi-square test.

2.2 IMMUNOISTOCHEMISTRY PLP, XBP1s/u STAINING

Sections were deparaffinised with heat at 55 °C in an oven for 30 minutes; then slides were degreased in two steps xylene 100% for 10 minutes, followed by a scale of ethanol solutions (100%,100%, 90%, 70%) for 5 minutes and finally in demiwater for 5 minutes. An antigen retrieval step was done in Citrate Buffer pH 6 (Sigma-Aldrich, Nottingham, United Kingdom) for 45 minutes; washed with PBS (Phospho-buffered saline- Fisher Scientific, Leicestershire, United Kingdom) 3 times for 5 minutes (= washing step). The endogenous peroxidase activity was blocked by incubating tissue in 3% H2O2 (Sigma, St. Louis, MO) for 15 minutes; washing step. The nonspecific antibody binding was blocked by preincubation with 3% normal GS (normal goat serum) for 20 minutes; sections were incubated overnight with primary antibodies described in the Table 2 at 4 °C. Biotin-labelled secondary antibodies (Vector Laboratories LTD, Peterborough, United Kingdom) were incubated for 2 hours; washing step; avidin-biotin complex (Vector kit, Vector Laboratories LTD, Peterborough, United Kingdom) was incubated for 1 hour; washing step; visualization step through DAB (diaminobenzidine tetrachloride-DAKO, Glostrup, Denmark); Cresyviolet counterstain was performed by 2 minutes incubation. Finally, tissue was dehydrated with graded ethanol and xylene prior to mounting with DPX (Thermofisher, Chesire, United Kingdom).

Primary Antibody	Company	Catalog No.	Host	Dilution
PLP	Abcam	AB183493	Rabbit	1:500
XBP1s	Cell Signaling	27901S	Mouse	1:250
XBP1u	Abcam	AB37151	Rabbit	1:500

Table 1 Primary antibodies used and respective dilution.
According to the datasheet of the antibodies provided by the companies, the suggested dilution (1:500), a more concentrated (1:250) and a more diluted (1:1000) dilution was used. The correct dilution was assessed through brightfield microscopy and negative controls stainings.

2.3 IMMUNOFLUORESCENCE XBP1s/u

For the evaluation of XBP1s and XBP1u protein isoforms in the brain tissue, a single fluorescence staining has been performed following the PLP staining protocol up to the primary antibody step overnight at 4 °C. AlexaFluor secondary antibodies (Table 3) were incubated for two hours, followed by a washing step, and finally the slides were mounted with mounting media with DAPI (4'6-Diamidino-2-Phenylindole - Thermofisher, Chesire, United Kingdom). The immunofluorescence staining was used only as a qualitative illustration; therefore, no quantitative analysis has been performed.

Secondary Antibody	Company	Catalog No.	Host	Dilution
AlexaFluor 647	Thermo Fisher	A21245	Goat anti Mouse	1:1000
AlexaFluor 488	Thermo Fisher	A11001	Goat anti Rabbit	1:1000

Table 2 Secondary antibodies used and respective dilution.
The assessment of the dilution of the antibodies was performed as for the primary antibodies dilution.

2.4 MICROSCOPY

BRIGHTFIELD

The determination of NAWM, lesions, and the antibodies dilution was performed with a brightfield microscope (DM5000; Leica Microsystems) using a 10× lens magnification.

CONFOCAL

The visualisation of XBP1s and XBP1u protein isoforms was done with a confocal microscope (Carl Zeiss) through Zen software, using a 40x oil lens magnification. The exposure time was 1000 ms and three channels were acquired, at 647 nm, 488 nm and UV (ultraviolet) in order to visualize respectively, the protein of interest, the background autofluorescence and the DAPI staining.

UCL IQ PATH

The slides were sent to UCL (University College London) at the Neuropathology division and were acquired with a standard brightfield microscope. An NDPI viewing software was used in order to download and view the images (https://www.ucl.ac.uk/ion/clinical-divisions/neuropathology/ucl-iqpath).

2.5 IHC ANALYSIS AND STATISTICS

WM, GM and lesion areas were analysed through Fiji software. The background was removed and the colours deconvoluted. Then “Moments” threshold was applied to images to eliminate non-specific staining. Standard scale bars (from pixels to μm) were applied to calculate the area of the images. The NAWM areas were selected according to the following criteria: lack of overlap between the regions and avoidance of folded tissue portions; no inclusion of GM areas (the distinction between WM and GM areas was identified by manual observation on brightfield microscopy). The first two criteria were the same also for the lesions and grey matter analysis. The measurements were performed through Fiji software and all data (area and % of myelin) were collected (Microsoft Office package) and statistically evaluated via GraphPad Prism7 software (One-Way ANOVA Dunnett’s multiple comparison test, with a single pooled variance for the NAWM comparison and Unpaired parametric t test for NAWM and lesions comparison). All the graphs include the mean and standard deviation. The stars present in the graphs represent the p value.

2.6 BREATH STUDY POPULATION

The study was meant to recruit 50 MS participants and 50 healthy controls. Unfortunately, due to the Novel Coronavirus pandemic, before the lockdown closure, we were able to collect 13 MS participants and 15 healthy controls, for a total of 28 participants. Primarily, participants who joined the previous breath study conducted at the Huddersfield University and Centre for Biomarker Research were contacted through email, also providing them with the results of the study and the new information sheet. The other MS participants were recruited from MS clinic at Seacroft Hospital in Leeds and at the MS society. The healthy controls were also recruited through advertisements at the University of Huddersfield and, for the most part, through family members and partners of MS participants. All the participants signed the consent forms before undertaking the breath sampling. Only the healthy controls were subjected to exclusion criteria, among which: no diagnosis of neurological disorders, blood-borne diseases, cancer and diabetes; no history of addiction from abuse substances or drugs; no pregnancies; no smoking or vaping. After the compilation of the questionnaire, the MS participants were divided both depending on the forms of MS according to the diagnosis (RRMS, PPMS, SPMS) and according to the drugs taken. The MS participants were between 28 and 73 years old, with a prevalence of females, while the controls were between 30 and 73 years old, with a prevalence of males (Fig.2.2).

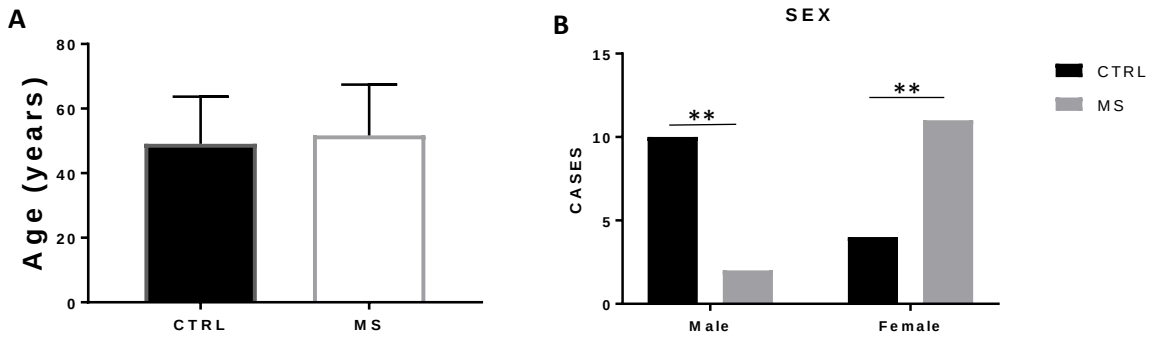


Figure 2.2 Breath biopsy cases details.
(A) Age of participants. Mann-Whitney two tailed test p = 0.7939 (B) Representation of the sex ratio of the participants, comprehending 10 male controls and 4 female controls; 2 MS males and 11 MS females. Fisher's exact test p = 0.0063.

2.7 BREATH BIOPSY TEST

Participants were allowed to eat and drink only water, for at least one hour before the test. A maximum of two participants were allowed to come to perform the sample collection within one hour, up to a total of 15 participants per round due to the availability of the Inert-coated metal thermal desorption tubes (C2-CAXX-5149 Markers International, Llantrisant, United Kingdom). In fact, the latter were a total of 60 tubes, and for each participant 4 tubes were required in order to collect the exhaled breath VOCs from the upper and lower airflows.

The ReCIVA Breath sampler machine (Owlstone medical ltd, Cambridge, United Kingdom) comprises a container where to set the 4 tubes, connected with the one-use ReCIVA face mask (01-0880 Owlstone medical ltd, Cambridge, United Kingdom) where the participants were asked to breath normally (Fig. 2.3). The participants breathed filtered air in order to avoid external VOCs sources, thanks to the presence of the CASPER Portable Air Supply, which is composed of an air pump that takes ambient air and forces it through a filter before supplying it to the sampling machine.

