DNA Methylation in Multiple Sclerosis

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Contents

Abstract Multiple Sclerosis (MS) is a leading cause of lifelong disability in young adults. The disease strikes individuals in their most productive years with incurable and progressive course that results in development of fatigue and accumulation of physical and cognitive disability. MS is characterized by autoimmune destruction of the myelin and subsequent neurodegeneration. This chronic disease of the central nervous system is likely triggered by environmental factors such as smoking, lack of sun exposure/vitamin D deficiency and infection, in genetically predisposed individuals, the strongest influence coming from *HLA-DRB1* variants within the HLA class II locus. However, the mechanisms underlying susceptibility to MS are still puzzling and specific clinical translations are lacking. Emerging evidence suggests the implication of epigenetic mechanisms such as DNA methylation in the pathogenesis of MS. In this chapter, we aimed to review findings from DNA methylation studies in MS and discuss their clinical relevance. We first present a critical overview of the outcomes of DNA methylation studies in immune cells and brain tissue from MS patients. We then discuss emerging evidence supporting a role of DNA

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S. Jurga, J. Barciszewski (eds.), *The DNA, RNA, and Histone Methylomes*, RNA Technologies, https://doi.org/10.1007/978-3-030-14792-1_8

methylation in mediating the effect from the major genetic risk variant *HLA-DRB1*15:01* and environmental risk factors, smoking and vitamin D deficiency, in MS. We also describe the potential of DNA methylation-based biomarkers and therapies for precision medicine in MS. We expect that the encouraging findings from DNA methylation studies in MS might open new avenues for a better understanding and treatment MS patients.

Keywords Multiple sclerosis · DNA methylation · Immune cells · Brain · HLA · Smoking · Vitamin D · Biomarkers · Therapy

1 Introduction

Multiple Sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS) characterized by autoimmune destruction of myelin and subsequent neuronal death (Compston and Coles 2008). MS is the leading cause on non-traumatic disability among young adults worldwide, affecting up to 200/100,000 individuals in Northern populations with nearly 70% of patients being women (female to male ratio ranging from 2:1 to 3:1) (Trojano et al. 2012; Bezzini and Battaglia 2017). At diagnosis (between 20 and 40 years of age), the majority of MS patients (80–90%) present with a relapsing-remitting (RRMS) form of disease characterized by repeated and transient episodes of neurological symptoms (relapse) followed by complete or partial recovery (remission) (Compston and Coles 2008). Current treatments are effective only in the early inflammatory RRMS stage, but they target broadly the immune system and pose serious safety concerns (Soelberg Sorensen 2017). Most RRMS patients (80%) will eventually convert to a secondary progressive stage (SPMS) with persistent neuronal loss and continuous accumulation of disability. Additionally, in ~10% of cases, patients will manifest a primary progressive form of MS (PPMS) already from onset. MS pathology is believed to be initiated by disruption of the blood-brain-carrier (BBB) and infiltration of peripheral immune cells into the CNS, resulting in confined areas of inflammatory demyelination and axonal injury, called lesions or plaques, which continuously arise in the CNS (Compston and Coles 2008). Variation of clinical symptoms between patients and during disease course is conditioned by anatomical localization and severity of the lesions and range from sensory, motor and visual deficit to fatigue and cognitive impairment. Thus, MS is a highly heterogeneous incurable chronic disease leading not only to personal debilitation but also to considerable economic and societal burden (Brundin et al. 2017).

Even though the exact cause of MS remains unknown, disease likely results from a complex interplay between genetic and environmental risk factors. The genetic basis of MS was first demonstrated in familial studies with an overall recurrence risk for monozygotic (MZ) twins of 18.2%, which significantly differs from the one for dizygotic twins (4.2%) and siblings (2.7%), and an overall genetic heritability estimated at 54% (O'Gorman et al. 2013). The first genetic association was established in the 1970s with the Human Leukocyte Antigen (HLA) region on

