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DNA METHYLATION CHANGES IN THE BRAIN WHITE MATTER OF MULTIPLE SCLEROSIS PATIENTS

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ABSTRACT

The literature implies that Multiple Sclerosis (MS) holds an epigenetic component that mediates the effects of its typical environmental risk factors on disease progression. Some of the major epigenetic modifications on DNA are the addition of methyl or a hydroxymethyl group to the C5 cytosine position, leading to 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC) synthesis. Interestingly, abnormal epigenetic modification patterns, such as the global DNA methylation loss in MS brain white matter, link to several neurodegenerative disorders. Abnormal DNA methylation/hydroxymethylation enzymes expression is also extensively documented in Alzheimer's disease and cancer. This doctoral research project traces the DNA methylation instability in MS white matter, back to the altered expression of the DNA methylation/hydroxymethylation enzymes.

INTRODUCTION

1. Multiple Sclerosis

Multiple sclerosis (MS) is a chronic and progressive disorder of the central nervous system (CNS) characterised mainly by autoimmune inflammation, myelin loss and neurodegeneration after demyelination.

In MS, myelinated nerves suffer from damages in discrete and scattered areas in the CNS, hence the name "multiple".

These lesions originate from the autoimmunity dysregulation where CNS myelin is recognised as foreign by the immune system, and it is subsequently destroyed by it.

The dispersed damaged regions where the myelin is attacked in the CNS and later lost around the axons evolve into "plaques".

Along with pathology progression and with the chronic inflammation establishment, these areas may assume features similar to scars, hence the term "sclerosis". These events lead

to a permanent neurological deficit, mainly due to a compromised axonal functionality.

Mild symptoms, such as muscle weakness or slight movement disorders, defines MS early stages. The later phases are characterised by progressive neurological deficits, such as paralysis and cognitive impairment, possibly leading to fatal complications such as sepsis or aspiration pneumonia (Dendrou, Fugger et al. 2015).

While most MS patients are not affected by a significant degree of disability, 60% of them might lose the capacity to walk 20 years after the onset of the disease, with significant implications for their quality of life and the socio-economic impact linked to their assistance. This latter aspect of the pathology is of particular relevance as MS is one of the most common neurodegenerative and debilitating diseases among young adults. It affects millions of individuals in the world and with a specific incidence among European and North American young adults (Browne, Chandraratna et al. 2014).

Nevertheless, since the etiologic basis and the pathogenesis of MS is mainly elusive, there is no significant progress in finding out a final cure (Tizaoui 2018).

1.1 Epidemiology

MS affects around 2.5 to 3 million people in the world (Hunter 2016), displaying a non-uniform geographical distribution as it is more widespread in regions that are distant from the equator (Khan, Amatya et al. 2014)

There is also a global latitude gradient in MS prevalence, as a higher MS incidence defines some geographical areas. Moreover, some ethnic groups result in being more affected than others, such as the Caucasian race with northern European ancestry. Within the same racial group, MS appears to be twice more common in females than in males, with an onset peak of around 30 years of age (Zheleznyakova, Piket et al. 2017).

1.2 Classification

MS classification is primarily based on pathology progression, as it commonly manifests itself following recurring and typical patterns.

At the onset of clinical signs, approximately 85 % of people with MS are diagnosed as relapsing/remitting, RRMS (relapsing/remitting multiple sclerosis). Characteristics of this MS type are the initial episodes of acute neurological dysfunction, followed by subsequent periods of partial or complete remission (Hottenrott, Dersch et al. 2017).

The relapses can last days or months and, in some cases, also years. During the remission period, also, even if the pathology appears clinically inactive, the injuries that occur in the CNS are detected by an instrumental examination such as the MRI scan (Magnetic Resonance Imaging). Sometimes remissions are less isolated over time, and the symptoms seem to become more severe with clinically detectable signs (Dendrou, Fugger et al. 2015) (Figure 1).

The residual portion of MS patients at the clinical onset, approximately 10-15%, are diagnosed as primary progressive, PPMS (Primary Progressive Multiple Sclerosis). A continuous and gradual progression of disability characterises this disease type, commencing from the initial manifestation of signs without any remission. If compared with RRMS, PPMS presents a later disease onset, between 30 and 50 years of age, without any gender discrepancy regarding its prevalence and frequency. Inflammatory alterations relate to an autoimmune activity against the CNS as revealed by cerebrospinal fluid (CSF) examination. Such immune variations are, for example, the oligoclonal bands (OCB), which are shared by both RRMS and PPMS forms (Hottenrott, Dersch et al. 2017)

Despite beginning with a period of relapses and remissions, about 50% of RRMS patients progressively lose this original pattern, thus developing into another MS form called

secondary progressive, SPMS (Secondary Progressive Multiple Sclerosis). The disease evolution from RRMS to SPMS occurs within 10-20 years from the initial onset, and, similarly to the PPMS type, SPMS patients showcase a progressive increase of neurological dysfunction.

In several cases, a preliminary clinical sign of MS remains as an isolated episode that is interpreted clinically as a separate condition, the Clinically Isolated Syndrome (CIS).

Individuals with CIS do not generally turn into MS patients, but they have a higher risk of developing MS. The CIS patient who endures more than one sign or symptom simultaneously, the multifocal case, is more likely to develop MS (Dendrou, Fugger et al. 2015).

Less frequent are the variants of MS, which generally present themselves as more aggressive than the most prevalent forms. For example, the Marburg disease, also known as acute, fulminant, or malignant MS, is defined by a severe and continuous degeneration of axonal myelin, often ending in death within one year after the first clinical manifestation (Capello and Mancardi 2004).

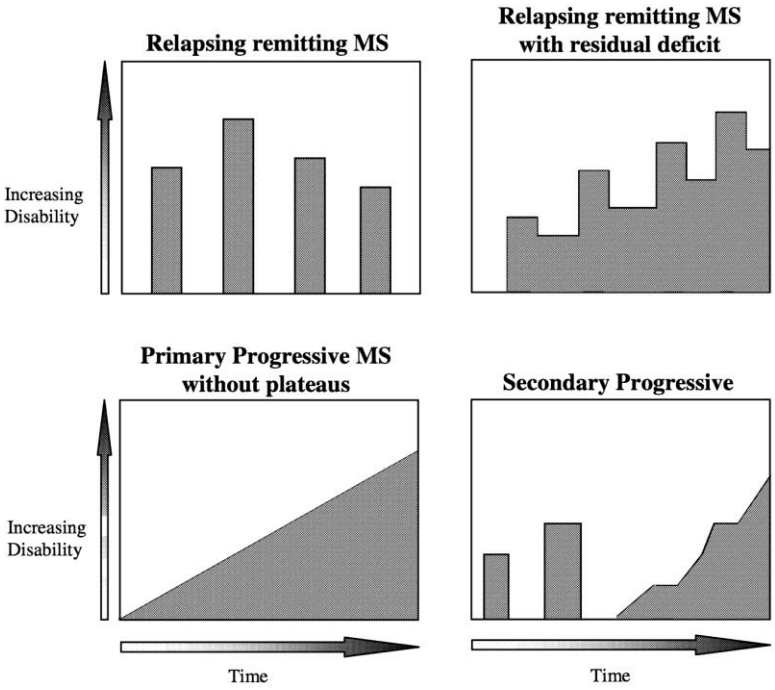


Figure 1. Spectrum of disease course. Spectrum of disease course: The RRMS (Relapsing Remitting MS) form can have two different courses: one in which remission phases alternate with disease manifestations; one in which the disease does not feature clear remissions. In the PPMS (Primary Progressive MS) form, the pathology is characterised by a continuous functional decline, starting from the onset of symptoms and does not present recurrences. Initial phases of remission characterise the SPMS (Secondary Progressive MS) form, later followed by a gradual neurological deterioration (Lublin and Reingold 1996).

1.3 Pathophysiology and pathogenesis

Demyelination areas localise in both the white and grey matter of the brain and spinal cord. The oligodendrocytes, the cells that exert a trophic effect on the axons and maintain the structural integrity of the myelin, are attacked by the immune system in MS. As a direct consequence of the damage, the compromised myelin sheath does not properly propagate the axonal action potential (Patel and Balabanov 2012). At the initial phases of the disease, both axons and neurons are still capable of preserving their functionality as well as their correct structure. With the disease progression, there is a gradual neuro-axonal integrity decline strongly related to the patient's degree of disability (Correale, Marrodan et al. 2019).

Interestingly, myelin degeneration may partially revert by spontaneous phenomena of re-myelination. Hence, oligodendrocyte precursor cells (OPCs) gather to the lesions where they start differentiating into functional oligodendrocytes. With myelin recovery, the conduction of nerve impulses re-establishes (Kirby, Jin et al. 2019). However, re-myelination mechanisms are efficient only during the early stages of the disease (Powers, Sellers et al. 2013). As well as the white matter, processes of demyelination localise in the grey substance but with a minor inflammatory component (Lucchinetti, Popescu et al. 2011; Nantes, Zhong et al. 2016).

The immune system, however, despite attacking the oligodendrocytes, recognise as non-self CNS-specific antigens that are components of the myelin. Indeed, it is possible to detect antibodies produced against these constituents in the lesions, the cerebrospinal fluid (CSF), but also the blood serum.

One of the first antibody raised against the myelin constituents is the one that recognises the MBP protein (Myelin Basic Protein), a fundamental component of the myelin sheath involved myelination (Stinissen and Hellings 2008; Zhou, Simpson et al. 2017).

Other autoantibodies recognise and attack specific-myelin proteins such as the Myelin Oligodendrocyte Glycoprotein (MOG), PLP (Myelin Proteolipid Protein) and AQP4 (Aquaporin-4) (McLaughlin and Wucherpfennig 2008; Lassmann and van Horssen 2011).

Also, MS autoimmunity directs against non-myelin targets. Other autoantibodies indeed attack the components of neurons such as neurofilaments, neurofascin, RNA-binding proteins, and potassium channels (Levin, Lee et al. 2013)

However, the processes that originate this autoimmunity in MS are not transparent yet. One possible hypothesis behind the aetiology of the autoimmunity is that an early CNS injury, not necessarily related to MS, abnormally exposes to the cell

surface some antigens that the immune system later identifies as non-self.

Another idea behind autoimmunity is related to a specific immune response referred to as "molecular mimicry". Contextually, the MBP itself retains on its structure a six amino acids peptide that is also present in the proteins of HBV virus (hepatitis B virus). Thus, once the immune system contains HBV infection, some unregulated responses might generate autoimmunity against myelin following antibody cross-reactivity (Fujinami and Oldstone 1985). Experiments demonstrated that rabbits injected with this HBV peptide exhibit pathological neural changes shared by the MS mouse model, the Experimental Allergic Encephalomyelitis (EAE) mouse. Besides, the mechanism of "molecular mimicry" could also explain the association between infection by microorganisms and the MS risk (Libbey, McCoy et al. 2007).

Regarding MS immune cells, T-lymphocytes seem to induce the early autoimmune inflammation that recognises "self" antigens as "non-self". As a result of this trigger, immune cells break through the blood-brain barrier (BEE) and invade the CNS. Along with these events in the brain, the peripheral circulation of RRMS patients present reactive T-cells against myelin. These circulating reactive T-cells against myelin might invade the brain as a result of functional alterations of the BEE

endothelial cells (Varatharaj and Galea 2017). It is observed that endothelial cells present leukocyte-adhesion molecules that allow these immune cells to pass through the BEE (Minagar, Maghzi et al. 2012). Inflammatory infiltrates of the CNS present other cell types of the peripheral immune system, such as B-lymphocytes, plasma cells, and macrophages

As well as T-cells, these cells attack myelin components and trigger autoimmunity along with the resident brain immune cells, such as microglia and dendritic cells. All these events trigger the mechanisms of demyelination or neurodegeneration, including oedema, phagocytosis processes, complement activation, cytokine production, as well as cytotoxic protein synthesis (Hausser-Kinzel and Weber 2019).

Interestingly, the inflammatory process of MS is dynamic as the cellular populations that invade the brain vary according to the phases of the pathology. The initial stages of the disease present a low degree of lymphocytic infiltration at the brain lesions, while microglia are activated. With disease progression infiltrations of T, B cells and macrophages increase as well as the activation of microglia. The direct consequence of such events is the progressive myelin atrophy, associated with axonal damage and neurological dysfunction.

Advanced stages of the disease are interested by inflammation contraction; however, microglia and macrophages still preserve their state of chronic activation (Dendrou, Fugger et al. 2015).

The cytotoxic effect of the inflammatory process is primarily associated with oxidative stress: macrophages and activated microglia produce reactive oxygen species (ROS) that further lead to demyelination and axonal damage. The advanced stage of the disease, where the inflammation process is moderate, the oxidative damage is caused by the release of iron from intracellular deposits with subsequent generation of ROS (Hametner, Wimmer et al. 2013)

However, despite the considerable amount of information related to MS immunity, the disease aetiology is still uncertain. However, tissue damage is considered to be secondary concerning the inflammation.