Figure 2.3 ReCIVA Breath Sampler machine.
(a) ReCIVA Face mask. (b) Air pump supply. (c) Breath desorption tubes (OwlstoneMedical, 2019).

The ReCIVA is connected with a USB cable to the laptop. The software is able to analyse real-time the CO2 levels, the pressure, the breathing rate, the collection time (generally around 10 minutes per participant) and the status of the collection, with the possibility of interruption in case participants need it (Fig. 2.4).

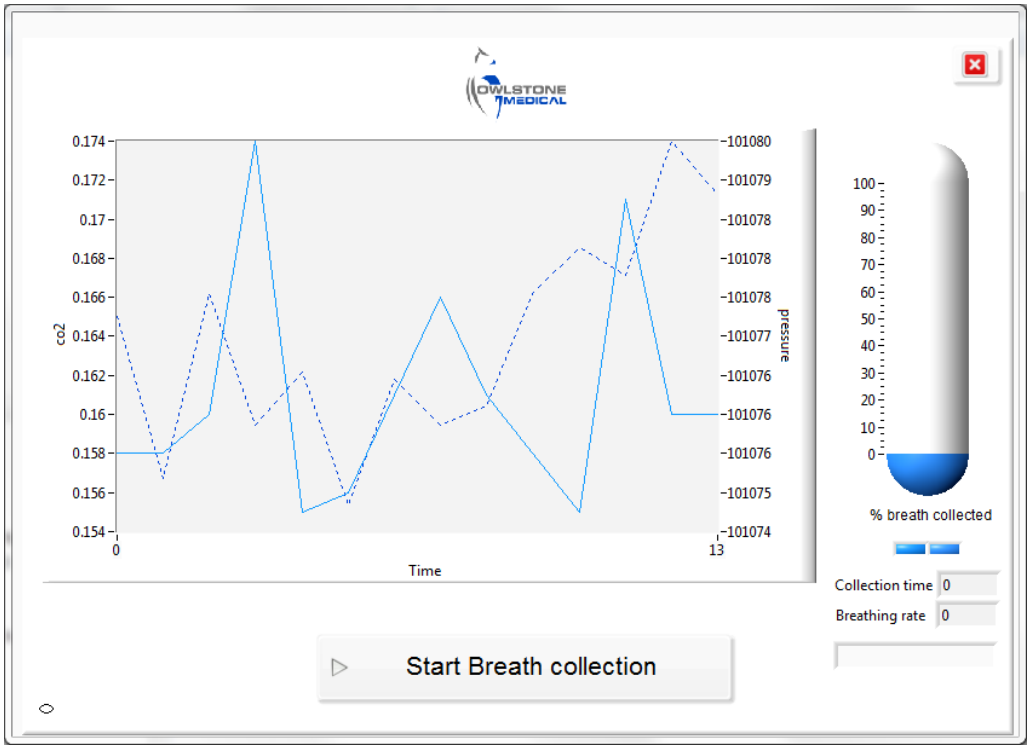


Figure 2.4 ReCIVA Breath software (OwlstoneMedical, 2019).

Inert-coated metal thermal desorption tubes used after the test were kept at -20°C for a maximum of 2 days before sending them for analysis.

2.8 VOCs ANALYSIS AND STATISTICS

Breath tubes were sent to Warwick University for VOCs analysis and tubes cleaning. An Inert-coated metal thermal desorption tubes (Markers International, Llantrisant, United Kingdom) was inserted in the auto-sampler (Markers International, Llantrisant, United Kingdom). The specific bar code present in each tube was inserted manually in the software. The Ultra GC run time was of 25 minutes, reducing the overall run time by employing a stand-by split of 150°C. The temperature was between 40°C and 280°C, increasing of 20°C by minute. A pre-purging step was applied for 1 minute. The inert-coated metal thermal desorption tube was warmed for 10 minutes at 250°C for desorption, while the trap purge was carried out for 1 minute and cooled to 30°C. The trap purging process was performed for 3 minutes at 300°C. The masses between 35-350 (atomic units) were analysed for 25 minutes by TOF-MS (Time-of-flight Mass spectrometry). The ionisation potential was -70V. To remove the background interference the TOF-DS software dynamic background subtraction (DBS) was used, together with the integration and deconvolution automatic system. To integrate it was set: Global Height Reject: 10000, Global Width Reject: 0.001, Baseline Threshold: 3, and Global Area Reject: 10000. To identify the compounds the list of the National Institute of Standards and Technology (NIST) was compared, with at least a match factor of 450.

To compare the VOC levels, the one-way ANOVA with Tukey's post-hoc test has been employed using GraphPad Prism 7 software. Only VOCs present in >1% were analysed.

RESULTS

3.1 XBP1S AND XBP1U IN MS FORMS

In this thesis *XBP1* gene products were evaluated in *post-mortem* brain tissue of MS patients and compared to the non-neurological control ones. The relevance of XBP1 as a biological marker for MS disease had been already evaluated by Wheeler and colleagues (Wheeler 2019), but the evaluation of both XBP1s and XBP1u protein isoforms has never been evaluated in three different regions, such as FC, TL and BG. As *XBP1* is a gene involved in the inflammatory pathway and since a strong component of MS aetiopathogenesis has an inflammatory source, the *XBP1* gene and its products could be investigated as putative biomarkers characterizing MS disease.

First, for qualitative purpose we wanted to image the XBP1 protein isoforms abundance in the tissue with a high magnification and we decided to perform immunofluorescence staining on MS tissue in the frontal cortex. As can be seen in Fig. 3.1 the XBP1u protein was detected at 488 nm and XBP1s at 647 nm with a 40x oil lens magnification. From the figure we can appreciate that XBP1u protein surrounds the nuclei (Fig.3.1 a), which it was what we expected, while for the XBP1s (Fig.3.1b) we can appreciate the non-perfect circular shape, highlighting the stress process inclusions.

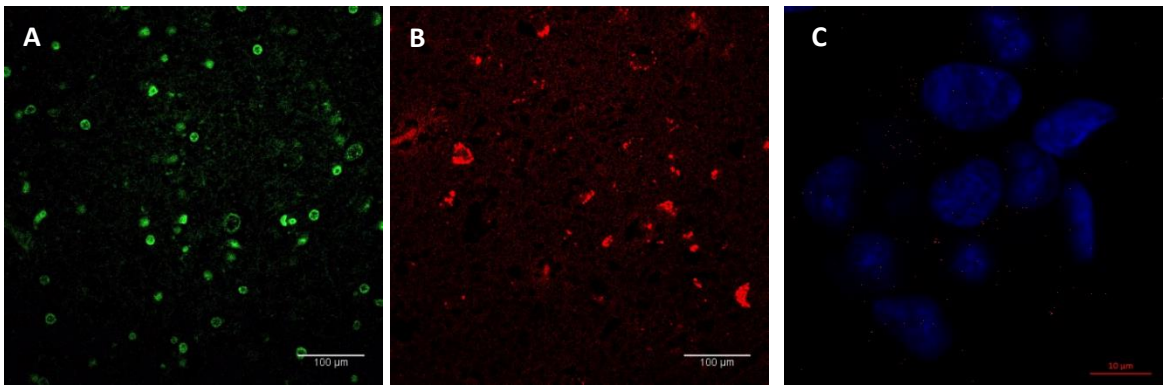


Figure 3.1 XBP1 images at confocal microscopy.
(A) XBP1u detected at 488 nm wavelength. (B) XBP1s detected at 647 nm wavelength. (C) Negative control, DAPI staining. Scale bar 100 um.

We validated the procedure with a PLP staining both (Fig.3.2a) as a positive control for the IHC technique and to discern the NAWM and the lesion areas. We found, as expected, a statistically significant difference between the amount of PLP present compared to the XBP1s and XBP1u levels (Fig.3.2 e, f). The PLP levels were, in fact, higher compared to the XBP1 isoforms (PLP vs XBP1s p=0.0241, PLP vs XBP1u p=0.0396) as PLP is the most abundant protein present in the myelin sheet, so more present compared to the *XBP1* products. XBP1s and XBP1u levels were evaluated in MS patients (n=8) and in controls (n=4) for the three areas FC, TL, BG through Ordinary One-Way ANOVA Dunnett's multiple comparison test, with a single pooled variance.