chromosome 6p21 (Jersild et al. 1975) and was later refined to the haplotype *HLA-DRB5*0101–HLA-DRB1*1501–HLA-DQA1*0102–HLA-DQB1*0602* (Fogdell et al. 1995) encoding HLA class II molecules involved in regulation of immune processes. Over the past decade, genome-wide association studies (GWAS) conducted in large case-control cohorts have enabled the identification of single nucleotide polymorphism (SNP) associated with the risk of developing disease at the population level. These collective efforts have made a breakthrough in decoding the genetic architecture of MS risk by identifying >200 MS-associated loci, with the strongest influence coming from the aforementioned *HLA-DRB1*15:01* variant (odd ratio, OR~3) (International Multiple Sclerosis Genetics et al. 2011, 2013). Yet, the functional interpretation of MS causal variants remains challenging as most of them are located in non-coding regions of the genome (Farh et al. 2015). Furthermore, a more complex pattern of inheritance is likely driven by parent-of-origin effects where the risk depends on whether the allele is inherited from the mother or the father (Ebers et al. 2004; Chao et al. 2009). Collectively, genetic data converge on a polygenic model of the risk of developing MS, with one locus conferring moderate effect and many loci of small effects. They further indicate that a limited part of the disease heritability can be explained by genetic variants, with population-based studies estimating significantly lower sibling relative risk compared to family studies (O'Gorman et al. 2013; Westerlind et al. 2014). This gap suggests a 'hidden' heritability which, together with a yet-unexplained rise in incidence of MS during the last decades, may be explained by non-genetic processes such as gene-environment interactions. Accordingly, vast epidemiological data support a role of environmental exposures and lifestyle habits in disease susceptibility. Compelling body of evidence associates tobacco smoking, Epstein-Barr virus (EBV)-mediated infectious mononucleosis, low vitamin D and sun exposure as well as obesity with susceptibility to develop MS (Olsson et al. 2017). Other environmental and lifestyle factors such as night shift work, seasonal change, alcohol and diet have also been reported to affect MS risk and warrant replication (Olsson et al. 2017). Interestingly, for most of the identified environmental factors such as sun exposure/vitamin D deficiency, mononucleosis, night shift work or high BMI, the childhood-adolescence period seems to represent a specific window of susceptibility in the risk to develop MS (Olsson et al. 2017). Moreover, gene-environment interactions have been shown to contribute to risk, as evidenced for interaction between smoking or EBV and MS-associated *HLA* factors (Hedstrom et al. 2011; Xiao et al. 2015). Jointly, known genetic and environmental factors and their interactions can explain a substantial fraction of disease risk (van der Mei et al. 2016). Yet, the mechanisms underpinning disease initiation and progression are poorly annotated and robust prognostic tools and more specific and potent therapies are lacking, thus posing major challenges for an efficient care of MS patients.

The low concordance rate of MS in MZ twins together with parent-of-origin effects, 'hidden' heritability and long-term impact of environmental risk factors suggest involvement of epigenetic mechanisms in disease pathogenesis. Epigenetics refers to mitotically (and meiotically) heritable changes in gene expression that do not entail variation in the DNA sequence. Epigenetic processes are therefore

primarily of non-genetic origin and cell type-specific, with non-shared environmental influence accounting for most of the variance (Busche et al. 2015). Epigenetic mechanisms refer to biochemical modifications of the genome, such as DNA methylation and histone posttranslational modifications, and their regulatory effects on chromatin dynamics and transcription. DNA methylation, the deposition of a methyl group to cytosine, mostly in the context of a CpG dinucleotide, is by far the most studied epigenetic modification in clinical studies of MS. Because DNA methylation inhibits gene expression when associated to promoter region of genes, hypermethylation in this region is considered as a mark of transcriptional repression. De novo deposition and maintenance of methylation are orchestrated by DNA methyltransferases DNMT3A/B and DNMT1, respectively, while active demethylation is catalyzed by members of the ten–eleven translocations (TETs) family of enzymes. Notably, the implication of epigenetics in MS is further supported by the identification of MS-associated genetic variation and transcriptional changes of genes encoding members of the DNA methylation machinery (Calabrese et al. 2014; Andlauer et al. 2016; Fagone et al. 2016). Moreover, early dysregulation of methionine metabolism (an essential metabolite in the methyl group transfer to DNA) has been recently proposed to impact DNA methylation patterns in MS as well (Singhal et al. 2018). Overall, DNA methylation dynamics is responsive to the environment and can lead to stable and heritable but reversible changes in gene-regulatory networks. Thus, DNA methylation studies represent a promising approach for improved understanding of MS pathogenesis and therapeutic opportunities. In this chapter, we will review the studies reporting DNA methylation alterations in MS patients and discuss the clinical translations of these findings.