1.4 MS risk factors

MS risk associates with both genetic factors as well as environmental agents. Concerning the genetic component, studies conducted on familial aggregation and twin pairs show that MS risk grows among relatives, as about 1.9-4.7% of patients have a family history of MS. In these cases, MS risk for a first-degree relative is 30 times greater than the general

population (O'Gorman, Lin et al. 2013), thus strengthening the idea of a relevant genetic component in MS.

Indeed, several genes are associated with MS susceptibility, such as some polymorphisms of the Major Histocompatibility Complex (MHC) (or human leukocyte antigen (*HLA*)) loci. One of these variants is the haplotype *HLA-DR15*, which associates with a three times higher MS risk (Schmidt, Williamson et al. 2007). The population of Sardinia that is known to have an increased risk of MS (Pugliatti, Sotgiu et al. 2001), curiously, manifests an association between MS and the *HLA-DR4* haplotype but not with *HLA-DR15* (Marrosu, Murru et al. 1997; Marrosu, Murru et al. 1998). GWAS (genome-wide association study) and meta-analysis allowed the identification of 350 MS-associated loci even outside the *MHC* (Wang, Pappas et al. 2011). Furthermore, whole-genome association studies have validated that 50 genes (Beecham, Patsopoulos et al. 2013) are strongly linked to MS susceptibility as well as to the severity of the pathology (Mowry, Carey et al. 2013).

However, although MS presents a clear genetic component, discrepancies in MS development have also been highlighted among members of the same family, thus implying the influence of other non-genetic factors. MS studies conducted on homozygous twins have highlighted susceptibility divergences as only one-third of the twin pairs are concordant

for MS development (Baranzini, Mudge et al. 2010). All these pieces of evidence demonstrate the critical influence of the environmental component on MS risk. To reinforce this statement it is essential to mention two consistent notions acquired on MS: on the one hand, migrants moving from a region of high MS risk to another associated with a lower risk showed a lower-than-expected disease rate; on the other hand, people moving from an area of lower risk to one of higher risk tended to maintain the lower MS risk of their country of origin. Migration studies also pointed to the importance of the first two decades of life on determining disease's risk on migrants: children of the migrants that moved to a high-risk area tended to acquire the MS risk rate featured in the host country. (Gale and Martyn 1995). Indeed, several environmental factors play a role in MS susceptibility, such as exposure to the Epstein-Barr virus (EBV), smoking, and vitamin D deficiency (presumed to be caused by low exposure to sunlight in areas distant from the equator or by an insufficient vitamin D intake). It is important to remark that vitamin D is considered beneficial in MS. Both low vitamin D intake or serum vitamin D levels are associated with MS risk as well as with an increased relapse risk and worsening of the disease (Munger, Levin et al. 2006; Mouhieddine, Darwish et al. 2019).

Vitamin D association with the pathology could demonstrate the prevalence of MS in high-latitude countries, featuring lower solar irradiation (Koch, Metz et al. 2013; Riccio and Rossano 2017). Concerning EBV infection, a higher IgG antibody raised against its nuclear antigens (Epstein-Barr NA; EBNA) is specifically associated with MS risk (Sundqvist, Sundstrom et al. 2012). Nevertheless, no association was identified concerning other EBV antigens or other viral infections (Ascherio and Munger 2007).

Smoking was associated with increased susceptibility to the development of MS as well as an increased frequency of relapses and the number of lesions (Pittas, Ponsonby et al. 2009). More recently, a significant relationship is emerging between the MS and the microbiota composition of the gut.

Indeed, evidence indicates that alterations in gut microbiota composition may result in disease (Kirby and Ochoa-Reparaz 2018).

Several studies have also demonstrated that the family microenvironment does not affect MS risk, thus inferring the importance of the environmental factors that operate at a population level (O'Gorman, Lin et al. 2013).

2. The epigenetic component of multiple sclerosis

MS is a common and complex disease in which the balance between susceptibility genes and the environmental factors determines the disease pathogenesis (Peedicayil 2016). However, several studies suggest the underlying contribution of the epigenetic component as well on MS risk. The fact that these mechanisms may be relevant in MS is due to several pathology's characteristics, such as the influence of geographic location, diet (e.g., vitamin D intake), and smoking.

Furthermore, the parent-of-origin effect (Ebers, Sadovnick et al. 2004), and the recorded increment in female-to-male diagnostic ratio suggest further the involvement of epigenetics in MS. These characteristics highlight the importance of the X-chromosome on MS risk and, since this genomic portion does not feature susceptibility genes, mechanisms such as X-chromosome inactivation or imprinting probably link it to the disease (Wallin, Page et al. 2004; Hirst, Ingram et al. 2009). Epigenetic mechanisms may help explain MS "missing heritability" (the inability of single genetic variations to explain phenotypes' heritability), the low concordance rate in identical twins, the variable clinical course, and the different gender prevalence. Many epigenetic alterations in MS have been identified yet, involving different tissues and organs, and they

lead to expression alterations of genes potentially relevant for MS (Lill 2014).

Several studies have already identified several epigenetic changes in histones, DNA methylation and microRNAs (miRNAs) involved in the MS pathogenesis (Huynh, Garg et al. 2014; Wu, He et al. 2017; He, Hu et al. 2018).

For example, MS brain sections not featuring plaque formation, the Normal Appearing White Matter (NAWM) samples, show increased citrullination of histone H3 (Mastronardi, Wood et al. 2006). Histone acetylation is found altered as well in the frontal lobes of MS patients (Pedre, Mastronardi et al. 2011). Epigenetic changes extend to DNA methylation too, hence affecting the expression of genes putatively involved in MS pathogenesis in NAWM samples (Huynh, Garg et al. 2014
). Epigenetic variations interest the immune blood cells as well where the expression of both histone deacetylases (HDACs) and miRNAs is found altered in relationship to MS pathogenesis (Ma, Zhou et al. 2014; Ciriello, Tatomir et al. 2018). Interestingly, even DNA methylation at the HLA-DRB1 locus is associated with MS susceptibility (Kular, Liu et al. 2018). However, among all the listed epigenetic variations implicated in MS pathogenesis, DNA methylation profiling offers a different chance to understand the genome activity of unexplained pathological conditions such as MS since both

stability and cell type-specificity characterise this epigenetic mark.

2.1 DNA methylation

DNA methylation, and in general epigenetic mechanisms, functions as a genetic annotation system that provides instructions for transcription regulation. This whole instruction pattern determines a stable gene expression profile of differentiated cells, and it establishes during development. However, methylation modifications also occur as a result of external environmental inputs, thus contributing to ageing as well as to a number of pathological conditions and disease predispositions.

From a chemical standpoint, DNA methylation is the covalent adding of a methyl group on the 5^{th} carbon of cytosine residues, mainly placed in the cytosine-guanidine dinucleotides (CpG dinucleotides), thus forming the 5-methylcytosine (5mC) nucleotide.

DNA methylation pattern establishes after the earliest phases of embryogenesis when almost all methyl groups are eliminated from the genomic cytosines in two events of global demethylation and re-methylation. The demethylated genome undergoes a first global re-methylation after fertilization, in a process called "de-novo methylation" for somatic tissue

differentiation, mainly by the activity of DNMT3A-B enzymes (DNA methyltransferase 3A and 3B). DNMT3A-B de-novo activity extends to the primordial germ cells (PGCs) development for establishing their sex-specific methylation pattern before birth (Greenberg and Bourc'his 2019).

The bulk of DNA methylation ensures genomic stability at non-coding regions, primarily repeat elements such as transposons and microsatellite DNA. Only a limited portion of DNA methylation is intended for gene expression in discrete positions such as promoters, enhancers or silencers, where it mainly works as a repressive signal.

Genomic DNA methylation is propagated during cell divisions by a maintenance mechanism involving the activity of DNMT1, an enzyme that preserves symmetrical DNA methylation throughout DNA replication (Klose and Bird 2006; Suzuki and Bird 2008).

Nowadays, 5mC is considered the fifth base of DNA which primary role is to maintain genome integrity and stability since it prevents transpositions, translocations and chromosomal aneuploidy. For instance, aberrant methylation of subtelomeres, which present high-density DNA repeats, may lead to certain hallmarks of genomic instability that are related to cell senescence. Proper DNA methylation of centromeric regions is necessary for their correct function and structure,

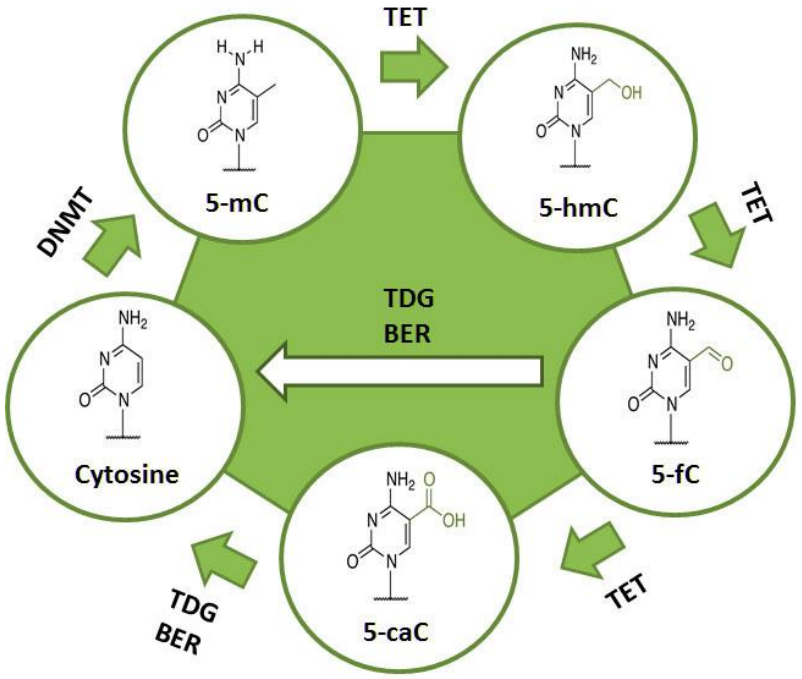


Figura 2. DNA methylation/demethylation. 5-methylcytosine (5mC) formation and its subsequent oxidation in the active demethylation pathway.

thus avoiding centromere perturbation that could lead to aneuploid cells (Deaton and Bird 2011; Defossez and Stancheva 2011).

Diverse mechanisms remove 5mC from the genome, sometimes influenced by tissue specificity. This process takes place in the absence of methylation on the newly synthesised DNA strands (passive demethylation), or by the activity of

specific enzymes (active demethylation). The passive process is probably due to the uncoupling of DNA replication and the maintenance methylation processes of DNMT1 that dilutes 5mC during cell divisions (Valinluck and Sowers 2007; Jin and Robertson 2013). The active demethylation mechanism, though, implies a series of enzymatic activities that remove methylation thanks to the sequential oxidations of 5mC. Indeed, 5mC oxidation proceeds throughout sequential steps: at first, oxidation converts 5mC into 5hmC (5-hydroxymethylcytosine), secondly into 5fC (5-formylcytosine) and lastly into 5caC (5-carboxylcytosine). After that, the machinery of base excision DNA repair (BER) delivers effective methylation removal as it recognises and targets 5fC and 5caC. Ten-Eleven Translocation (TET) family of enzymes, which accounts for the actual 5mC oxidations, comprises three members, TET1, TET2, and TET3 (Wu and Zhang 2017). Despite having similar chemical activities, the three proteins present structural differences. In fact, TET1 and 3 contain a DNA binding domain at their N-terminal, a ZF-CxxC (zinc finger-CxxC) domain, not featured by TET2. Indeed, the TET2 DNA binding domain exists as a separate gene, called IDAX, and it is important for TET2 activity regulation (Dunican, Pennings et al. 2013). TETs physiological role involves epigenetic reprogramming during embryogenesis,

development, stem cell differentiation, and cell transformation (Dawlaty, Breiling et al. 2014). Regarding 5hmC, this nucleotide is mainly present in embryonic stem cells and the CNS, thus suggesting that 5mC oxidation products do not function only as demethylation intermediates. Indeed, recent 5hmC mappings describe a highly site-specific distribution in promoters, gene bodies, and intergenic areas near genes which positively correlates with active transcription at these loci, leading to the possibility that 5hmC is the sixth base of DNA with its own functions (Pastor, Pape et al. 2011; Xu, Wu et al. 2011; Song, Yin et al. 2017). 5hmC is indeed involved in regulating genes with synaptic function in mouse and human brain (Khare, Pai et al. 2012). Differently from 5mC, 5hmC seems to have a different role in transcription regulation because it prevents the recruitment of transcription repressors, which often contain methyl-binding domain (MBD-domain), as well as the DNMTs (Branco, Ficz et al. 2011). As the knowledge on 5hmC roles are at an embryonal state, a large number of data are still emerging. Recent pieces of evidence explain that both 5fC and 5caC may influence the substrate specificities and transcriptional fidelity of RNA polymerase II. However, further structural study is required to explain the molecular impacts of 5fC or 5caC modifications on gene expression (Kellinger, Song et al. 2012).