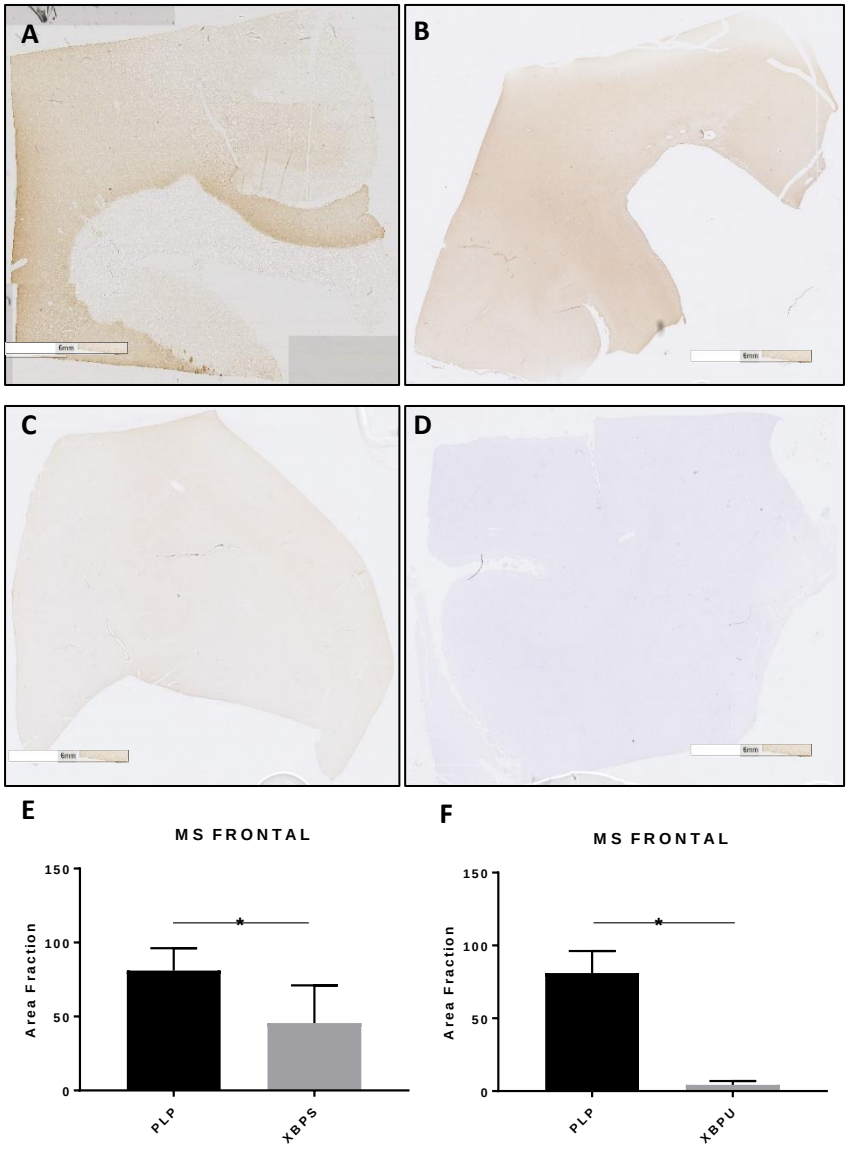
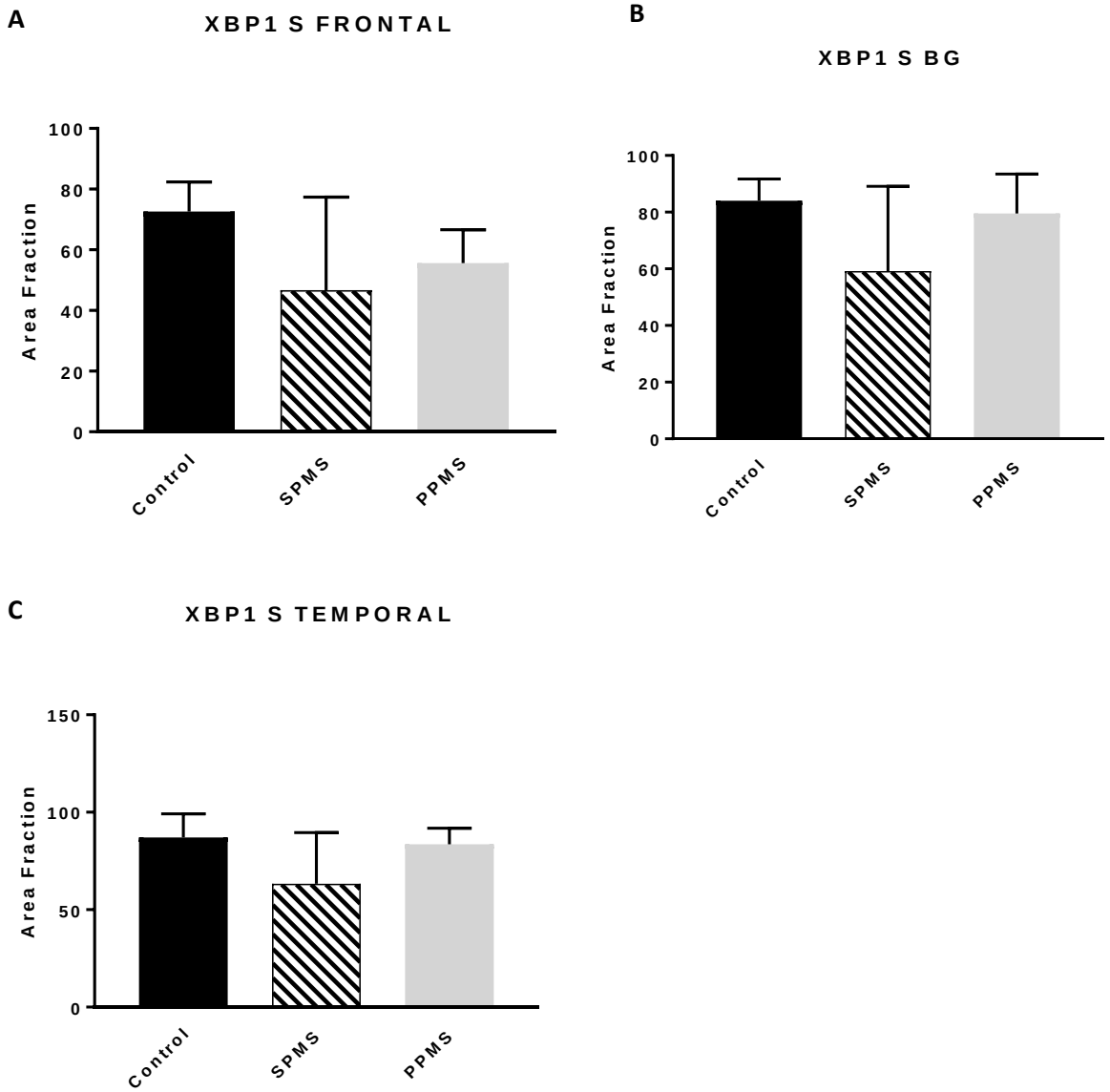


Figure 3.2 IHC technical standardization.
Representation of IHC stainings acquired at brightfield microscopy. Scale bar 6 mm. (A) PLP staining (B) XBP1s staining (C) XBP1u staining (D) Negative control. (E) All the graphs include the mean and standard deviation. The stars present in the graphs represent the p value. PLP and XBP1s levels in MS

in frontal cortex Paired parametric two-tailed t test p=0.0241. (F) PLP and XBP1u levels in MS in frontal cortex Paired parametric two-tailed t test p=0.0396.

Surprisingly, the XBP1s levels, the isoform responsible for the folding and the degradation of the unfolded proteins, were not differing in all the cases and regions considered (Fig.3.3 a, b, c). On the contrary, the XBP1u levels, responsible for the negative feedback, were lower in MS patients than in controls in frontal cortex (CTRL vs SPMS p=0.0193, CTRL vs PPMS p=0.0286), in basal ganglia (CTRL vs SPMS p=0.009, CTRL vs PPMS p=0.0202) and in temporal lobe (CTRL vs SPMS p=0.0005, CTRL vs PPMS p=0.0003) (Fig.3.3 d, e, f). Both SPMS (n=4) and PPMS (n=4) have been evaluated as we wanted to ascertain whether a difference between the two different forms of the disease emerged.