2 DNA Methylation Studies in MS

2.1 DNA Methylation in Peripheral Immune Cells

Findings from immunological, genetic and histopathological studies of patients with MS have revealed a crucial role of immune cells in the pathogenesis of MS. The strongest genetic influence comes from the *HLA* class II region, which encodes essential molecules for antigen presentation by antigen presenting cells (APCs, such as macrophages) and antigen recognition by pathogenic T helper (Th) cells. MS is regarded as $CD4^+$ Th cell-driven disease with predominant Th1- and Th17-mediated proinflammatory processes. Accordingly, experimental autoimmune encephalomyelitis (EAE), an MS-like animal model, can be induced by passive transfer of activated CNS antigen specific $CD4^+$ T cells (Ben-Nun et al. 1981) and pharmacological treatment of RRMS patients with inhibitor of lymphocytes migration showed efficacy in reducing inflammation and disease activity (Polman et al. 2006; Kappos et al. 2010). In that context, DNA methylation studies in MS have aimed to explore the molecular mechanisms underlying MS immunopathogenesis by profiling whole blood, blood peripheral mononuclear cells (PBMCs) and sorted $CD4^+$ and $CD8^+$ T

cells, the majority of them in case-control cohorts. Details about the cohorts and main findings are described in Table 1.

Studies addressing global methylation exploit the fact that methylation measured at repetitive elements, such as Alu repeats and long interspersed nucleotide elements (LINE-1), jointly representing one third of the entire genome, can serve as a surrogate of total genomic methyl cytosine. Increased global DNA methylation in blood cells and sera has been reported in RRMS patients compared to controls (Neven et al. 2016; Pinto-Medel et al. 2017; Dunaeva et al. 2018). LINE-1 methylation further correlates with either motor disability, measured as Expanded Disability Status Scale (EDSS) status (Neven et al. 2016) or IFN-treatment duration (Pinto-Medel et al. 2017). Given that loss of global methylation typically leads to chromosomal instability, loss of imprinting and activation of transposable element, these data suggest an increased genome stability in MS patients. However, such interpretation should be considered with caution as global methylation methods do not reveal locus-specific changes. This is important in light of the consequences of the locus-specific genome instability on reactivation of specific endogenous retroviruses, which has been observed in MS patients (Morris et al. 2018).

Gene-candidate approaches have examined DNA methylation at *a priori* selected candidate genes involved in inflammatory processes and/or MS susceptibility. They have focused on promoter regions and thus inferred the putative impact of the observed changes on transcription. Results from promoter profiling of eight neuroinflammatory genes in whole blood from RRMS patients and healthy donors showed increased methylation levels at *RUNX3*, *CDKN2A*, *SOCS1*, and *NEUROG1* genes implicated in neuroglial and T cell differentiation, most of them being reported as dysregulated in MS patients (Sokratous et al. 2018). The negative regulator of proinflammatory signaling *SHP-1* gene displayed hypermethylation correlating with reduced transcript levels in peripheral blood leukocytes from MS patients compared to controls (Kumagai et al. 2012). Interestingly, studies have shown that T cells from RRMS patients exhibit hypomethylation of CpGs in the two previously identified MS risk loci, vitamin D receptor (*VDR*) and IL2 receptor (*IL2RA*) genes compared to controls (Ayuso et al. 2017; Field et al. 2017). Lower methylation levels at these regions correlate with MS-specific increased *IL2RA* and *VDR* expression in T cells and blood leukocytes, respectively. Of note, *IL2RA* expression by $CD4^+$ T cells has been shown to be regulated by vitamin D, further supporting its relevance in MS susceptibility (Berge et al. 2016). Finally, we have shown that $CD4^+$ T cells from RRMS patients exhibit hypomethylation of the *VMP1/MIR21* locus compared to SPMS patients and healthy controls. Lower methylation associated with upregulation of the microRNA (miRNA) *miR-21* and concomitant downregulation of its target genes, important in cell apoptosis and proliferation, in $CD4^+$ T cells (Ruhrmann et al. 2018). This data highlights an interplay between epigenetic mechanisms where DNA methylation changes at restricted CpGs of a miRNA can lead to perturbed expression of multiple genes involved in immune processes. Thus, even though most of these studies are biased towards pre-selected candidates, they reveal functionally relevant changes in methylation which could contribute to enhanced inflammation in MS. Of special notice, similar changes of some candidate genes, such as *RUNX3*, *NEUROG1,*