2.2 DNA methylation in multiple sclerosis patients

Early indication that associated 5mC to multiple sclerosis was the study of *PAD2* (Peptidyl Argininedeiminase 2) gene promoter in MS NAWM.

Since the underlying evidence of such investigation was the detected global methylation loss in NAWMs by about 2/3 compared to the average control level, the promoter of *PAD2* gene, an enzyme that converts arginine into citrulline residues, helped to interpret the eventual impact of the global methylation variation on brains' MS pathogenesis (Mastronardi, Noor et al. 2007).

This study displays a similar methylation reduction rate on *PAD2* promoter, thus causing the increment of *PAD2* transcript and protein. As a direct outcome of PAD2 upregulation in NAWM, MBP undergoes an abnormal gain of citrulline residues, thus leading to myelin structural destabilisation (Moscarello, Wood et al. 1994; Kim, Mastronardi et al. 2003).

It is essential to remark that this chemical conversion exclusively hits the MS-CNS, and it is not found in other organs or neurodegenerative diseases (Mastronardi, Noor et al. 2007).

Epigenetic alterations in MS characterise the cellular components of the peripheral circulatory system. The same *PAD2* gene shows an altered epigenetic control even in

PBMCs from MS patients. As similarly to brain tissues, the *PAD2* promoter presents a reduced methylation rate that causes increased expression of the enzyme in peripheral blood cells. This mechanism might explain the development of autoimmune responses against MBP protein at the level of the central nervous system as it firstly develops in the periphery of the body (Calabrese, Zampieri et al. 2012).

Recent genome-wide sequencing studies on MS NAWMs did not confirm the previously detected global methylation loss. Instead, differentially methylated regions (DMRs) are scattered on the whole NAWMs genome and feature both hypermethylation and hypomethylation. RNA sequencing analyses conducted on these samples also confirmed that the observed DMRs occur and modulate the expression of specific gene categories involved in modulating the vulnerability of MS brains (Huynh, Garg et al. 2014).

On the one hand, hypermethylation seems to hit and repress genes that have a potential protective effect for the brain such as *BCL2L2* and *NDRG1*, two oligodendrocyte survival genes; on the other hand potentially detrimental genes for brain's health, such as the ones that encode cysteine proteases (*LGMN* and *CTSZ*), are hypomethylated and aberrantly upregulated. This notion is something that needs further exploration as eventual MS epigenetic targets may provide attractive new

targets for therapeutic strategies since the epigenetic modifications are susceptible to reshaping. A recent investigation in the MS hippocampus showed that demyelination processes coincide with other MS-specific DMRs, not shared by the previous studies.

However, deregulations in the expression of methylation/demethylation enzymes reflect on hippocampus health as they influence the transcription of genes that are crucial for maintaining the brain's health. For example, hypomethylation of *AKNA*, a gene involved in microglia regulations, promotes the expression of tumour necrosis factor-alpha (TNF-α), thus leading to neuronal death. On the contrary, *WDR81*, a neuronal survival gene, is hypermethylated and transcriptionally silenced in the MS hippocampus (Chomyk, Volsko et al. 2017).

The role of methylation in MS pathogenesis does not limit to local genomic changes, but it links to variations in the DNA methylation/demethylation machinery as well.

It is reported that TET2 modulates T-cells proliferation and differentiation in the murine model of MS. Once TET2 upregulates via 5-aza-2′-deoxycytidine (decitabine), a compound that hinders DNA methylation, the neurological worsening of the animals is prevented (Wang, Wang et al. 2017). As further evidence pointing to alterations in the

methylation/demethylation in MS is that even several *TET2* polymorphisms have been associated with disease's pathogenesis (Ottone, Cicconi et al. 2012; Ziliotto, Marchetti et al. 2019). Moreover, *TET2* is among the MS susceptibility genes discussed earlier (Beecham, Patsopoulos et al. 2013).

Noteworthy in this scenario is the fact that both genetic and epigenetic variations seem to associate *TET2* to MS susceptibility. Similarly, even the major MS risk factor, the haplotype *HLA-DR15*, is known to be affected by both genetic and epigenetic changes in the context of MS pathogenesis (Kular, Liu et al. 2018). Consequently, the patients that do not present MS-associated genetic polymorphisms they would instead probably retain epigenetic variations on these genes, hence leading to as similar consequences determined by their genetic variants.

Other studies have also demonstrated that DNMTs are involved in the regulation of myelination. DNMT1 and DNMT3A are indeed involved in the modulation of oligodendrocyte differentiation and survival (Moyon, Ma et al. 2017).

PBMCs from MS subjects show that the expression of DNA methylation-associated enzymes, TET2 and DNMT1, is significantly reduced along with aberrant methylation profiles at their promoters. Moreover, 5hmC was reduced in PBMCs from MS subjects, probably as a direct consequence of the

altered expression of the TET2 enzyme (Calabrese, Valentini et al. 2014). Recently, it was discovered that the methylation/demethylation machinery is also altered in the demyelinated brain lesions as they present TETs downregulation, DNMTs upregulation as well as a hydroxymethyl residues reduction (Chomyk, Volsko et al. 2017).

As a further demonstration that epigenetic mechanisms are involved in MS is the fact that the literature reports an increasing number of amelioration of the disease via 'epigenetic drugs.' In this direction, the EAE animal model is essential as it opens up more opportunities for designing therapeutic strategies for MS. Trichostatin A (TSA), vorinostat and valproic acid (VPA), three HDAC inhibitors, were shown in several studies to attenuate spinal cord inflammation and disease severity as well as preventing demyelination, neuronal and axonal loss in EAE mice (Camelo, Iglesias et al. 2005; Xuan, Long et al. 2012; Ge, Da et al. 2013). EAE models confirmed the effects of methylation inhibitors. Treatment with 5-aza-2′-deoxycytidine has an immunosuppressive activity on CNS and prevents EAE disease severity and manifestations (Chan, Chang et al. 2014). Despite all these data recounted to possible epigenetic therapies, the utilisation of epigenetic drugs for MS is currently at an embryonal state. However, as these

novel therapies have already confirmed their effectiveness in the treatment of cancer and autoimmune diseases, this innovative approach will be hopefully beneficial for people affected with MS.

AIM OF THE WORK

The possibility that eventual epigenetic aberrations exert a causal role in MS is a hypothesis yet. This PhD thesis aimed to fill this informational gap by tracing the epigenetic imbalance in non-affected brain tissues from MS patients back to the altered expression of the DNA methylation/demethylation enzymes.

MS is a common and heterogeneous disease in which various genes and environmental determinants contribute to its pathogenesis. However, the low concordance rate in identical twins, the mutable clinical progression, and the distinctive sex prevalence imply the association with epigenetic phenomena. Many studies revealed alterations in DNA methylation, histones, and microRNAs (miRNAs) involved in MS NAWM (Normal Appearing White Matter) samples.

The study was carried out on autoptic samples of white matter not compromised by the MS pathological autoimmunity (NAWMs). This choice was necessary to avoid potential confounding effects linked to the extreme morphological heterogeneity and cellular composition that characterise the demyelination plaques (Lucchinetti, Popescu et al. 2011; Zheleznyakova, Piket et al. 2017). Furthermore, these brain sections have been selected as they present constitutive

anomalies not determined by MS autoimmunity. We analysed epigenetic variations in the context of two severe forms of the disease, the secondarily progressive form (SPMS), and the primary progressive form (PPMS). A group of control individuals, not affected by MS or other neurodegenerations, allowed the detection of MS-related epigenetic alterations. The other MS forms, such as the relapsing-remitting form (RRMS), have not been analysed as these patients turn into SPMS ones before the age of death. The experimental plan structures into four main objectives:

a. To determine the overall levels of the different epigenetic forms of cytosine such as 5mC, 5hmC, 5fC, and 5caC (dot-blot assays).
b. To measure the expression of the main enzymes involved in DNA methylation (DNMT1, DNMT3A, DNMT3B) and DNA demethylation/oxidation (TET1, TET2, TET3, TDG, and IDAX) (qRT-PCR technique).
c. To observe local epigenetic variations in specific gene loci, putatively associated with MS (MeDip - qRT-PCR technique).

MATERIALS AND METHODS

1. Origin of brain tissue samples

UK Multiple Sclerosis Society Tissue Bank provided the brain samples of normal-appearing white matter from MS subjects.

For each patient, the analysis was conducted on three adjacent and cryopreserved NAWM sections of 2x2x1 cm from the cerebral frontal lobe.

These tissue fragments have been cut into smaller samples, weighing about 500 µg each, and stored at -80°C in the preservative solution Allprotect Tissue Reagent (Qiagen) to avoid tissue degradation and preserve RNA and DNA molecules.

The autopsy report has listed the information related to age, sex, disease duration, and disease form. The histopathological analysis of the patients' report confirmed that any disease signs,

demyelination events, or ongoing inflammations affect the samples.

2. DNA purification

Genomic DNA was purified using the optimised protocol described by Saldanha et al., as large amounts of lipids characterise the NAWM sections (Saldanha, Gannicliffe et al. 1984).

The procedure for extracting DNA from tissue starts with mincing 0,5-1 g of brain tissue in the specific buffer (10mM Tris-HCl pH 8, 5 mM EDTA, 400 mM NaCl, 1% SDS), with a ratio of 1:10 w/v (g/ml), and incubating it at 60° C for 30 min. The enzyme RNase A (Sigma), 20 ng/µl, ensured the removal of RNA contaminations on to the lysis by incubating the mixture for 1 hour at 37 ° C.

Successive removal of proteins was performed by Proteinase K addition, reaching the final suspension concentration of 0.1 µg/µl (Sigma) and leaving the sample at 60° C for about 16 hours.

The mixture is then extracted two times by adding an equal volume of Phenol: Chloroform: Isoamyl Alcohol 24: 25: 1 (Sigma). This suspension undergoes vortexing and centrifugation at maximum speed for 15 min at room

temperature (RT) to recover the aqueous phase containing the nucleic acid.

To precipitate DNA from the aqueous phase, we added sodium acetate 3 M pH 5.2 (1/10 of the mixture volume) and pure ethanol (2.5 times the suspension volume).

The samples were then incubated at -80 °C for one hour, to optimise DNA precipitation, and subsequently centrifuged at 13000 rpm for 10 min at 4 °C to recover the nucleic acid. The DNA underwent salt removal with ethanol 75%, dehydration at room temperature, and resuspension in TE solution (10 mM Tris-HCl pH 8, 1 mM EDTA). Purity and integrity of the extracted DNA was checked by spectrophotometry and agarose gel electrophoresis.

3. Dot blot technique

DNA samples are firstly denatured at 95 °C for 15 min. After three serial 1:1 dilutions in TE (1-0,25 µg), we immobilised DNA on a nitrocellulose filter (Hybond-N, GE Healthcare) via the Bio-Dot apparatus (Bio-Rad) and analysed in duplicate. DNA from two different cell types, HEK 293T cells, and peripheral blood mononuclear cells (PBMC), were used to check antibody specificity. Synthetic DNA sequences (Epigentek) retaining one specific cytosine modification allowed the evaluation of the antibody specificity.

The nucleic acids are then exposed to UV rays to fix them to the membrane. Once completed the DNA fixation, the filter undergoes incubation in a blocking solution (5% fat-free milk, 0.05% Tween 20 in PBS) for 1 hour. After the blocking phase, the membrane was probed for 1 hour with specific primary antibodies: anti-5mC 1:1000 (Epigentek), anti-5hmC 1: 5000 (Active Motif), anti-5fC 1:250 (Active Motif), anti-5caC 1:250 (Active Motif). Once completed the primary antibody incubation, the membrane is later washed three times in PBS 0.05% Tween 20 for 15 minutes to probe it with appropriate secondary antibodies for 1 hour. All the secondary antibodies are anti-IgG mouse/rabbit conjugated to HRP enzyme (horseradish peroxidase, horseradish peroxidase) (Santa Cruz Biotechnologies). The filter is then washed three times for 15 minutes in PBS 0.05% Tween 20 with to remove the secondary antibodies. The Pierce ECL Plus Western Blotting Substrate kit (Thermo Fisher Scientific) allowed the detection of the antigen-antibody complexes via a chemiluminescent reaction.

Exposition, for 1-5 min, with photosensitive photographic plates (Amersham Hyperfilm ECL) captured the light produced by the membranes.

Filter colouration with a nonspecific nucleic acid dye, 0.02% methylene blue in 0.3 M of sodium acetate (pH 5.2), for 15 min checked eventual loading differences.