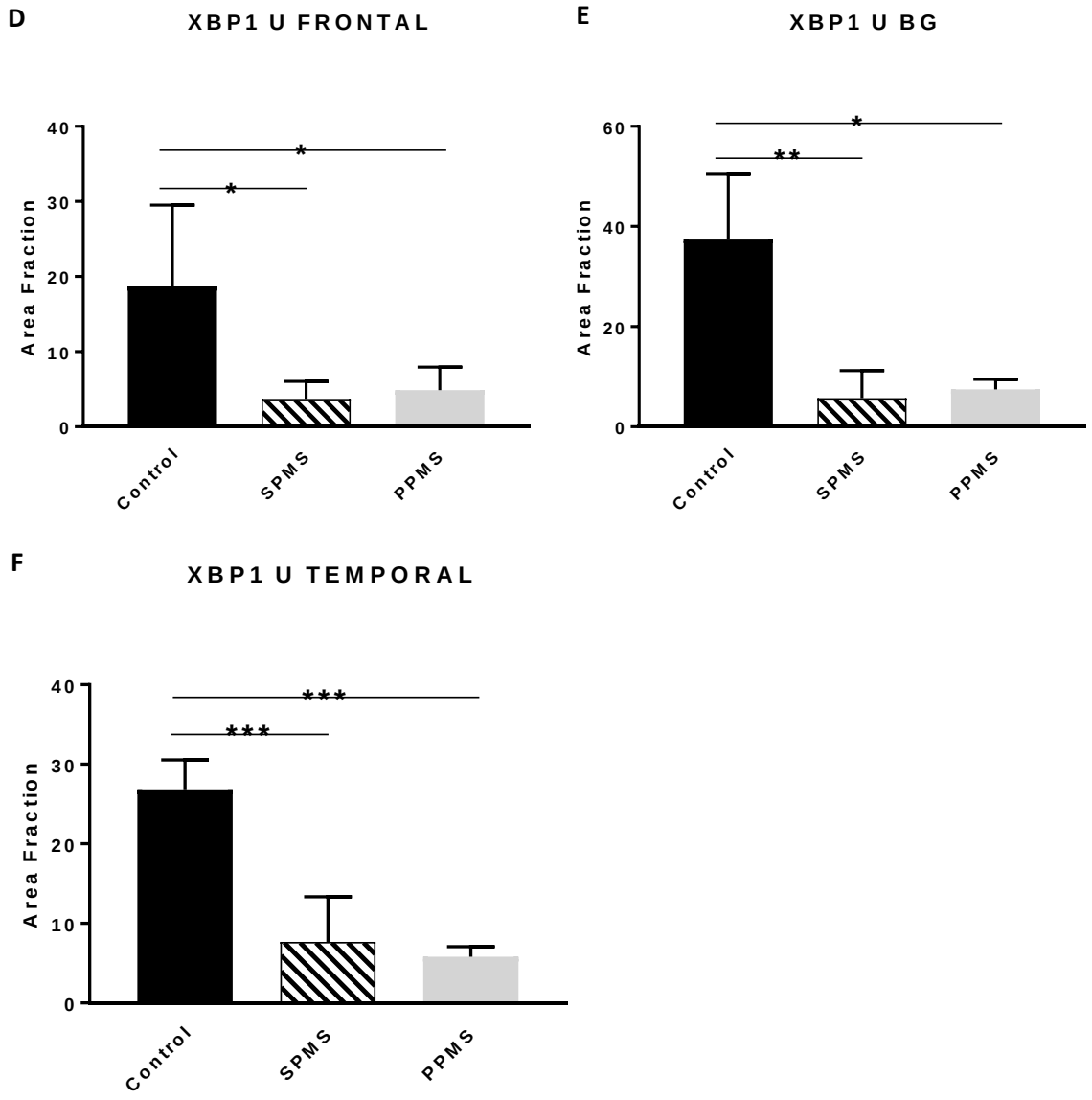
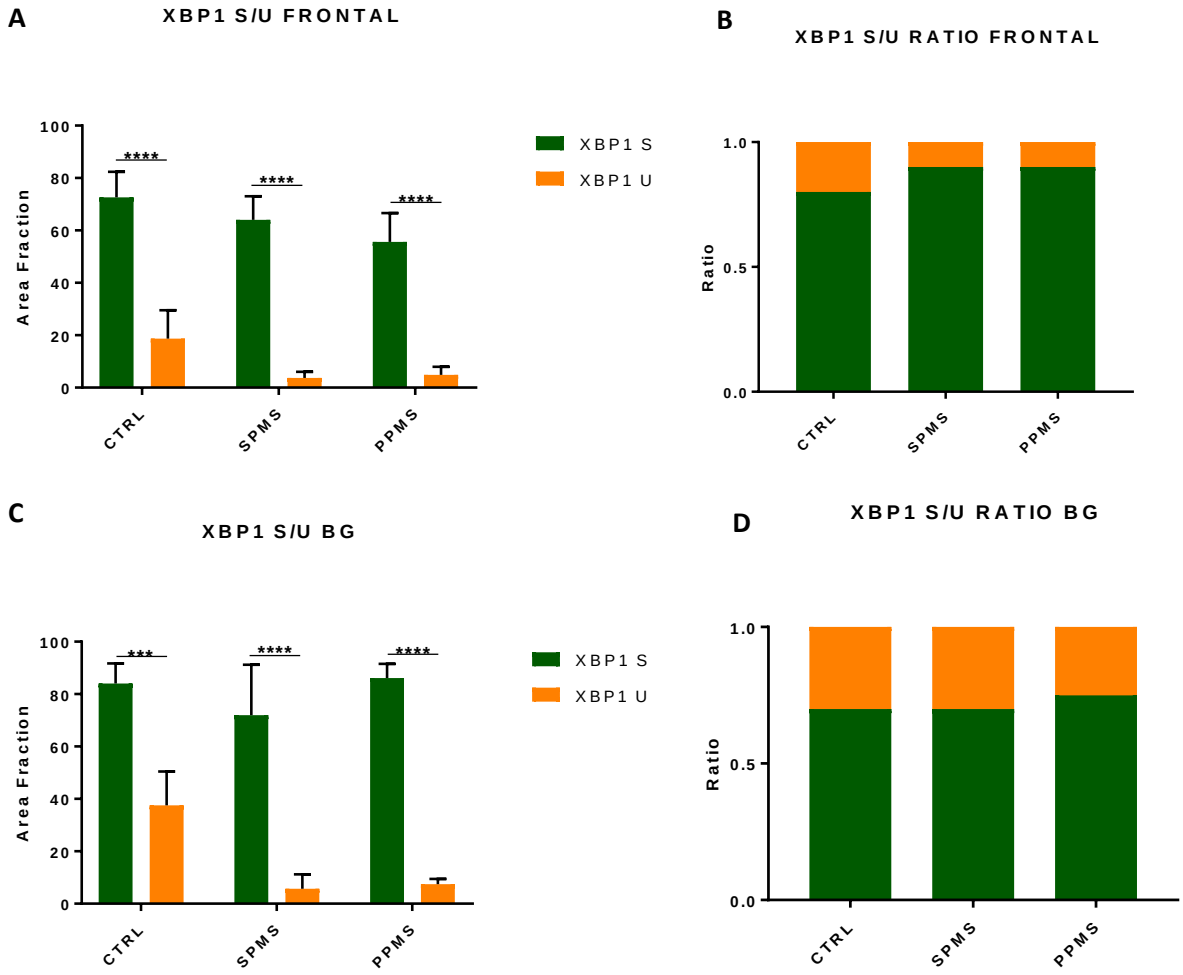


Figure 3.3 XBP1 expression in Multiple sclerosis cases and controls.
Dunnett's multiple comparison test. (A) XBP1s levels in frontal cortex p=0.2037. (B) XBP1s levels in basal ganglia p=0.2147, (C) XBP1s levels in temporal lobe p=0.2099. (D) XBP1u levels in frontal cortex CTRL vs SPMS p=0.0193, CTRL vs PPMS p=0.0286. (E) XBP1u levels in basal ganglia CTRL vs SPMS p=0.009, CTRL vs PPMS p=0.0202. (F) XBP1u levels in temporal lobe CTRL vs SPMS p=0.0005, CTRL vs PPMS p=0.0003.

3.2 XBP1 S/U RATIO

According to the results shown above, we found that XBP1u levels were lower in MS patients compared to the control ones. As the XBP1u works as negative feedback regulator for the XBP1s protein production, we decided to look at the ratio between the two isoforms. The XBP1s and XBP1u levels were compared in order to determine if one isoform was more present respect to the other, showing an unbalance in the regulatory feedback between XBP1u and XBP1s. The isoform ratio test was performed in MS patients (n=8) and in controls (n=4) for the three areas FC, TL, BG through Ordinary One-Way ANOVA Tukey's multiple comparison test. Interestingly, the XBP1s levels were significantly higher respect to XBP1u levels in all the three areas considered (Fig.3.4 a, c, e) supporting the data previously found (Fig.3.3), which support the hypothesis that the XBP1u levels are too low to exercise their effect as negative feedback regulator.