Table 1 DNA methylation studies in Multiple Sclerosis

Tissue	Cohort (F/M)	Meth	Findings[a]	Reference
Global methylation approaches				
WB	51 RRMS (38/13), 137 HC (73/37)	Pyroseq	Global hypermethylation (Alu, LINE-1, SAT-α) in MS. Correlation between Alu, LINE-1 DNA methylation and EDSS score but not MS course.	Neven et al. (2016)
Serum	24 untreated RRMS (17/7), 24 HC (19/5)	BS-seq, MSP	Cell-free circulating DNA displays hypermethylation of L1PA2 sub-family of LINE-1 fragments.	Dunaeva et al. (2018)
Buffy coat	54 untreated RRMS (36/18), 36 IFN-treated RRMS (21/15), 25 HC (14/11)	LINE-1 assay	Slight global hypermethylation in MS. Negative correlation with duration of IFNbeta treatment	Pinto-Medel et al. (2017)
Candidate-gene DNA methylation approaches				
WB	50 pairs of MZ twins discordant in MS (35/15)	MSP	No difference of *MHC2TA* pIV promoter methylation between discordant MZ twin pairs.	Ramagopalan et al. (2009)
WB	Benign cohort: 48 RRMS (EDSS $\leq$ 3). Malignant cohort: 20 PPMS (EDSS $>$ 6)	Pyroseq	No difference at *HLA-DRB1*1501* and *HLA-DRB5* methylation in benign vs. malignant MS.	Handel et al. (2010)
Buffy coat	7 PPMS, 50 RRMS, 12 SPMS (49/20), 19 HC (10/9)	Cloning BS-seq	Hypermethylation of *SHP-1* promoter 2 in MS vs. HC. No correlation with MS clinical parameters.	Kumagai et al. (2012)
PBMCs	39 RRMS, 1 SPMS (32/8), 40 HC (30/10)	EpiTyper, dot blot	Downregulation of *TET2* and *DNMT1* gene expression in MS. Hypermethylated CpGs in *TET2*. Reduced global 5hmC level and slightly increased global 5mC in MS.	Calabrese et al. (2014)
PBMCs	31 RRMS, 1 SPMS (22/10), 30 HC (15/15)	Cloning BS-seq	*PADI2* hypomethylated promoter correlation with upregulated gene in MS. No correlation with clinical parameters.	Calabrese et al. (2012)

(continued)

Table 1 (continued)

Tissue	Cohort (F/M)	Meth	Findings[a]	Reference
T cells, PBL	23 RRMS (14/9), 12 HC (8/4)	Cloning BS-seq	*VDR* alternative promoter hypermethylation in MS, no correlation with clinical parameters. *VDR* mRNA upregulation in PBLs of MS.	Ayuso et al. (2017)
WB, PBMCs, NAWM	PBMCs: 28 RRMS, 10 HC, WB: 14 MS, 14 HC, NAWM: 8 MS, 6 HC	EpiTyper, 450K	No difference in *IL2RA* methylation in PBMCs and NAWM in MS vs. HC. After mixed-tissue deconvolution: 1 hypomethylated T cell-specific DMP at *IL2RA* promoter correlating with increased *IL2RA* expression in T cells in MS vs. HC.	Field et al. (2017)
$CD4^+$ T	DC: 12 RRMS (9/3), 8 SPMS (4/4), 12 HC (8/4). VC: 30 RRMS (22/8) 11 SPMS (8/3), 12 HC (5/7), 9 INDC (7/2)	450K, pyroseq	Hypomethylation of *VMP1/MIR21* locus in RRMS (compared to HC and SPMS) and association with lower miR-21 expression.	Ruhrmann et al. (2018)
WB	66 RRMS (33 rel, 33 rem, 44/22), 33 HC (22/11)	MS-MLPA	Hypermethylation of *RUNX3, CDKN2A, SOCS1, and NEUROG1* in MS vs. HC. No difference inbetween relapse vs. remission.	Sokratous et al. (2018)
Genome-wide DNA methylation approaches				
$CD4^+$ T	2 RRMS, 1 SPMS pairs of discordant MZ twins (2/1)	RRBS	Two common DMPs (*TMEM1, PEX14*) between two twin pairs.	Baranzini et al. (2010)
$CD4^+$ T, $CD8^+$ T	30 treated RRMS (26/4), 28 HC (15/13)	450K	$CD4^+$: 74 DMPs (35 genes) in RRMs vs. HC: 19 *HLA*-DMPs, 55 non-*HLA* DMPs. Correlation of *HLA-DRB1* DNA methylation with *HLA-DRB1*1501* haplotype. $CD8^+$: 79 non-MHC DMPs (51 genes). No overlap with $CD4^+$ T cells.	Graves et al. (2014), Maltby et al. (2015)