The GS-800 Calibrated Densitometer (Bio-Rad) quantified the methylene blue signals. For the following quantification procedure, all density values underwent subtraction from the background (signal of a sample without nucleic acid). The ratio between the "specific" cytosine variant light signal (immunological detection) and the "nonspecific" colourimetric blue signal (methylene) related to a signal strength value of each DNA modifications. Each sample's ratio was matched to a control one, to which the ratio value was arbitrarily associated with 1.0, to provide the comparison of the obtained signals.

4. Extraction of total RNA

The RNeasy Lipid Tissue Kit (Qiagen) allowed total RNA extraction from the samples. DNase addition that removed DNA contaminations allowed a better purification of RNA. Isolated RNAs were evaluated for their concentration, purity and integrity in the same way as DNA.

5. Gene expression quantification (RT-qPCR)

5.1 Reverse transcription (RT)

RT-qPCR was used to assess transcription levels for the studied genes. 1.5-2 µg of RNA for each sample was converted into the complementary cDNA by reverse transcriptase reaction (RT), according to the manufacturer's protocol for the

High-Capacity RNA-to-cDNA Kit (Thermo Fisher Scientific). Negative controls have been obtained through reactions performed in the absence of both RNAs or reverse transcriptase enzyme. The reaction was carried out with a Gentaur XP thermocycler following the steps, times and temperatures as indicated in Table 1. Storage at -20 ° C of the samples occurred at the end of the reaction.

5.2 Gene expression evaluation (qPCR)

qPCR assays tested the expression of the genes of interest in triplicate on 20 ng of cDNA relative to the initial RNA quantity.

The amplification comprehended the TaqMan gene expression fluorescent probes (Thermo Fischer Scientific) and the reagent solution Taqman Universal Master Mix (Thermo Fisher Scientific). The qPCRs were then loaded in 96-well optical plates and evaluated with the iCycler iQ (Bio-Rad) thermal cycler following the steps, times and temperatures listed in Table 2.

Table 1

	Step 1	Step 2	Step 3
Temperature (°C)	37°C	95°C	4°C
Time	60 min	5 min	∞

Table 2

	Step 1	Step 2	step 3 (x 40 cicli)	
Temperature (°C)	50°C	95°C	95°C	60°C
Time	2 min	10 min	15 sec	1 min

TaqMan gene expression assays were performed for *TET1* (Hs04189344_g1); *TET2* (Hs00325999_m1); *TET3* (Hs00896441_m1); *IDAX* (Hs00228693_m1); *TDG* (Hs00702322_s1); *DNMT1* (Hs00945875_m1); *DNMT3A* (Hs01027162_m1); *DNMT3B* (Hs00171876_m1); *GUSB* (Hs00939627_m1); *GAPDH* (Hs02786624_g1); *XPNPEP1* (Hs00958021_m1); *AARS* (Hs00609836_m1); *HPRT1* (Hs02800695_m1); *ACTB* (Hs01060665_g1); *OSP* (Hs00194440_m1); *RBFOX3* (Hs01370654_m1); *GFAP* (Hs00909233_m1); *CD45* (Hs02519237_s1); *CD68* (Hs02836816_g1); *TNF*-α (Hs00174128_m1); *IL1B*

(Hs01555410_m1); *CXCL13* (Hs00757930_m1); *AIF1* (Hs00610419_g1); *GAS6* (Hs01090305_m1); *HLA-DPB2* (Hs01678253_m1).

For the proper comparison between different qPCR experiments, one sample was used in each plate as a calibrator to normalise the different amplifications (reference). Calibration curves estimated the amplification efficiency of each of the primer/probe pairs (serial dilutions 1:1 of the reference cDNA sample to obtain five dilution points containing 100 - 6.25 ng). The expected qPCR efficiencies were between 90 and 98%.

Among a list of housekeeping genes, *HPRT1* (hypoxanthine phosphoribosyltransferase 1), *AARS2* (alanyl—tRNA synthetase 2), *ACTB* (beta-actin), *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase), *GUSB* (glucuronidase beta) and *XPNPEP1* (X-prolyl aminopeptidase 1) it was determined which are the most suited for the expression normalisation. Two algorithms, geNorm (Vandesompele, De Preter et al. 2002) and NormFinder (Andersen, Jensen et al. 2004), have been used for such purpose. They both concluded that the geometric mean between the expression level of *GUSB* and *XPNPEP1* being the optimal normalisation strategy to carry out expression analysis.

Table 3

GeNorm		NormFinder	
Transcript	**Stability**	**Transcript**	**Stability**
HPRT1	1,794	***AARS2***	0,461
AARS2	1,771	***ACTB***	0,449
ACTB	1,501	***HPRT1***	0,421
GAPDH	1,408	***GAPDH***	0,407
GUSB	1,339	***GUSB***	0,289
XPNPEP1	1,168	***XPNPEP1***	0,135

GeNorm and NormFinder identify the optimal normalisation gene among a set of candidates. Stability values of the analysed candidate genes. The represented numerical value is inversely proportional to the stability detected among the analyse genes.

The normalised gene expression in all samples referred to the expression of the same loci in the reference sample, to which the value 1.0 was arbitrarily assigned.

6. DNA immunoprecipitation (DIP)

DNA immunoprecipitations were performed to determine eventual locus-specific CpG methylation alterations on MS susceptibility genes. The same antibodies for the dot-blot assays applied for this other technique. The three sample

cohorts (CT, SPMS and PPMS) of genomic DNA were tested via a "pooling sample method" as this strategy is less expensive and it allows faster enrichment evaluations: 12 samples were pooled to form one cohort with 1 µg of genomic DNA each, thus having pooled cohorts of 12 µg. Each sample pool was sonicated (40% amplitude; 0.5 cycles) 16 times with pulses of 10 seconds and pauses of 1 minute in 400 µl of TE. After the sixteenth sonication, a 1% agarose gel electrophoresis checked the genomic DNA for the proper fragmentations (1000 bp-300 bp). Fragmented DNA is then denatured at 95° C for 10 minutes and ice-cooled for 10 minutes to allow the antibody hybridisation with the DNA. Each antibody was incubated with 5 µg of DNA in a solution containing TE and the IP buffer (10 mM Na-phosphate, 0.14 M NaCl, 0.05% Triton x-100). 2 µg of sonicated DNA have been stored at -80° C as they serve as the INPUT for qPCRs. On the one hand, 5 µg of the fragmented DNA incubated with the specific antibody for the four cytosine modifications (anti-5mC; anti-5hmC; anti-5fC; anti-5caC); on the other hand, 5 µg incubated with a non-specific IgG antibody as an isotype control.

The DNA-antibody hybridisations were conducted overnight on a rotating platform at 4° C. The antibody-DNA complexes are then recaptured with an agarose resin (Merck Millipore Protein A Agarose for the rabbit isotypes; Merck Millipore

Protein G Agarose for the mouse isotypes). The incubation of antibody-DNA complexes with the resins was carried out for 2 h on a rotating platform at 4° C. Once the recapture is completed, the resins are washed with cold IP buffer and later digested with proteinase K (Sigma-Aldrich) to solubilise the captured DNA fragments. For each digestion, addition of 7 µL of proteinase K (10 mg/ml) to the resin and then incubation for 2 hours on a rotating platform at 55° C occurred. The samples underwent a double organic extraction, to remove protein contaminations, with an equal volume of phenol:chloroform: isoamyl alcohol 25:24:1. DNA fragments precipitation is carried out with two volumes of pure ethanol, 400 mM of NaCl and 0.5 µg/µL glycogen (Bio Protech), then cooled on ice for 30 minutes. Precipitated DNA was later recovered by centrifugation at 14,000 rpm for 30 minutes at 4° C. The supernatant is discarded, and the sample was dehydrated at room temperature and resuspended in 50 µl of nuclease-free water. qPCR assays evaluated the enriched fractions by using site-specific primers: the experiments tested 14 µl of immunoprecipitations and 10 ng of the input sample. Each reaction was carried out using specific primers (0.4 µM) and the reagent SYBR Premix EX Taq (Takara) under the experimental conditions suggested by the manufacturer. The

following equation: 100 * 2 ^ (Ct input - Ct (IP)) calculated the enrichment value in the immunoprecipitated samples.

Ct input defines the average value of the cycle threshold for the input samples, while Ct (IP) the average value of the cycles threshold for the immunoprecipitated sample. Table 4 shows the sequence of primers in PCR assays. Before running the actual experiments, synthetic sequences for 5mC/5hmC cytosine variants (Zymoresearch) checked the technique efficiency as well as the antibody specificity. These DNA molecules hold the same nucleotide sequence with the only difference being that each one contains either 100% unmodified cytosines, 5-methylcytosines, or 5-hydroxymethylcytosines. Based on the amount genomes present in 12 µg, we added an appropriate amount of DNA standard so that it would have been considered as a single copy of a gene.

7. RT-qPCRs for the expression evaluation of the candidate genes

Along with the locus-specific evaluations of the enrichments, the expression of these genes was evaluated as well. The amplification was carried out starting from 20 ng of cDNA (relative to the amount of RNA subjected to retro-transcription) using specific primers (0.4 µM) and the reagent SYBR Premix

Table 4

Primer	Forward-Primer	Reverse-Primer
GPC5	TTTCACCGTGTTGACCAGGTT	AAATATGGTATACAGGCCGGG
IFI30	ATTTCTTCCAATAGGGCTGG	AAGACTCACCACCCCAACTTT
KIF1A site 1	TTTCCTGGGGCTGCAGAGA	GGTTTCCACCTTCCCATCA
KIF1A site 2	TCTGCCAAGGAAGGGAGGA	TGATGCGGGAGATGTACGA
KIF1A site 3	AGCGAGCGTTGTTGGAAAA	TGACAAAGGTGCGTTCGTAA
LEPR site 1	TGTTGAAAACCCAAGTCCCA	TGGCTTGGACTTTGGATGAA
LEPR site 2	GAGGCCTTTAAAAATTAGCCA	CGCTGGGTGATATGGAAGA
SYK	AAGAAGGGAAAGCCACAGCAT	TTCTGCCCTCCTGCTTCCT
GAPDH	TACTAGCGGTTTTACGGGCG	TCGAACAGGAGGAGCAGAGAGCGA
Spike-in	GAATCAAATTTGGCCGCA	GCCCTTTCGTCTTCAAGAAT

Primer sequences for the analysis of the immunoprecipitation enrichment of the candidate gene loci.

EX Taq (Takara) in the experimental conditions suggested by the manufacturer. Each sample was measured in triplicate, and the amplification was conducted in 96-well optical plates with an ICycler IQ (Bio-Rad) thermal cycler. For the calculation of the expression, the procedure already described in paragraph 3.5.2 has been adopted. The sequences of the used primers are shown in Table 5.

Table 5

Primer	Forward-Primer	Reverse-Primer
GPC5	GCCAGGATATGCAGCAGTTT	TTGTTTGATGAGAGTTTCAAGGG
IFI30	TGACCATTGTCTGCATGGAA	TCCATGATAGTGTCTGGCGA
KIF1A	TGAGACAGGCAGCCATATCC	GAGACAAGATGTTGGGGTCG
LEPR	AACCTTCAATTCCAGATTCGC	TCTGGAACTGGGAGACTGACA
SYK	GTGAAGCAGACATGGAACCTG	AGCTTCTCCAGCTGAGGCTT

Primer sequences for the expression analysis of candidate gene loci.

8. Statistical analysis

The characteristics of the population were studied using the mean and standard deviation (SD) for continuous variables (age, disease duration, post-mortem interval) and frequencies (%) for categorical variables (gender, disease activity). Eventual differences in these characteristics between cohorts were tested with the one-way analysis of variance tests (ANOVA) (continuous variables) or the chi-square test (prevalence, for categorical variables). The differences in the measured variables between the experimental groups were analysed with the Kruskal-Wallis (KW) and generalized linear model (GLM) tests. The paired comparisons (between the individual groups of samples) were performed with the Mann-

Whitney and Bonferroni methods. The GLM analysis was used to evaluate the influence of potential confounding variables on the differences between the experimental groups. The association between variables was analysed with Spearman and Pearson linear correlation.

All statistical analyses were performed using the SPSS software (SPSS Inc., Version 23.0).

RESULTS

1. Subjects and tissue

The experiments compared NAWMs from 29 patients with clinically definite MS, 15 SPMS and 14 PPMS, with NWMs (Normal White Matters) from 13 control subjects not-affected by neurodegenerations (Table 6). As the autopsy reports show, the three sample cohorts do not present any differences in regards to age, gender, postmortem interval (PMI: the elapsed time since a subject has died), disease duration (DD) or disease activity presence at the time of death.

Active patients are the ones that displayed ongoing demyelinations as revealed by the histopathological examination in the autoptic reports.