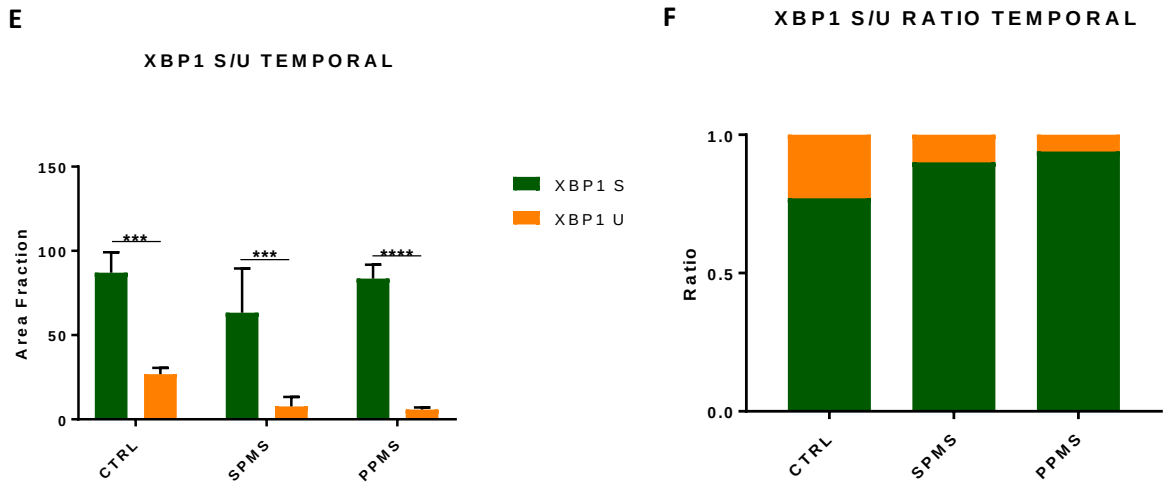
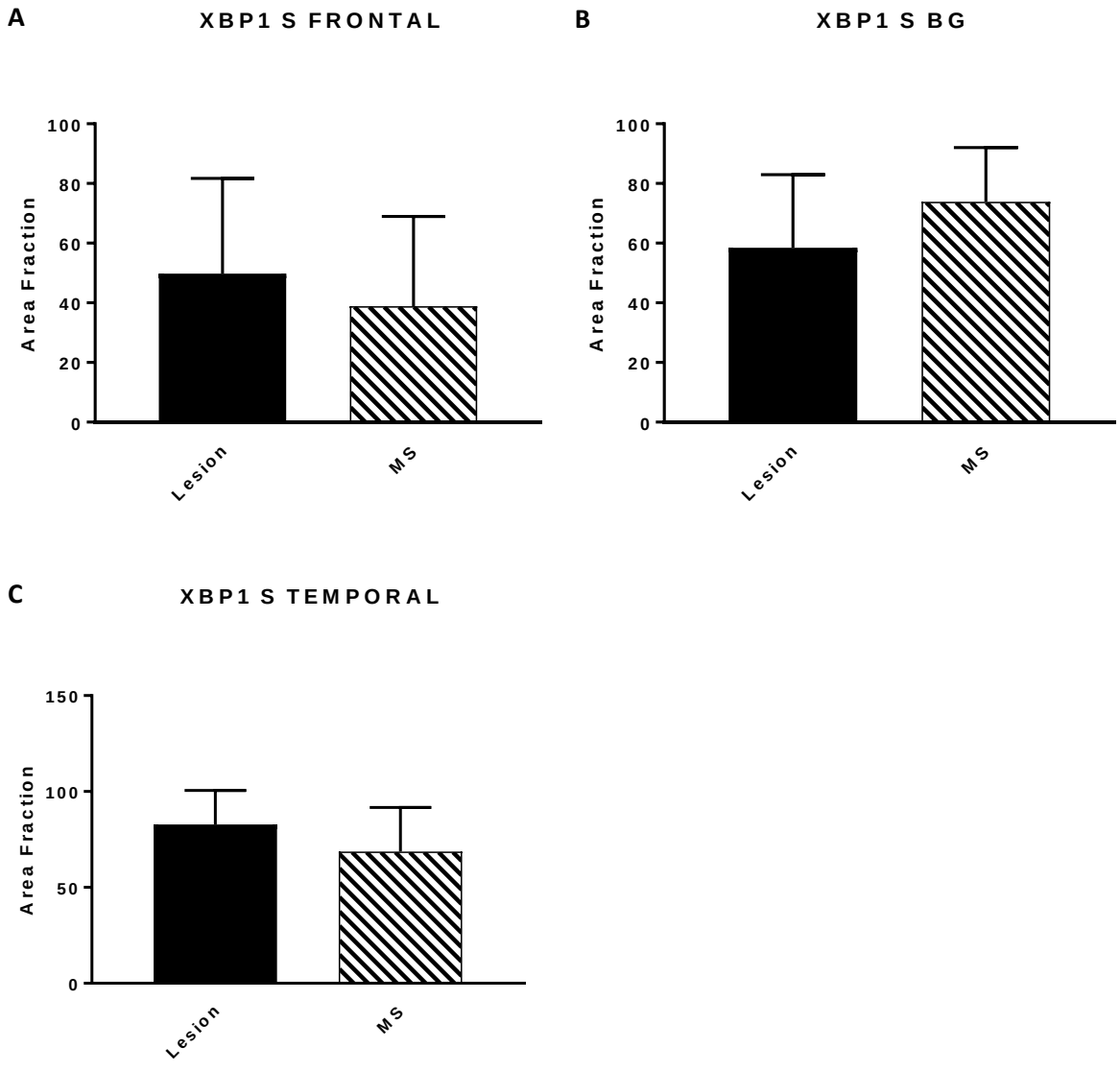


Figure 3.4 XBP1 s/u ratio in all Multiple sclerosis forms and controls.
Tukey's multiple comparison test (A) XBP1s vs XBP1u levels in frontal cortex CTRL p< 0.0001, SPMS p< 0.0001, PPMS p< 0.0001. (B) Percentage ratio distribution of the XBP1 isoforms in frontal cortex in the three cases. (C) XBP1s vs XBP1u levels in basal ganglia CTRL p= 0.0005, SPMS p< 0.0001, PPMS p< 0.0001. (D) Percentage ratio distribution of the XBP1 isoforms in basal ganglia in the three cases. (E) XBP1s vs XBP1u levels in temporal lobe CTRL p= 0.0004, SPMS p= 0.0002, PPMS p< 0.0001. (F) Percentage ratio distribution of the XBP1 isoforms in temporal lobe in the three cases.

3.3 XBP1 S AND XBP1 U LESIONS

As the distinctive features of MS are the lesions where the focal demyelination occurs, we decided to explore the presence of the XBP1 isoforms in these areas. However, the low number of the lesions present (n=5) for FC, BG and (n=2) for TL, do not allow to underline a difference in the expression of both XBP1 protein isoforms detected and analysed with Unpaired parametric t test (Fig.3.5 a, b, c, d, e, f).