(continued)

Table 1 (continued)

Tissue	Cohort (F/M)	Meth	Findings[a]	Reference
WB, $CD4^+$ T, $CD8^+$ T	16 RRMS (16/0), 14 HC (14/0)	450K	No genome-wide DMPs. Nominally significant CpGs: predominant hypermethylation in $CD8^+$ specifically. Two common DMPs (at *TMEM48 and APC2*) in $CD4^+$, $CD8^+$ T cells and WB. No difference between different disease duration.	Bos et al. (2015)
PBMCs	14 RRMS (9/5), 8 PPMS (6/2), 8 HC (6/2)	450K	136 DMPs between RRMS, PPMS and HC: 30 DMPs (17 genes) in RRMS vs. HC, 67 DMPs (25 genes) in PPMS vs. HC and 51 DMPs (22 genes) in PPMS vs. RRMS, respectively. Most PPMS-DMPs are hypermethylated.	Kulakova et al. (2016)
$CD4^+$ T	28 untreated RRMS (28/0), 22 HC (22/0)	450K	153 genes with DMRs: *HLA-DRB1* hypomethylated, *SNORD1A*, *SHTN1*, *MZB1* and *TNF* displayed DMRs at TSS region.	Maltby et al. (2017)
WB	Selected cohort: 50 MS (19 current, 9 past, 22 never-smoker, 50/0); Broad cohort: 132 MS (33 current, 34 past, 65 never-smoker, 90/42), 135 HC (34 current, 31 past, 70 never-smoker, 100/35).	450K, pyroseq	Effect of smoking dependent on smoking load and time since cessation. 58 DMPs (29 genes) in current vs. never-smokers with MS, including 8 unreported DMPs. Reversible changes with time post cessation. *AHRR* gene: correlation with expression in PBMCs. Effect of smoking load interacts with MS disease.	Marabita et al. (2017)
WB, $CD4^+$ T, $CD8^+$ T, $CD14^+$,	WB: 140 MS (98/42), 139 HC (104/35); $CD14^+$: 23 MS (15/8), 13 HC (9/4), $CD4^+$: 21 MS (14/7), 12 HC	450K, pyroseq, BS-seq, RNAseq, GWAS	Hypomethylation of *HLA-DRB1* exon 2 mediate genetic risk from *HLA-DRB1*15:01* and a novel protective	Kular et al. (2018)

(continued)

Table 1 (continued)

Tissue	Cohort (F/M)	Meth	Findings[a]	Reference
CD19$^+$ B, PBMCs	(8/4), CD8$^+$: 15 MS (6/9), 14 HC (9/5), CD19$^+$: 17 MS (9/8), 12 HC (6/6).		variant through change of *HLA-DRB1* expression.	
CD4$^+$ T	7 (4/3) baseline and 6 month after DMF treatment	EPIC	974 DMPs after treatment, 97% hypermethylated	Maltby et al. (2018)
CD8$^+$ T, CD4$^+$ T	Combined cohorts including samples from (Bos et al. 2015; Maltby et al. 2015, 2017): CD4$^+$: 94 RRMS (94/0), 94 HC (94/0), CD8$^+$: 68 RRMS (68/0), 57 HC (57/0)	450K, EPIC	No genome-wide DMPs in CD8$^+$ but CpGs hypermethylated in MS. DMR: 2 intragenic DMR in both CD4$^+$ and CD8$^+$: *HLA-DRB1* (hypomethylated), *SLFN12* (hypermethylated). DMR in CD4$^+$ only: intergenic (*MOG/ZFP57* and downstream *SLFN12*), *NINJ2* (hypermethylated). Correlation with expression in WB.	Rhead et al. (2018)