Being the samples size not large enough to determine if the normal distribution characterises the measured values, we applied both non-parametric and parametric statistical

Table 6

	Controls	SPMS	PPMS	*P*
N.	13	15	14	
Age (years)	73,08 ± 10	64,47 ± 13,43	66,07 ± 16,81	0,216[§]
% Females (n)	46 (6)	60 (9)	57 (8)	0,745[‡]
PMI (hours)	18,00 ± 6,22	17,73 ± 7,12	18,92 ± 5,74	0,346[§]
DD (years)		31,86 ± 11,49	30,36 ± 13,66	0,769[$]
% Active patients (n)		60 (9)	50 (7)	0,588[‡]

Patients data survey. The reported values are mean ± SD. Percentages (number) describe female prevalence and active patients at the time of death. The reported p-values refer to the following statistical tests: one-way [§]ANOVA; [‡]Chi-squared test; [$]Student's t-test.

procedures to verify the occurrence of differences between subjects groups.

The statistics tested the impact of these parameters on the examined variables by using two approaches: the Generalized Linear Model (GLM) and the Kruskal-Wallis test (KW).

2. NAWMs' cell composition and inflammation

Before running the experiments, some preliminary tests checked the white matter samples for variations in their cell composition, the eventual presence of immune cells and the

inflammation rate via the expression evaluation of specific markers (qPCRs).

RBFOX3, *OSP* and *GFAP* transcripts have been measured as markers for neurons (Gusel'nikova and Korzhevskiy 2015), oligodendrocytes (Michalski, Keck et al. 2018) and glial cells (Yang and Wang 2016), respectively. *CD45* (the common leukocyte antigen) and *CD68* (cluster of differentiation 68) worked as leukocyte and macrophage markers (Altin and Sloan 1997; Chistiakov, Killingsworth et al. 2017). The expression of several inflammation factors, involved in MS pathogenesis, has been measured as well (*AIF1*, *CXCL13*, *GAS6*, *HLA-DPB2*, *IL-1β*, *TNF-α*) (Khademi, Kockum et al. 2011; Rossi, Motta et al. 2014; Rossi, Studer et al. 2014; Bellan, Pirisi et al. 2016; Chinnasamy, Lutz et al. 2016; Zrzavy, Hametner et al. 2017). As Table 7 shows, the analysed samples do not present differences in terms of cell composition, immune cells presence or inflammation.

Table 7

	Cohorts				
	Median (IQR)				
	CT	SPMS	PPMS		
Stat	***a***	***b***	***c***	***P* (KW)***	***P* (GLM)[§]**
OSP	2.730 (6.415)	4.000 (2.439)	3.693 (6.978)	0,517	0,626
RBOFX3	1.025 (2,133)	2.166 (2,640)	1.027 (1,090)	0,402	0,384
GFAP	0.382 (0.490)	0.397 (0.516)	0.385 (0.398)	0,918	0,996
CD45	0.693 (1.318)	0.59 (0.608)	0.845 (1.356)	0,591	0,475
CD68	0.847 (1.410)	0.628 (0.812)	0.940 (0.957)	0,390	0,179
TNF-α	0.533 (0.602)	0.439 (0.410)	0.578 (0.704)	0,537	0,724
IL-1β	0.046 (0.096)	0.177 (0.167)	0.058 (0.104)	0,121	0,742
CXCL13	0.562 (0.855)	0.464 (0.631)	0.508 (0.677)	0,870	0,511
AIF1	1.023 (1,762)	0.905 (1,504)	0.768 (0,979)	0,638	0,594
GAS6	0.674 (0,524)	1.033 (0,861)	0.654 (0,859)	0,872	0,054
HLA-DPB2	1.026 (1,055)	1.054 (1,617)	1.516 (1,126)	0,160	0,129

NAWMs' (Normal Appearing White Matter) cell composition and inflammation. *KW test: non-parametric comparison with the Kruskal-Wallis test. Comparison data for pairs derived from the Mann-Whitney U test. The reported data corresponds to median and interquartile range (IQR). [§]GLM: comparison by generalized linear model. The model includes the effects of age, gender and PMI as covariates.

Table 8

	Cohorts				
	Median (IQR)				
	CT	SPMS	PPMS		
Stat	***a***	***b***	***c***	***P* (KW)***	***P* (GLM)[§]**
5mC	1.347 (0.548)	1.228 (0.916)	1.265 (0.259)	0,898	0,161
5hmC	1.730 (0.300)[b,c]	1.570 (0.620)[a]	1.255 (0.525)[a]	**0,002**	**0,008**
5fC	0.294 (0.126)	0.291 (0.099)	0.268 (0.116)	0,203	0,594
5caC	4.295 (1.999)	2.939 (2.764)	3.360 (3.876)	0,149	**0,047**

	Cohorts				
	Median (IQR)				
	CT	SPMS	PPMS		
Stat	***a***	***b***	***c***	***P* (KW)***	***P* (GLM)[§]**
TET1	1.431 (1.215)	1.316 (1.270)	1.228 (0.866)	0,919	**0,040**
TET2	8.45 (8.345)[b, c]	3.810 (2.08)[a]	4.84 (1.705)[a]	**0,001**	**0,013**
IDAX	4.176 (2.998)[b]	6.353 (5.108)[a, c]	4.441 (2.551)[b]	**0,006**	**0,004**
TET3	1.425 (0.595)	1.769 (0.840)	1.697 (0.845)	0,534	0,719
DNMT1	0.540 (0.261)	0.587 (0.141)	0.484 (0.330)	0,315	0,487
DNMT3A	2.955 (0.807)	2.603 (0.899)	2.419 (1.080)	0,621	0,417
DNMT3B	0.072 (0.067)	0.050 (0.048)	0.041 (0.032)	0,073	**0,039**
TDG	0.404 (0.197)	0.394 (0.205)	0.432 (0.220)	0,677	0,255

3. Decrease of 5-hydroxymethylcytosine in NAWM samples

The isolated DNA from NAWM samples were used to examine the relative cytosine modifications (5mC; 5hmC; 5caC; 5fC) content via dot-blot assays.

The dot-blots also examined the DNA of HEK-293-T cell line and PBMC cells from blood; the former worked as negative controls and the latter as a positive control for the antibody specificity (data not shown).

Overall, the experiments showed that 5hmC levels were significantly reduced in the MS groups compared to the controls (Table 8), especially in the PPMS group than in the SPMS group (Figure 3). Nevertheless, the MS cohorts do not deviate from the controls in terms of 5mC contents (Table 8). Interestingly, MS samples also show a reduction of both 5fC and 5caC contents that reach a sufficient statistical significance only with the GLM test (Table 8).

Cytosine modifications' levels and methylation/ demethylation enzymes' expressions. *KW test: non-parametric comparison with the Kruskal-Wallis test. Comparison data for pairs derived from the Mann-Whitney U test. The reported data corresponds to the median and interquartile range (IQR). The values in bold indicate the groups with significant differences (P <0.05). §GLM: comparison by the generalized linear model. The model includes the effects of age, gender and PMI as covariates.

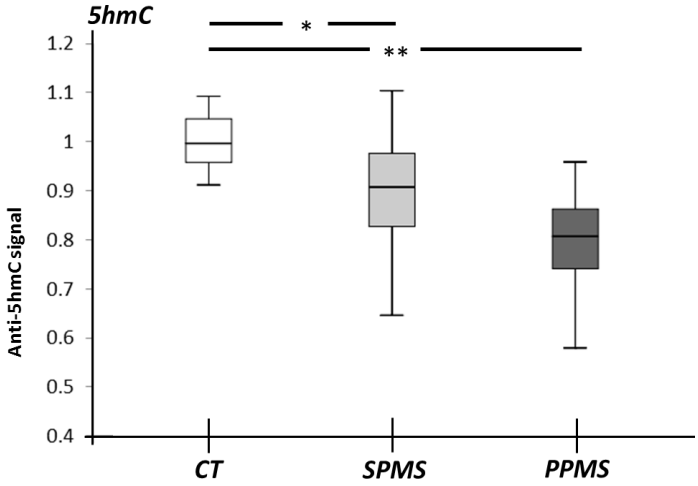


Figure 3. 5hmC reduces in SPMS (Secondary Progressive MS) and PPMS (Primary Progressive MS) NAWMs (Normal Appearing White Matter). Global 5hmC level as detected by Dot-blot assay in control (CT), SPMS and PPMS NAWM specimens. The relative levels were re-calculated taking the 5hmC levels in control as 1. The box plot displays the medians and the distribution of the measurements. The statistical significance refers to the Kruskal-Wallis test followed by the Mann-Whitney U test for the comparison between groups. * $p < 0.05$, ** $0.01 < p < 0.05$.

4. *TET2* and *IDAX* genes are transcriptionally deregulated in MS NAWMs

We, consequently, checked the MS NAWM samples for defects in DNA methylation/demethylation machinery. qPCR experiments assessed the expression of the maintenance/de novo DNA methylation enzymes, *DNMT1*, *DNMT3a* and

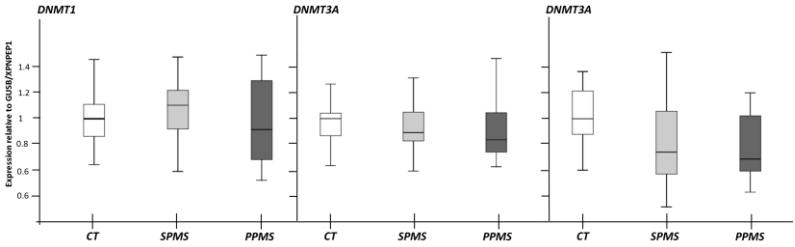
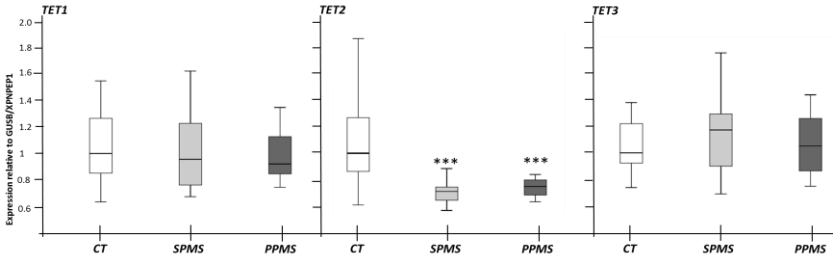


Figure 4. Methylation enzymes gene expression. *DNMTs* family gene expression was measured by qPCR. To normalise the expressions, *GUSB* and *XPNPEP1* geometric mean was used and the relative levels were calculated taking the control as 1. The box plot displays the medians and the distribution of the measurements.

DNMT3b, as well as the proteins accountable for DNA demethylation, *TET1*, *TET2/IDAX*, *TET3* and *TDG*.

The geometric mean of *GUSB* and *XPNPEP1* transcript levels normalised the expressions. The expression results of the methylation/demethylation machinery revealed that in NAWMs, the expression of *TET2* is down-regulated in both MS cohorts (A). Furthermore, the protein that recruits *TET2* on DNA, *IDAX*, is up-regulated only in SPMS patients. All the methylation proteins do not vary significantly in as well as no discrepancies feature *TET1* and *TET3* transcripts.

A



B

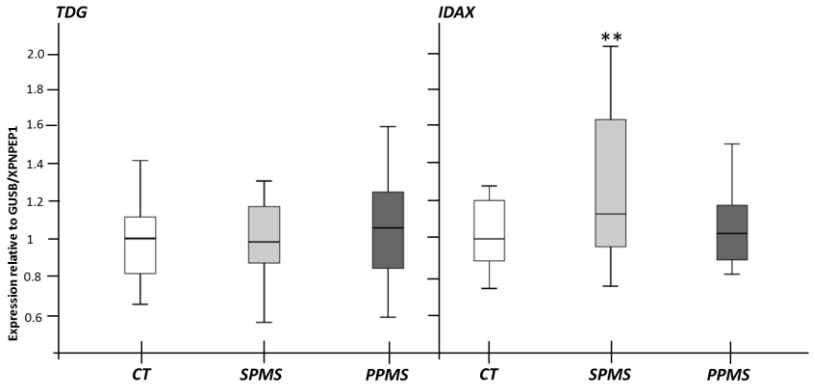


Figure 5. Demethylation enzymes gene expression. *TETs* family gene expression was measured by qPCR (A). The DNA binding protein of *TET2* (*IDAX*) and *TDG* transcripts were also measured as well (B). To normalise the expressions, *GUSB* and *XPNPEP1* geometric mean was used and the relative levels were calculated taking the control as 1. The box plot displays the medians and the distribution of the measurements. The statistical significance refers to the Kruskal-Wallis test followed by the Mann-Whitney U test for the comparison between groups. * $p < 0.05$, ** $0.01 < p < 0.05$.*** $p < 0.01$.