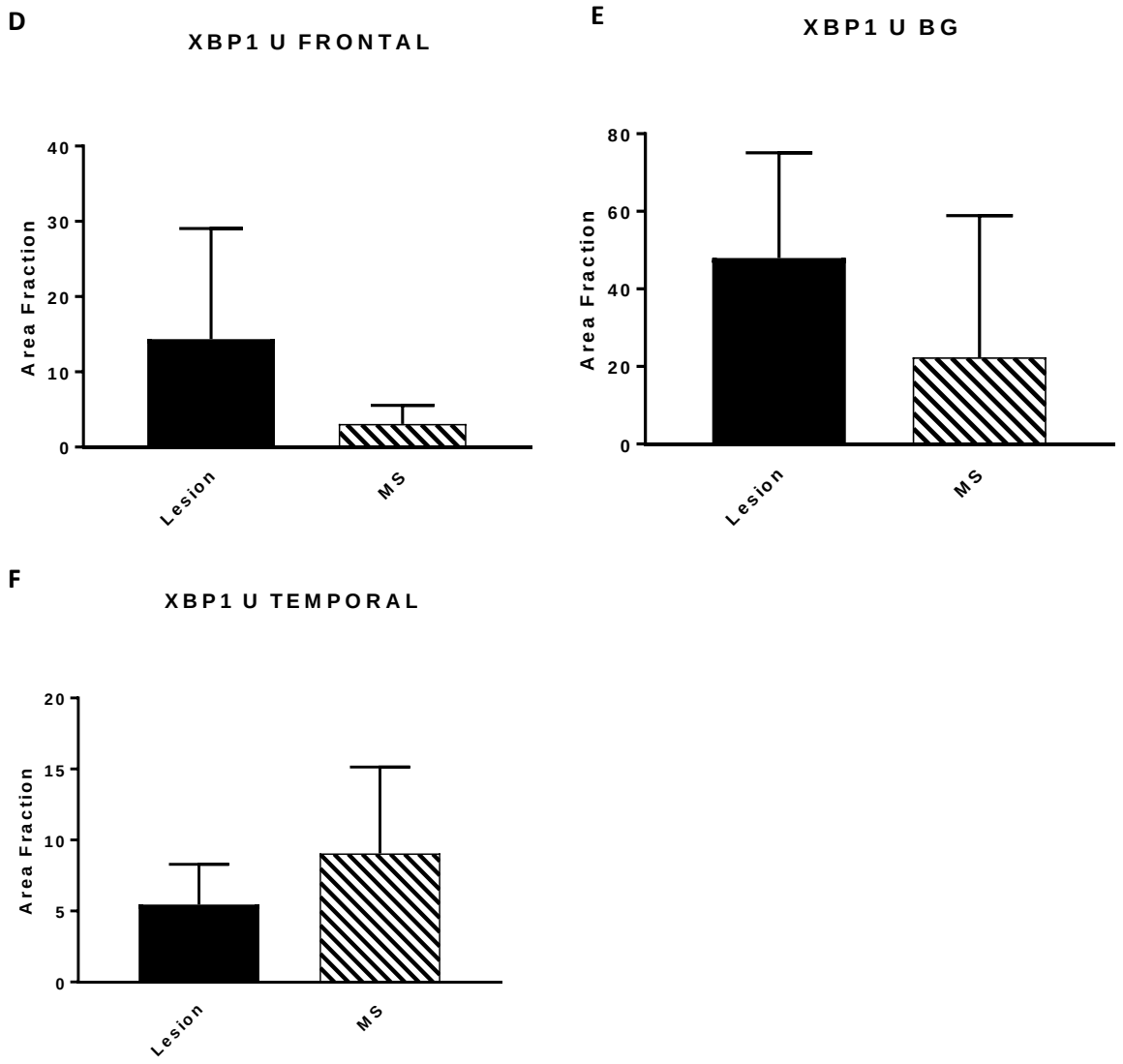


Figure 3.5 XBP1 expression in Multiple sclerosis lesions.
Unpaired parametric t test (A) XBP1s levels in frontal cortex lesions p= 0.5954. (B) XBP1s levels in basal ganglia lesions p= 0.2904, (C) XBP1s levels in temporal lobe lesions p= 0.5627. (D) XBP1u levels in frontal cortex lesions p= 0.1299. (E) XBP1u levels in basal ganglia lesions p= 0.2435. (F) XBP1u levels in temporal lobe lesions p= 0.4041.

To determine if the presence of an isoform over the other was reflecting what we found for the NAWM (Fig.3.4), we decided to apply the same ratio analysis used for the NAWM areas also to the lesion areas. A statistically significant difference can be appreciated considering the expression of XBP1s in the lesions (here denominated LES S) in TL compared to the expression of XBP1u in the lesions (LES U), analysed with Unpaired parametric t test, but due to the lower number of lesions present we feel that this analysis should be reinforced widening the n size (Fig. 3.6e).

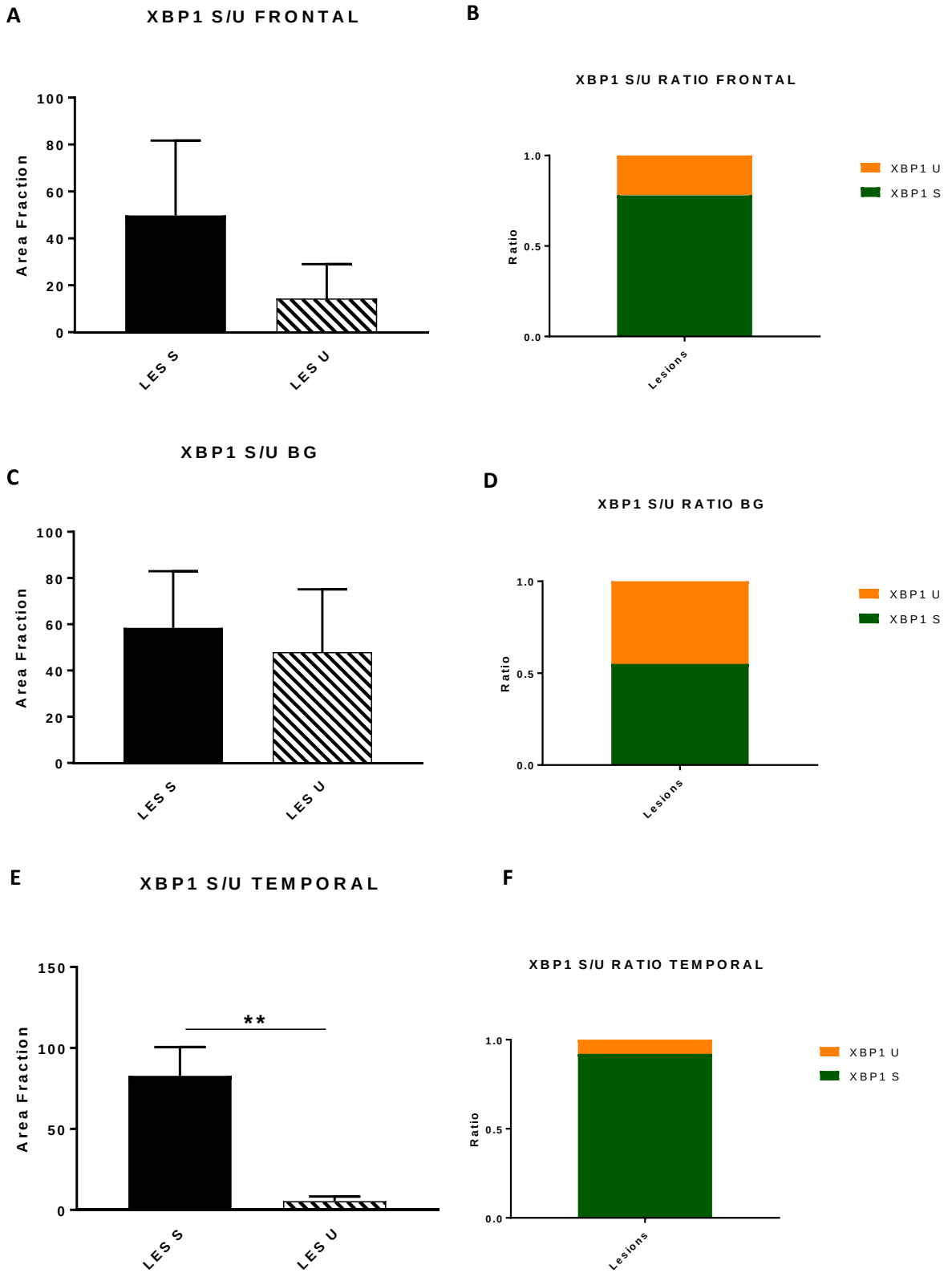


Figure 3.6 XBP1 s/u ratio in lesions.
Unpaired parametric t test (A) XBP1s lesion (LES S) and XBP1u lesion (LES U) levels in frontal cortex lesions p= 0.0544.(B) Percentage ratio distribution of the XBP1 isoforms in frontal cortex lesions (C) XBP1s and XBP1u levels in basal ganglia lesions p= 0.5393. (D) Percentage ratio distribution of the XBP1 isoforms in basal ganglia lesions. (E) XBP1s and XBP1u levels in temporal lobe lesions p= 0.004. (F) Percentage ratio distribution of the XBP1 isoforms in temporal lobe lesions.

3.4 VOCs IN MS AND HEALTHY CONTROL PARTICIPANTS

We implemented a breath biomarker assay with the ReCIVA technology. The technique allows to test 5 participants per hour, as the collection time amounts to only 10 minutes, during which the airflow pressure and the CO2 levels can be monitored in real-time. As can be seen in Fig. 3.7 at least 44 compounds of interest can be collected in a single test.