5. Relationship between 5hmC, *TET2* and *IDAX* with NAWMs' cell composition and inflammation

The markers for inflammatory events, cell composition and immune cells presence exhibit a significant positive correlation between them, while they correlate negatively with the oligodendrocyte (OSP) marker (data not shown). This data confirms the well-known relationship between inflammation and the loss of CNS myelin-forming cells, even in the plaque-free areas of the brain. Despite having diverse significances in regards to these markers, with the GLM test, only the neuronal marker, *RBFOX3*, manifests an association with 5hmC (partial correlation with age, gender and groups as control variables, data not shown). Besides, the Wald Chi-Square tests and the GLMs showed that none of the measured markers removes the discovered differences. Therefore, patients' groups exert the strongest effect on determining the 5hmC, *TET2* and *IDAX* variations (p-values after GLM's correction: 5hmC, <0.001; *TET2*, <0.001; *IDAX*, 0.005).

Table 9

Source	5hmC			TET2			IDAX		
	Wald Chi-Square	df	Sig.	Wald Chi-Square	df	Sig.	Wald Chi-Square	df	Sig.
Cohort type	137,586	2	**0,000**	39,027	2	**0,000**	10,499	2	**0,005**
Age	0,303	1	0,582	2,484	1	0,115	0,817	1	0,366
Gender	21,833	1	**0,004**	1,481	1	0,224	1,333	1	0,248
OSP	3,62	1	0,057	16,709	1	**0,000**	0,027	1	0,870
RBFOX3	57,106	1	**0,000**	4,696	1	**0,030**	1,42	1	0,233
GFAP	0,009	1	0,925	0,652	1	0,419	0,098	1	0,755
CD45	11,36	1	**0,001**	0,797	1	0,372	8,814	1	0,003
CD68	38,488	1	**0,000**	2,918	1	0,088	14,755	1	**0,000**
TNF-α	2,092	1	0,148	1,225	1	0,268	12,06	1	0,001
IL-1β	0,46	1	0,498	0,366	1	0,545	13,705	1	**0,000**
CXCL13	9,211	1	**0,002**	0,107	1	0,744	0,069	1	0,793
AIF-1	1,945	1	0,163	0,006	1	0,938	8,823	1	**0,003**
GAS6	0,317	1	0,573	1,946	1	0,163	5,136	1	**0,023**
HLA-DPB2	49,381	1	**0,000**	15,383	1	**0,000**	7,482	1	**0,006**

Association between inflammation events and cell compositions on 5hmC, *TET2* and *IDAX*. The Wald Chi-Square test examines and weights whether the independent variables have a significant relationship with a tested dependent variable (5hmC, *TET2*, *IDAX*). Along with the markers of inflammation and cell composition, the analysis accounted for patients' age and gender. The reported values refer to the Generalized Linear Model (GLM) test.

6. Association of 5hmC, *TET2* and *IDAX* levels with the pathological activity and duration.

The MS samples were then stratified based on the presence or absence of signs of active demyelination in NAWMs. Though this cohort arrangement corresponds to higher levels of markers associated with inflammation and reduced oligodendrocyte marker levels (*OSP*) in the active NAWMs, none of these changes are statistically significant except *GAS6*, both in KW and GLM tests, and *TNF-α* solely after GLM analysis (Table 10).

No significant differences feature the groups with active and inactive demyelination concerning 5hmC, *TET2* and *IDAX* levels. Furthermore, 5hmC, *TET2* or *IDAX* parameters did not associate with disease duration (data not shown).

7. Association between 5hmC contents and *TET2-IDAX* enzyme expression

Since TET2 and IDAX lead to 5hmC formation (Wu and Zhang 2017), their expression (Table 8 and Figures 5) might associate to the observed 5hmC levels between sample groups (Table 8 and Figure 4). This hypothesis was tested by correlation analysis carried out on the whole population (Table 11). No association between 5hmC and *IDAX* or *TET2* was

Table 10

	Disease activity			
	Inactive	**Active**	***P* (KW)***	***P* (GLM)[§]**
5hmC	1.635 (0.825)	1.190 (0.5)	0,323	0,666
TET2	4.22 (3.297)	4.150 (1.2)	0,981	0,738
IDAX	5.231 (4.749)	5.790 (4.754)	0,323	0,903
OSP	4.6 (5.270)	3.56 (3.024)	0,755	0,946
RBFOX3	1,007 (2,46)	2,433 (1.51)	0,286	0,816
GFAP	0.404 (0.410)	0.334 (0.346)	0,548	0,728
CD45	0.524 (0.640)	0.740 (0.825)	0,183	0,253
CD68	0.655 (0.785)	0.628 (1.04)	0,548	0,632
TNF-α	0.276 (0.518)	0.488 (0.795)	0,167	**0,013**
IL-1β	0.077 (0.138)	0.144 (0.175)	0,126	0,092
CXCL13	0.433 (0.519)	0.605 (0.564)	0,456	0,619
AIF-1	0.645 (2.27)	0.688 (0.658)	0,808	0,147
GAS6	0.569 (0.35)	1.235 (0.55)	**0,003**	**0,003**
HLA-DPB2	1,166 (1.28)	1.756 (5.90)	0,547	0,718

Comparison of selected parameters between the samples with inactive and active MS. *KW test: non-parametric comparison with the Kruskal-Wallis test. Comparison data for pairs derived from the Mann-Whitney U test. The reported data corresponds to the median and interquartile range (IQR). The values in bold indicate the groups with significant differences ($P < 0.05$). [§]GLM: comparison by the generalized linear model. The model includes the effects of age, gender and PMI as covariates.

Table 11

		5hmC		
		Spearman rho	**Pearson r**	**Partial Correlation§**
5hmC	**Correlation Coefficient**	1	1	1
	Sig. (2-tailed)	-	-	-
TET2	**Correlation Coefficient**	0,218	0,313	-0,115
	Sig. (2-tailed)	0,166	0,044*	0,539
IDAX	**Correlation Coefficient.**	0,221	0,219	0,316
	Sig. (2-tailed)	0,159	0,164	0,083
IDAX+TET2	**Correlation Coefficient**	0,608	0,566	0,373
	Sig. (2-code)	**< 0,001**	**< 0,001**	**0,039****

Correlation between 5hmC and *TET2*, *IDAX* and *TET2+IDAX*. §Partial correlation measures the degree of association between the variables and 5hmC considering as confounding variables age, gender, PMI and cohort type. The significances refer to the correlation tests (Spearman, Pearson and Partial correlation).

found, except for a slight Pearson correlation between *TET2* and 5hmC. All of the correlation tests, however, show that the

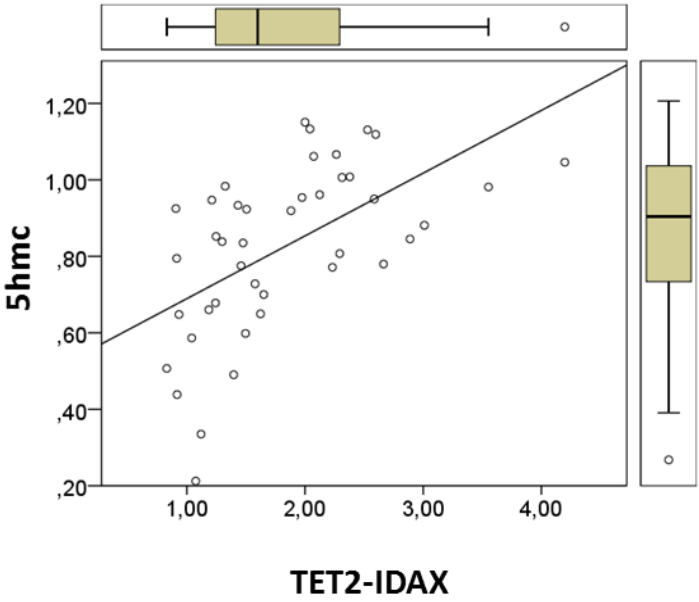


Figure 6. Correlation between 5hmC and the *IDAX-TET2* sum. Interpolation graph and regression line between 5hmC values and the *IDAX-TET2* sum.

sum of *TET2* and *IDAX* expressions strongly associate to the observed 5hmC variations (Table 11 and Figure 6). Lastly, neither *IDAX* nor *TET2* show any further association with other epigenetic modifications (data not shown).

8. Locus-specific analysis of 5mC oxidation derivatives

The following experimental step was to observe whether the global 5hmC reductions in MS NAWM reflect in a locus-specific manner on the methylation profile of MS-related genes. Therefore, we investigated the local pattern of 5mC, and

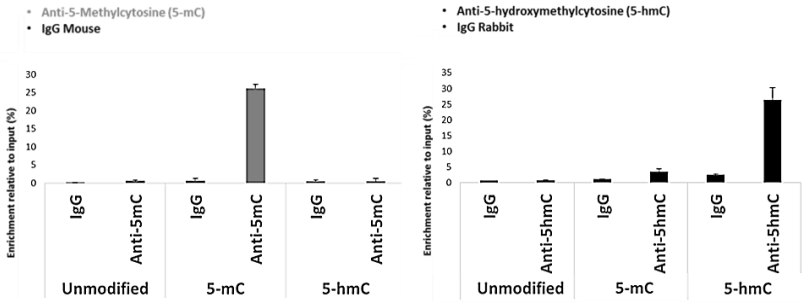
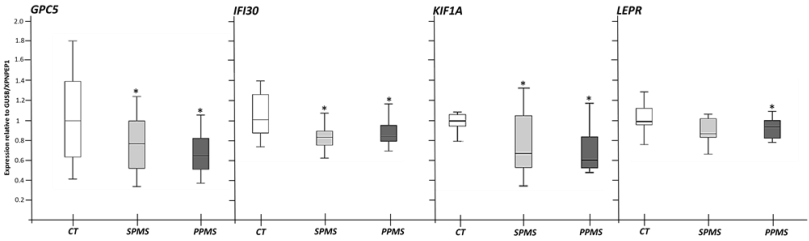


Figure 7. DNA immunoprecipitations on standard sequences. The spike-in DNA standards demonstrated both the technique efficiency as well as the preferential binding of the antibodies (anti-5mC; anti-5hmC) to 5mC-standard and 5hmC-standard, respectively. Each experiment was executed along with a mock-IP (normal rabbit for anti-5hmC and normal mouse for anti-5mC).

its oxidation derivatives (5hmC, 5fC, 5caC), as well as the expression levels of such genes. We carried out this analysis via DNA immunoprecipitation assays. Both the efficiency of the DNA immunoprecipitations and the antibody specificity were checked by running the experimental procedure on spike-in control DNA standards that hold the same nucleotide sequence with the only difference being that each one contains either 100% unmodified cytosines, methylated cytosines or hydroxymethylated cytosines.

The preliminary results on the standard sequences display that the antibodies recognise the cytosine variants which are specifically raised against, thus confirming the DNA immunoprecipitation procedure being reliable and precise (Figure 7). We then concentrated our following experiments on both the expression and the methylation pattern of genes implicated in brains' MS pathogenesis. The investigated positions should be mainly 5hmC enriched, as the DNA immunoprecipitations allow a feasible methylation pattern study on such sites. An exhaustive oxidative demethylation analysis with this technique qPCR, indeed, is not achievable on positions that are supposedly not 5hmC modulated/enriched. (Huynh, Garg et al. 2014; Ellison, Bradley-Whitman et al. 2017). The information on 5hmC enriched positions was obtained from healthy white matter hydroxymethylation datasets (Sanchez-Mut, Heyn et al. 2017). Through a database analysis, five genes have been identified that respected such criteria: kinesin family member 1A (*KIF1A*), Interferon Gamma-Inducible Protein 30 Preproprotein (*IFI30*), Glypican Proteoglycan 5 (*GPC5*), Leptin Receptor (*LEPR*) and Spleen Tyrosine Kinase (*SYK*) (Figure 8). Firstly, we verified the eventually altered expression of such genes in our samples as a further demonstration of their putative involvement in MS. The expression of *GAPDH* gene, which is not involved in MS

A



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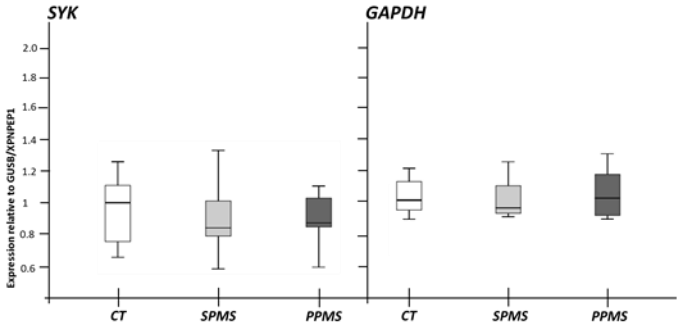

Figure 8. MS related gene expression. Level of the transcripts of the *KIF1A*, *LEPR*, *IFI30*, *GPC5*, *SYK* and *GAPDH* genes evaluated in real-time RT-PCR in the CT (Control), SPMS (Secondary Progressive MS) and PPMS (Primary Progressive MS) samples. To normalise the expressions, *GUSB* and *XPNPEP1* geometric mean was used and the relative levels were calculated taking the control as 1. The box plot displays the medians and the distribution of the measurements. The statistical significance refers to the Kruskal-Wallis test followed by the Mann-Whitney U test for the comparison between groups. * $p < 0.05$, ** $0.01 < p < 0.05$.*** $p < 0.01$.

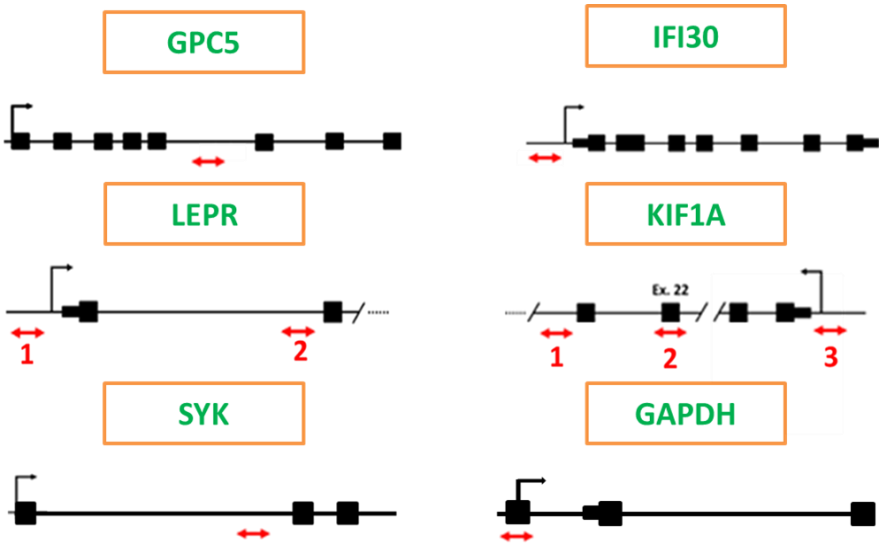

Figure 9. Positions assayed for their methylation content on MS-related genes. DNA immunoprecipitations investigated the methylation pattern on a total of nine positions (three positions for *KIF1A* and two for *LEPR*) for cytosines' modifications presence.

pathogenesis, was tested as well. Figure 8 shows that four selected genes deregulate in our NAWM samples, except for *SYK*. Furthermore, we confirmed that *GAPDH* expression does not change in MS NAWM samples (Figure 8).

After analysing the expression of these genes, we determined if the local 5mC contents are varied in the selected positions. The experiments tested a total of nine positions (three positions for *KIF1A* and two positions for *LEPR*) for 5mC nucleotide presence.

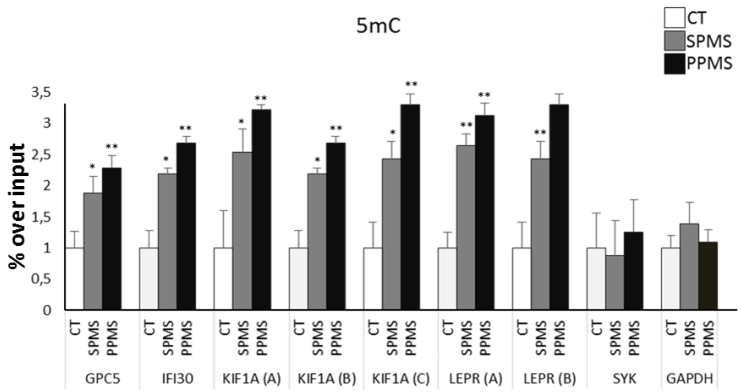

Figure 10. 5mC enrichments Methylation enrichments (MeDIP) of *GPC5, IFI30, KIF1A, LEPR, SYK* and *GAPDH* in the three sample pools representative of the three cohorts. The data, percentage over input where the control cohort was taken as 1, are represented as mean ± S.E.M. of three experimental replicates. The significance refers to the ANOVA test followed by the Bonferroni test for the comparison between the MS and CT group. * $p < 0.05$, ** $p < 0.01$.

The 5mC-immunoprecipitations show that the MS samples present a local 5mC increase on all genes except for *SYK* and *GAPDH* loci. This observation confirms that the variations in methylation content is shared only by the genes that change in their transcription (Figure 8). Interestingly, this increment is particularly evident in the PPMS cohort (Figure 10).

Since the detected increase of 5mC on these positions is likely due to a defective demethylation mechanism in MS samples, it

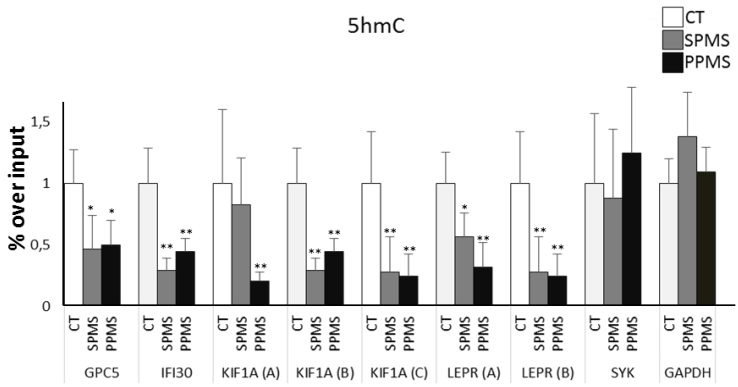

Figure 11. 5hmC enrichments. Hydroxymethylation enrichments (hMeDIP) of *GPC5*, *IFI30*, *KIF1A*, *LEPR*, *SYK* and *GAPDH* in the three sample pools representative of the three cohorts. The data, percentage over input where the control cohort was taken as 1, are represented as mean ± S.E.M. of three experimental replicates. The significance refers to the ANOVA test followed by the Bonferroni test for the comparison between the MS and CT group. * $p < 0.05$, ** $p < 0.01$.

is reasonable to assume that a parallel decrement of 5hmC may also take place on the studied genes, thus confirming further the global hydroxymethylation trend.

To confirm this idea, we also assayed these genomic sites for the presence of 5hmC. The experiments show that the 5mC accumulation in the four genes is accompanied by a loss of 5hmC, which is more evident in the PPMS cohort as well. Coherently with the genes that do not vary 5mC contents,

GAPDH and *SYK* do not show any 5hmC decrement. This data further demonstrates that a defect in the global hydroxymethylation content (Figure 11) may reflect changes in specific positions that are associated with genes involved in MS. The detection of 5mC gain on the altered genes, along with the 5hmC decline, lead us to further enrichment analysis. As the methylation mechanism is supposedly defective in the NAWMs, it is conceivable that including 5fC and 5caC may endure some fluctuations in their content. DNA immunoprecipitation experiments on these two cytosine variants explored this hypothesis. The obtained results pointed to an evident reduction of 5fC, especially in the PPMS group, while the 5caC decrement was weaker by comparison and barely reaching statistical significance (Figure 12).

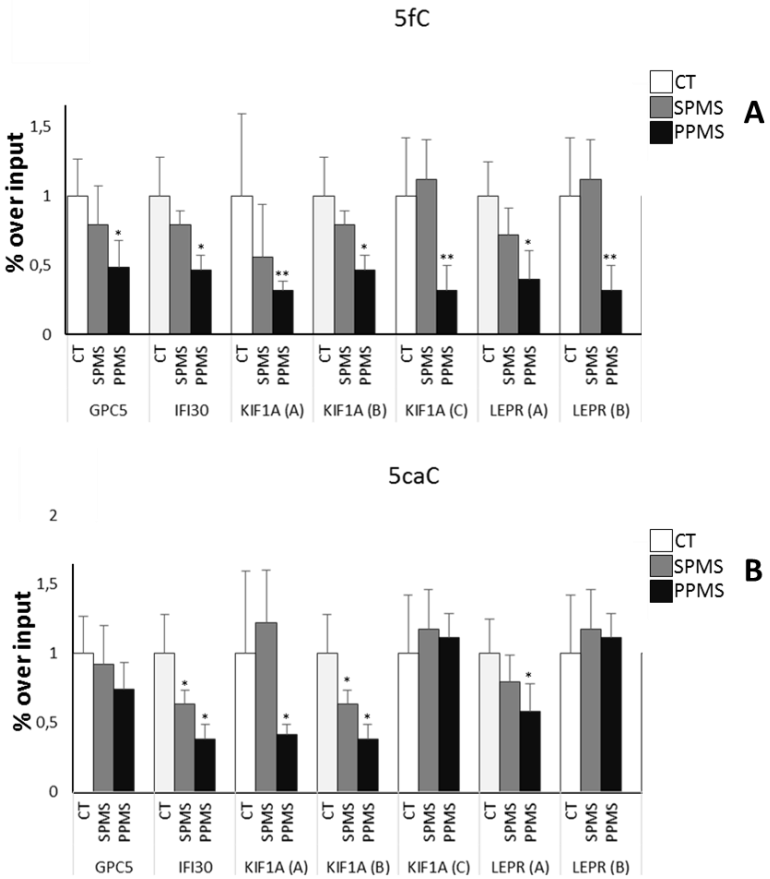

Figure 12. 5fC and 5caC enrichments. 5-formylcytosine (A) and 5-carboxylcytosine (B) enrichments of *GPC5*, *IFI30*, *KIF1A*, *LEPR*, *SYK* and *GAPDH* in the three sample pools representative of the three cohorts. The data, percentage over input where the control cohort was taken as 1, are represented as mean ± S.E.M. of three experimental replicates. The significance refers to the ANOVA test followed by the Bonferroni test for the comparison between the MS and CT group. * $p < 0.05$, ** $p < 0.01$.

DISCUSSION

MS aetiology has yet to be determined, but the interest in the epigenetic events that link to this disease is considerably growing. Indeed, a number of epigenetic changes that relate to MS seem to characterise both the central nervous system (CNS), the organ that suffers the most from the consequences of the disease, as well as other districts or tissues such as the blood immune cells (PBMCs) (Calabrese, Valentini et al. 2014; Huynh, Garg et al. 2014; Chomyk, Volsko et al. 2017).

These epigenetic variations concern both the chromatin structure, through aberrant histone modifications, as well as DNA modifications, through abnormal presence in its DNA cytosine variants (5mC, 5hmC, 5fC, and 5caC). Their direct consequence is the transcriptional alteration of genes that are associated with the pathogenesis of MS. Although the not so clear relationship between MS and its epigenetic component,

the literature suggests that MS development probably links to environmental risk factors such as smoking, vitamin D deficiency and Epstein-Barr virus infections. In this scenario, the epigenetic variations may mediate the causal effects of these factors on MS establishment (Huynh and Casaccia 2013). However, since both the origin of these variations and their actual relevance in MS pathogenesis are mostly subtle, the possibility that the associated epigenetic aberrations may exert a causal role in MS remains a hypothesis yet.

This investigation aims to find the contextual data to fill the informational gap by tracing the epigenetic imbalance observed in MS brains back to the altered expression of the DNA methylation/demethylation enzymes. Indeed, both abnormal remodelling in the epigenetic profile as well as in the expression of the DNA methylation/demethylation proteins is well documented in other neurodegenerative disorders (Ellison, Bradley-Whitman et al. 2017).

Besides, a previous scientific result from our group revealed that PBMCs from MS patients exhibit a global loss of DNA hydroxymethylation that associates with the down-regulation of TET2, an enzyme accountable for methyl removal from cytosines (Calabrese, Valentini et al. 2014).

The data presented in this doctoral thesis work show for the first time that brain areas not damaged by MS autoimmunity,

the brain normal-appearing white matters (NAWMs), are also characterised by a 5hmC deficiency that associates with the aberrant expressions of two components of the DNA demethylation machinery, TET2 enzyme, and its modulating protein IDAX.

Despite not having the characteristic scars that are the main consequential feature of MS, these brain sections have been analysed because several studies have highlighted that constitutive anomalies are already in the NAWM, thus affecting the brain haemodynamic and microstructure (Zhong and Lou 2016). Also, the structure of NAWMs farther from the lesions may feature blood-brain barrier alterations, fibrinogen leakage and an initial micro-plaque formation (Vrenken, Geurts et al. 2006).

Thus, it is plausible to expect that changes in the epigenetic profile of MS may also mark the brain regions not yet damaged by the inflammation and they might play a prodromal role in the disease establishment before the autoimmune phenomenon takes place.

We observed that DNA from NAWM specimens displays a global 5hmC drop in both MS cohorts if compared to the control subjects. Interestingly, this decline is more noticeable in the primary progressive MS patients (PPMS), which clinically feature the constant accumulation of disabilities from

the first onset, than in the secondary progressive MS subjects (SPMS), that usually start with the less severe relapsing-remitting MS type (Dendrou, Fugger et al. 2015). Hence, the detected epigenetic differences may also propose a link between disease progression and the 5hmC loss in NAWM samples.