	458266	458244	458250	458242
Compound Name	**Area %**	**Area %**	**Area %**	**Area %**
[(4-Hexylbenzene-1,3-diyl)bis(oxy)]bis(trimethylsilane)	0,72	1,19	0,58	0,92
1,3-Pentadiene, (E)-	1,30	0,23	2,51	
1,4-Pentadiene				
1-Hexadecanol				
1-Hexanol, 2-ethyl-	0,01	0,40	0,91	
3,4-Dimethylcyclohexanol	0,59	1,84	0,30	0,25
4-Iodoimidazole				5,36
4-Octene, (E)-		1,52		
4-Penten-2-ol	7,47			
5,9-Undecadien-2-one, 6,10-dimethyl-, (E)-				
Acetaldehyde		0,81		1,45
Acetamide, 2,2-dichloro-	6,40			
Acetic acid	11,82	3,46	12,97	6,56
Acetone	30,02	21,76	45,36	16,75
Acetophenone	1,42	0,88	1,12	0,68
Argon	0,02	0,03	0,03	11,36
Benzaldehyde	2,35	2,17	1,88	2,34
Benzene	0,02	2,20	0,09	3,77
Butane, 1-methoxy-				
Carbon dioxide	0,81	1,97	7,31	4,00
Carbon disulfide		1,45		0,56
Cyclotetrasiloxane, octamethyl-	1,57	5,40	0,88	6,57
Cyclotrisiloxane, hexamethyl-	1,55	6,53	1,24	11,67
Decanal	4,45	0,58	1,43	0,18
Decane		1,13		0,17
Dimethyl ether	0,37	0,34	0,19	0,36
D-Limonene		0,10	0,59	
Docosanoic acid, docosyl ester		1,21		
Dodecane	0,28	1,52	0,44	0,34
Hexadecane	0,32	1,18	0,16	
Hydrogen chloride	0,24	1,27	0,28	0,50
Isopropyl Alcohol	0,02	9,02		3,93
Methanephosphonofluoridic acid, TBDMS derivative		0,29		2,08
Methanesulfonic anhydride	0,12		0,35	4,26
Methanesulfonyl	0,44	1,91		
Nonanal	6,08	1,86	2,25	0,21
Octanal	1,13	0,60	0,61	0,40
Phenol	1,34		2,20	0,54
Propanediamide, 2-				
Propanoic acid	0,13			
Propanoic acid, 2-hydroxy-, propyl ester			1,19	
Silanol, trimethyl-		1,99	0,27	1,70
Undecanal	1,27	0,16	0,22	

Figure 3.7 VOCs detectable in a single test.
In yellow there are highlighted the VOCs present as percentage in a number comprised between 1 and 10, while in orange the VOCs present over 10. The first two columns on the x axis (identified with the code numbers 459266, 458244) are the two tubes that collect the upper airways flow, while the last two columns (identified with the code numbers 458250, 458242) are the two tubes that collect the lower airways flow.

From the questionnaire given to the participants, we highlighted 10 RRMS and 3 SPMS participants. Unfortunately, due to the novel Coronavirus pandemic, the analysis of the MS participants and controls has been temporarily held off.

DISCUSSION

4.1 LEAD BIOMARKERS IN MS FIELD

CXCL13

The discovery of B-cell follicles in *post-mortem* human brains (Magliozzi, et al., 2013) opened up the possibility to the "*inside-out"* theory of MS together with the presence of CXCL13 chemokine in these ectopic follicle structures. The role of this chemokine, in fact, is to activate the CD4+ T cells in order to form the germinal centre (Zotos et al. 2010; Crotty 2012; Victora and Nussenzweig 2012) and directs the B cells into secondary lymphoid organs (Cyster 2005; Okada and Cyster 2006). The role of CXCL13 as biomarker has become prominent after finding the higher expression of CXCL13 in the CSF of MS patients in all the clinical forms (CIS, RRMS, SPMS, and PPMS) in comparison to healthy controls (Stilund et al. 2015). The levels of CXCL13 correlate also with increased relapse rate, EDSS (Expanded Disability Status Scale) score, and lesion burden (Khademi et al. 2011; Matute-Blanch et al. 2017; Paul et al., 2019). CXCL13 is a promising disease activity biomarker, as it is able to distinguish between the different forms of MS disease. In particular, CXCL13 levels increase in RRMS relapses (Khademi et al. 2011) and in CIS cases who later, during time, have been confirmed to become MS cases, compared to CIS patients who did not convert (Brettschneider et al. 2010; Khademi et al. 2011). However, it is not a clinical validated biomarker as the higher CXCL13 expression in the CSF has been found also in patients with encephalitic viral infections (Khademi et al. 2011).

CNS NEUROFILAMENTS

Another biomarker found in the CSF of MS patients are the CNS neurofilaments. These structures include heavy, medium, light chains and α-internexin filaments, and their expression its higher after an axonal insult. In particular, it has been found a higher expression of light-chain neurofilaments in CSF of MS patients compared to healthy control ones, and their expression is correlated with active lesions present in the brain of MS participants, detected through MRI scan (Bielekova and McDermott 2015). Light-chain neurofilaments can be considered as a prognostic biomarker, as their expression is higher in CIS patients (Disanto et al. 2015). Overall, the higher levels of light-chain neurofilaments in the CSF predict the conversion from a mild form of MS into a more severe one, as demonstrated for the progression during a year from CIS form to clinically defined MS disease (Martínez et al. 2015) and the progression to SPMS (Salzer et al. 2010). Light-chain neurofilaments can also be potentially considered as a treatment-response biomarker as there is a reduced level in the CSF after treatment with fingolimod, natalizumab, and rituximab (Gunnarsson et al. 2011; Romme Christensen et al. 2014; Kuhle et al. 2015). Before considering light-chain neurofilaments as a clinical biomarker there is the need to validate them in longitudinal cohort studies in order to determine if the expression level of light-chain neurofilaments before drug treatment is a predictive marker or if the level change is dependent on treatment efficacy.

GLIAL FIBRILLARY ACIDIC PROTEIN (GFAP)

The abnormal expression of proteins in the neuronal or glial cells can be a stronger hallmark for a neurodegenerative disease. For example, in the astrocytes an intermediate filament protein called GFAP (Jacque et al. 1978) is mainly expressed. The higher levels of GFAP in CSF of MS patients have been demonstrated to correlate with astrocyte damage (Rosengren et al. 1995; Malmestrom et al. 2003; Norgren et al. 2004) as occurs for the XBP1s incremental expression in MS astrocytes (Wheeler 2019).

MICRORNAS (miRNAs)

Other promising biomarkers in the MS field, that can be found in different sample types, are the miRNAs, a class of single-strand short non coding RNAs. They modulate gene expression, regulate the development and differentiation programs, the proteins synthesis in neuron (Ota et al. 1990; Pette et al. 1990) apoptosis, metabolism and angiogenesis (Zhu et al. 2013; Qu et al. 2014; Paul et al., 2019). Due to their role, miRNA expression has been correlated with several diseases, including cancer, neurodegeneration and autoimmunity (Paul et al., 2019). Regarding MS, the expression of the miRNAs has been evaluated in numerous human body fluids, among which whole blood, including peripheral blood mononuclear cells (PBMCs) (Otaegui et al. 2009; Fenoglio et al. 2011; Martinelli-Boneschi et al. 2012; Keller et al. 2009; Cox et al. 2010) and *post-mortem* brain tissues (Junker et al. 2009). The large variety of the miRNAs showed a dysregulation during relapses (Otaegui et al. 2009) and increase in PBMCs from RRMS patients compared to healthy controls (Fenoglio et al. 2011). The miR-326 expression in PBMCs has been found to associate with a worsening in the clinical condition both of MS patients and their equivalent in a murine experimental model, the experimental autoimmune encephalomyelitis (EAE) mice (Zahednasab and Balood 2014; Paul et al., 2019). The detection of miRNAs in the previously mentioned sample types proposes them as accessible candidates for the biomarker field but with the need to be further explored.

KIR4.1 ANTIBODIES

An IHC study (Srivastava et al. 2012) revealed the presence of IgG1 and IgG3 antibodies binding glial cells of post-mortem MS brain tissue. The target of these antibodies is the potassium channel KIR4.1, with a particular tropism for the end feet of the astrocytes. These antibodies have been detected not only in post-mortem human brains, but also in the serum of nearly half of MS patients examined (total n=397). As only part of MS patients had this autoantibody response (Srivastava et al. 2012) the KIR4.1 is still a controversial biomarker to be considered, together with the lack of reproducibility of this study, investigated by several other groups (Brickshawana et al. 2014; Nerrant et al. 2014; Brill et al. 2015; Chastre et al. 2016; Pröbstel et al. 2016). However, this might be explained due to the fact that Srivastava et al focused mostly on the lower-glycosylated isoform of KIR4.1 expressed in oligodendrocytes (Marnetto, Valentino, Caldano, & Bertolotto, 2017). Taken together, all these results do not exclude the possibility to considerate the KIR4.1 channel as an emerging biomarker candidate in *post-mortem* brain of MS patients.