However, 5-hydroxymethylcytosine contents in both MS groups are much more dispersed than the control ones, thus hinting that the 5hmC evaluated level may be related to inter-individual differences exclusively among MS samples. The possible lack of sample homogeneity, regarding the examined anatomical brain site, could explain this difference. This circumstance, however, is not seen among the control samples despite sharing with the two MS cohorts the same experimental sampling premise. Besides, MS presents itself with a plethora of forms and neurological symptoms as a result of different levels of immune cell infiltrations, diverse damaged areas as well as various damage's entities. Consequently, the observed 5hmC deviations could be produced by such tissue contrivances that are shared only by the MS samples. However, the Wald-Chi square in the GLM revealed that inflammation and cell composition did not significantly alter the variations of 5hmC, *TET2* and *IDAX* among patients' type.

Moreover, the identified loss of 5hmC does not associate with a simultaneous global increase in 5fC and 5caC presence, thus suggesting the possibility that the 5-hydroxymethylcytosine reduction does not link to an enhanced active demethylation process in the MS NAWMs.

Instead, it is more likely to hypothesise that the detected 5hmC losses associate with the failure of MS NAWMs to convert 5mC in its subsequent demethylation intermediates (5hmC, 5fC, and 5caC).

Indeed, a not-significant reduction trend marks MS NAWMs for both 5fC and 5caC presence. This implies that the removal of each demethylation intermediates follows a contemporary content increase in the precursor 5mC, hence causing DNA hypermethylation. However, the tests do not reveal any significant difference in the 5mC content between sample groups.

The incapacity to detect any 5mC differences between sample groups could be explained by the fact that the production of 5hmC, 5fC, and 5caC starting from 5mC affects discrete genomic regions, such as promoters, which generally represent less than 1% of the total 5mC of the genome (Schubeler 2015). A plausible 5mC increment, due to defective demethylation, would not have been globally detected by applying the dot-blot method. A site-specific investigation of these changes is then

required to confirm that, once 5hmC content reduces, a lateral accumulation of 5mC occurs in MS brains.

Accordingly, a genome-wide methylation/hydroxymethylation profiling will also be needed to connect a functional meaning to the observed global 5hmC alterations, as they may modify the expressions of particular genes implicated in MS pathogenesis. Indeed, genome-wide DNA methylation/hydroxymethylation analysis in healthy brains have determined that 5hmC signal scatters in intragenic regions (gene bodies) and proximal positions to the transcription start site of actively expressed genes. 5hmC genomic pattern positively correlates too with histone modifications of euchromatin, which enriches actively transcribed genes, while it negatively relates to repressive histone transcription modifications (Shen and Zhang 2013).

This study reports some preliminary information on the functional association between the observed 5hmC drop and the transcriptional alteration of specific genes that are putatively involved in MS. The obtained data suggest a functional link between methylation remodelling and MS, as a local reduction of 5hmC associates with transcriptional deregulation of MS-related genes. Indeed, loss of 5hmC and the subsequent increment of 5mC feature exclusively in the transcriptionally altered genes. In correspondence, a decrement

in 5fC enrichment is evident in the before-mentioned deregulated genes. 5caC, however, exhibits a less evident decrement in MS cohorts. This data confirms that the detected 5hmC epigenetic drop in MS relates to a supposed pathological inability to exert demethylation/5hmC synthesis where the genome requires it.

As a reinforcing evidence linking DNA 5-hydroxymethylation epigenetic variations to MS is the fact that two genes that have a weak or no relationship with the disease, *GAPDH*, and *SYK*, do not showcase a different expression in NAMWs as well as a differential 5mC/5hmC amount if compared to the cohort group. Significantly, the genes that confirmed this link are also associated to single nucleotide polymorphisms directly implicated in MS susceptibility as well as therapy response, as in the case of *GPC5* (Baranzini, Mudge et al. 2010; Shin, Kim et al. 2013), *IFI30* (Isobe, Madireddy et al. 2015)) and *LEPR* (Farrokhi, Dabirzadeh et al. 2016).

Regarding *KIF1A*, mutants for this gene impact the SNC as they display motor and sensory changes with significant repercussions on synaptic vesicle density, neuronal stimulation and neurotransmission (Dutta, Chang et al. 2011). Other *KIF1A* mutations are known to produce progressive encephalopathy and brain atrophy (Esmaeeli Nieh, Madou et al. 2015). Coherently with the current literature not associating SYK to

MS, except for one study that suggests this protein as a therapeutic target in MS (Liao, Hsu et al. 2013), our data display that *SYK* gene did not feature transcriptional variation as well as subsequent 5mC/5hmC changes. These results suggest that an epigenetic alteration in the 5hmC pattern may play a role in the condition pathogenesis through the alteration of MS-involved gene expressions. However, explicit assumptions about the functional consequences of the 5hmC reduction in MS are however impossible to formulate since the role played by this cytosine modification in the adult human brain has not been fully elucidated yet.

Many studies hypothesise that 5hmC may be required in governing various cognitive functions in MS. In mice, for example, DNA hydroxymethylation machinery depletions lead to some hallmarks that are generally shared among MS patients such as weakened learning and memory as well as chronic depression (Rudenko, Dawlaty et al. 2013). Moreover, 5hmC deficiency is also observed in the CNS of other neurodegenerative conditions such as Huntington's disease and Alzheimer's disease. The global 5hmC impairment observed in these two pathological contexts has been linked to altered regulation of intracellular signalling pathways that are known to be implicated in the pathogenesis of these diseases (Wang, Yang et al. 2013; Condliffe, Wong et al. 2014).

The possibility that the described 5hmC decrease associates with a 5mC oxidation/demethylation inhibition may be explained by the concomitant repression of *TET2* transcription that is similar between the two MS cohorts. Indeed, this protein belongs to the TET family of enzymes, which converts in a stepwise manner 5mC into 5hmC, 5fC, and 5caC. Among the members of this enzymatic group, however, only TET2 does not retain on its structure the DNA binding domain that is usually featured in these enzymes, which is in trans-supplied by the *IDAX* gene instead (Dunican, Pennings et al. 2013).

Interestingly, MS subjects manifest a differential expression of *IDAX* as well, notably in the SPMS group where this transcript is detected up-regulated. The differential expression of *TET2* and *IDAX* could describe why 5hmC decreases in both MS cohorts, lesser in the SPMS cohort if compared to PPMS. Since the SPMS subjects do not begin with the progressive form, but they evolve into it later in life, it is possible to assume that *IDAX* up-regulation may act as a sort of "epigenetic failsafe mechanism", which balances the downregulation of *TET2* expression, thus limiting the side effects of an eventual 5hmC decrement. As further evidence of this theory, is the fact that IDAX is the actual DNA binding protein that detached itself from TET2 structure during evolution. IDAX, it is also known to modulate TET2 activity by both recruiting the enzyme on

DNA and even by eliciting its degradation via caspase activation (Dunican, Pennings et al. 2013).

In contrast, *TET2* deficiency is not compensated by *IDAX* up-regulation in PPMS subjects, thus causing a more evident 5hmC loss in the patients that start with the severe form of this pathology (Figure 3). This may explain why 5hmC levels positively associate, in all the performed correlation tests, with the sum of *IDAX*-*TET2* level of transcription in each individual. If individually considered, *IDAX* and *TET2* do not correlate with the measured levels of 5hmC in our sample groups.

Moreover, the association of *TET2*-*IDAX* deregulations with both MS and 5hmC variations is independent of inflammation events likewise from cell composition changes which, although detectable yet in MS NAWMs, did not associate directly with MS. Although the detected statistical interactions positively associate with the inflammation markers with each other and with the loss of oligodendrocytes, none of these markers showed mean differences between controls and MS individuals.

We next evaluated the impact of the disease's activity at the time of death.

No association was also found between 5hmC, *TET2* or *IDAX*, and the disease's activity. This further suggests that the

measured variations of the methylation/demethylation factors in MS NAWMs are independent of demyelination despite the MS active patients showing some inflammation. In fact, some of the inflammation markers increase in patients with ongoing demyelination. Interestingly, *GAS6* expression significantly increases in patients with active disease (both in the Kruskal-Wallis and the GLM tests). This result is relevant as GAS6 is known to exert a protective role against demyelination for its trophic effect for neurons and oligodendrocytes, as well as being a microglia anti-inflammatory factor (Bellan, Pirisi et al. 2016). Hence, it is likely coherent to remark that the scar-free NAWM samples from active patients they also feature *GAS6* up-regulation.

Regarding TET2 activity in MS, we think that its deregulation may be an early pathological event in NAWM as it characterises brain positions that are disease-free without previous plaque establishment. Additionally, diminished TET2 protein expression marks non-cerebral tissues in MS patients, such as peripheral blood cells (Calabrese, Valentini et al. 2014). As additional confirmation of the potential prodromal role of TET2 in MS, recent genome-wide association studies have found out *TET2* as one of the novel non-MHC susceptibility loci linked to MS (Beecham, Patsopoulos et al. 2013).

Another recent discovery has further elucidated the role of TET2 in MS. In such study, the enzyme is downregulated in demyelinated regions of the MS hippocampus, in correspondence with an apparent decrement of 5hmC (Chomyk, Volsko et al. 2017). Considering the autoimmune nature of MS, the decrease of both *TET2* and 5hmC could deregulate the inflammatory modulation of this disease. Indeed, TETs enzymatic family is known to suppress the production of pro-inflammatory cytokines (Neves-Costa and Moita 2013) as well as promoting the transcription of anti-inflammatory cytokines (Ichiyama, Chen et al. 2015). Indeed, the latter research group has highlighted that in EAE mice, T-cells raise the expression of anti-inflammatory cytokines, such as IL-10, via an active DNA demethylation process mediated by TET2.

They also observed that the mouse model for multiple sclerosis magnified its neurological severity when the *TET2* gene was knocked out, as a further explanation that this protein is involved in regulating immune responses.

These investigations suggest that the reduced expression of *TET2* in NAWM samples could be a crucial factor in rendering MS white matter more defenceless to autoimmunity. Brain astrocytes may be implicated as well in modulating the inflammation responses through the production IL-10, which is

under the control of TET2 activity (Villacampa, Almolda et al. 2016).

TET2 is not only crucial for modulating inflammation in the brain, but it is also involved in maintaining the proper CNS microstructure. Several studies highlight the function of TET2 for oligodendrocytes survival and differentiation, as well as being required for the myelination processes in the mouse (Zhao, Dai et al. 2014). Its deficiency in the white matter of MS patients could promote demyelination, making the oligodendrocytes more sensitive to inflammatory damage, as well as imperilling the ability of the brain to re-establish the myelin on the axons.

Curiously, the literature also hints that MS patients have a higher than expected risk of Acute Myelocytic Leukemia (AML) (Martinelli, Cocco et al. 2011), a cancer which its poor prognosis is known to be linked to *TET2* mutation (Chou, Chou et al. 2011).

An engaging research project conducted on mice displays an age-dependent TET2 and 5hmC loss in the adult hippocampus, hence impairing the animals' learning and memory. The results further demonstrated that the exposure of aged animals to young blood (parabiosis) recovers TET2 and 5hmC in the old brains, re-establishing the cognitive functions of the animals (Gontier, Iyer et al. 2018).

It is possible to think that the loss of TET2 in MS brains, eventually driven by an unidentified blood factor, may determine the CNS ageing-related degeneration.

It is known that the white matter from healthy aged patients shares a lot of typical structural hallmarks with the MS NAWMs, such as scattered shrinkage of myelin and remodelling in the brain's haemodynamic (Haider, Zrzavy et al. 2016).

Despite all this information and data, however, future experimental strategies should investigate in more detail how the deregulations of the hydroxymethylation machinery reshape the pathogenesis of MS.

Contextually, the future investigation will focus at pursuing the following aims: 1) linking these epigenetic alterations to a specific cell type of the NAWM (oligodendrocytes, astrocytes, and microglia cells) to better understand their impact on the unhealthy brain; 2) mapping on the whole genome the 5hmC fluctuations associated with MS to identify the genes that suffer from this epigenetic deregulation; 3) investigating the underlying causes of *TET2* and *IDAX* deregulation. Indicative for this purpose is that *TET2* undergoes transcriptional silencing, via DNA hypermethylation, in MS and several other pathologies (Calabrese, Valentini et al. 2014; Bahari, Hashemi et al. 2016).

Further research will also aim at better clarifying whether such epigenetic deregulations may be related to MS environmental risk factors. The current evidences are striking as some of the major MS risk factors, such as smoking and EBV infection, are known to be powerful epigenetic drivers. Smoking, for example, is known to remodel the chemical modifications of the histones, DNA methylation profile and the expression of non-coding RNAs, to probably increase the susceptibility to various pathologies, including cancer (Koturbash, Beland et al. 2011).

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