TOB-1

As previously described, there is no evidence for a specific gene causing MS, but there are potentially risk genes that make person more exposed to develop it, namely the ones that have a direct or indirect role in the immune system such as *XBP1* or *TOB-1*. The *TOB-1* gene inhibits T-cell proliferation and its downregulation is directly correlated with an enhancement of the immune response, facilitating the conversion from CIS form to clinically defined MS disease (Corvol et al. 2008; Paul et al., 2019).

GUT MICROBIOME

The gut microbiome is a recent hot topic that has been discovered playing a role in several diseases including cancer, type 1 diabetes, IBD, rheumatoid arthritis and MS (Scher et al. 2013; Kostic et al. 2014; Alkanani et al. 2015). For what concerns the MS field, the alteration of the gut microbiome of EAE mice leads to the CNS autoimmunity (Lee et al. 2011) and the ingestion of commensal microbiota protects against the disease exacerbation (Ochoa-Repáraz et al. 2010). In a NGS study (Jangi et al. 2016), the gut microbiome of MS subjects was compared to the healthy controls one, an alteration of bacteria population and has been found in the MS participants, correlated with an increase in the expression of genes active in IFN (Interferon) signalling, dendritic-cell maturation and nuclear factor (NF)-κB signalling pathways in circulating T cells and monocytes (Paul et al., 2019; Brown et al., 2020). In the same study, patients treated with IFN-β and glatiramer acetate show a higher expression of two types of bacteria compared with all the other MS participants. MS patients of the second cohort tested with a breath biopsy procedure showed in their breath a higher presence of methane compound compared to non-neurological subjects, that can correlate with an increase of the Methanobrevibacter genus in the gut of MS patients of the first cohort (Jangi et al. 2016). There is the need of further studies in order to understand if the alterations in the gut microbiome are a predictive factor or subsequential cause of MS disease.

4.2 XBP1 EXPRESSION IN MS PATIENTS

XBP1 is a gene involved in the immune system function and ER stress. Its products are balanced in order to prevent the triggering of the unfolded protein response and to maintain the cytoplasm homeostasis (Issenman & Jaffer, 2004). Our findings, even if at the proteomic level, show a decrease of XBP1u protein in MS cases and the unbalance in the XBP1s/u ratio. As previously described, these results are in line with other neurodegenerative diseases pattern, in which XBP1 and XBP1u levels were decreased in blood of bipolar disorder (Bengesser et al., 2018), ALS and AD patients (Montibeller & de Belleroche, 2018). Contrarily to other neuro-disorders, while the XBP1s/u ratio in the bipolar disorder was not significantly altered (Bengesser et al., 2018), our results suggest a decrease in the XBP1u levels. Regarding the expression of both isoforms of XBP1 protein in the lesion area, the n size needs to be increased to reinforce a statistically significant difference between the two. Nevertheless, they were both upregulated in the FC area, mirroring the evidences found by Wheeler et al (Wheeler 2019). Overall, the main finding of this thesis is the reduction of XBP1u levels, which is considered a negative feedback regulator for XBP1s production, responsible for the UPR. Contrarily to our study, Kim et al found an increased expression of XBP1s but looking at mRNA levels in Schizophrenia patients (Kim et al., 2019). The differences with other studies highlight the need to investigate deeper the role of these proteins in MS disease, expanding the research focus on the grey matter areas and increasing the n size.

4.3 VOCs AND THEIR POTENTIAL

The selectivity and specificity rate of VOCs and the ease of use in performing the breath biopsy allow to employ this technique as a powerful tool to identify diagnostic biomarkers. As previously mentioned regarding the MS field, the technique used to assess MS is the lumbar puncture, a painful technique which requires highly qualified medical personnel to perform it. The potentiality of VOCs has already been demonstrated in lung cancer, since they were employed to discern cancer patients from healthy ones (Krilaviciute et al., 2015), in IBD (Arasaradnam et al., 2016) and in AD (Mazzatenta et al., 2015). The emerging role in the MS field is promising and needs to be further explored, the preliminary results (Ionescu et al., 2011) (Broza et al., 2017) and Dr McHugh's studies reinforce the possibility to further employ VOCs and breath biopsy, as it is reliable, quick and easy to perform. Unfortunately, due to the novel Coronavirus pandemic, the analysis of the tests performed in this thesis could not have been done, but the positive answers from the participants experiencing the test suggest that the breath biopsy is a non-invasive procedure that should be further explored in order to make it a clinical routine practice.

4.4 IHC AS BIOMARKER DETECTION TECHNIQUE

IHC is a technique based on the antibody-antigen recognition principle, that allows to visualize targets, such as proteins, in a colorimetric or fluorescent way (Brandtzaeg, 1998). It is a multi-step procedure involving fixation and permeabilization steps, implemented by Coons et al, in 1941 (Coons, Creech, & Jones, 1941). Routinely, IHC is used to detect specific proteins, phosphoproteins and/or cell types, with the final outcome of identifying the physiological state of the tissue/cell, evaluating the disease characteristics and compare them with healthy ones, and contribute to the characterization of the aetiopathogenesis process behind the disease of interest (Gurcan et al., 2009). The IHC provides information not only on the condition of the tissue, but also on the position of the target analysed, its expression and possible spatial connection between several targets (Jones, 1941). This analysis allows to implement the biomarker involvement in the disease screening as a prognostic, diagnostic and treatment-response tool, and it has already been employed in cancer research (Irshad et al., 2014). In fact, IHC is used to identify the subtype of tumours and to discern between benign and malignant cell types (Leong & Wright, 1987). According to the American Society of Clinical Oncology (ASCO) and College of American Pathologists (CAP), in order to test the presence of human epidermal growth factor receptor-2 (HER2) in breast cancer, a standard IHC routine with score system is recommended (Wolff et al., 2014). In the biomarker field, IHC has been proven to have both a prognostic and a treatment-therapy value for epidermal growth factor receptor (EGFR), programmed death ligand-1 (PD-L1), anti-FGFR (fibroblast growth factor receptor) and anti-cMET (hepatocyte growth factor receptor) (Redman, Crowley, Herbst, Hirsch, & Gandara, 2012). Ultimately, IHC confirms RNA and protein signals detected with other molecular techniques, providing assurance for otherwise questionable targets (Chae et al., 2017). The usage of IHC in biomarker research is due to the availability of the technique, the meaningful results, the timing required (usually 2 days) and the cost efficiency compared to molecular platforms (Mino-Kenudson, 2017). In this thesis, the role of IHC in the discovery of XBP1 impairment in MS disease was fundamental and allowed to detect not only the position of the proteins and their expression, but also to visualize them in the brain. Therefore, even if lot of effort needs to be done in order to standardize the quantitative analysis, the IHC remains a valuable tool in the research for predictive biomarker.

4.5 IHC LIMITATIONS

Although IHC has numerous advantages, the use of this technique needs to be performed being aware of pros and cons. One of the main limitations is to translate the qualitative staining images into quantitative analysis. Therefore, lots of efforts have been done in order to create an automation system, not only for the time required in order to manually quantify the presence of biomarkers, but also to create a standard methodology that will improve accuracy and sensitivity (Sheikhzadeh, Ward, van Niekerk, & Guillaud, 2018). The skills of the personnel required to perform and interpret IHC stainings, is another variability in the reproducibility of the results (Jaffer & Bleiweiss, 2004). As the procedure is based on the antibody-antigen recognition, is important to notice the possibility of false positives, as the antibody may recognize multiple epitopes, lacking on specificity (Yaziji & Barry, 2006). Finally, adequate tissue handling and selection of tissue samples are the key points to successfully perform an IHC, not only for predictive biomarkers, but also for protein expression in general (Mino-Kenudson, 2017).

4.6 FUTURE EXPERIMENTS

The increment of the n size for the *XBP1* study is necessary in order to validate the difference between the XBP1 isoforms and will enhance knowledge of the presence of these proteins not only in other brain areas such as the NAGM and the lesion areas, but also in other CNS structures such as astrocytes, oligodendrocytes and so on. With regards to the Breath biopsy project, the methodology implementation will be a solid basis to carry on the study first with a small cohort, then to England population, hoping to bring it forward to the European one.

4.7 CONCLUSIONS

This thesis has added the foundation to better explore the role of XBP1 proteins in the MS disease, highlighting the difference in expression of the two isoforms and the dysregulation of protein levels between MS patients and healthy controls. Regarding the breath biopsy study, the usage of VOCs as diagnostic biomarkers has already been demonstrated and needs further insights in the MS area. However, the usage of breath biopsy in clinical practice is a promising tool looking at the positive feedbacks received by MS participants in the present study.

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