[a]Of note, described are findings deemed significant by the original study using originally reported criteria for significance, which vary widely

WB whole blood, *PBMCs* peripheral blood mononuclear cells, *PBL* peripheral blood leukocytes, *MS* Multiple Sclerosis, *RRMS* relapsing-remitting MS, *SPMS* secondary progressive MS, *PPMS* primary progressive MS, *HC* healthy controls, *INDC* inflammatory neurological disease control, *F/M* female/male number, *EDSS* expanded disability status scale, *MZ* monozygotic, *DMF* dimethylfumarate, *DMP* differentially methylated position, *DMR* differentially methylated region, *GWAS* genome-wide association study, *RRBS* reduced representation bisulfite sequencing, *450K* Infinium HumanMethylation450 BeadChip, *pyroseq* pyrosequencing, *BS-seq* bisulfite cloning-sequencing, *MSP* methyl sensitive PCR, vs. versus

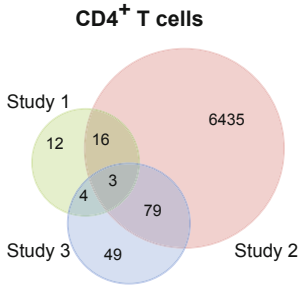
MIR21 or *IL2RA*, could be identified in genome-wide investigations of specific cell types, described below (Bos et al. 2015).

The recent progress in genome-wide methylation analyses has advanced the field beyond candidate gene approaches by enabling investigation of the methylome landscape of patients. A seminal genome-wide study has investigated the CD4$^+$ T methylome of three MZ twin pairs discordant for MS and found only two common DMPs between twin pairs, which is not unexpected given a small and heterogeneous cohort (Baranzini et al. 2010). The development of array-based technologies such as Infinium HumanMethylation BeadChip arrays has allowed cost- and time-effective DNA methylation profiling of blood cells from case-control cohorts, the large majority comparing RRMS patients with healthy controls. These epigenome-wide association studies, referred to as EWAS, have revealed that epigenetic alterations occur at multiple loci throughout the genome of immune cells, reporting detailed

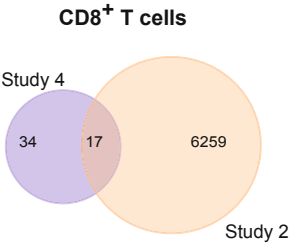
mapping of differentially methylated single CpG positions (DMPs) or regions (DMRs), their effect sizes (represented by Δβ-values) and directionality (Table 1). However, EWAS conducted in Norwegian and Australian cohorts have yielded various results to date, discrepancies that can be further reflected by the low overlap between studies (Bos et al. 2015). Indeed, no common DMPs were observed between the three existing studies focusing on $CD4^+$ T cells from RRMS compared to controls (Graves et al. 2014; Bos et al. 2015; Maltby et al. 2017). Overall, three annotated genes, *HLA-DRB1* and *HLA-DRB6* genes from the MS risk *HLA* class II locus as well as the RNA-editing *ADARB2* gene, overlap between the studies, although with different reported CpGs (Fig. 1a). Among common DMPs reported by at least two studies, 12 of them displayed consistent changes (i.e. same directionality) (Fig. 1b). They map to immune genes, i.e. *HLA* class II genes (*HLA-DRB1*, *HLA-DRB5*, *HLA-DRB6)*, TGF-β induced gene *TGFBI,* as well as the ribosomal kinase *RPS6KA2* gene and the protein-ubiquitin ligase *FBXO27* gene. In $CD8^+$ T cells, while only one intergenic DMP overlaps between the two existing studies (Bos et al. 2015; Maltby et al. 2015), 17 common genes were found to exhibit at least one differentially methylated CpG, although at different locations, in the two studies (Fig. 1b). They are involved in phagocytosis (*MEGF10*, *BAI1*), cell proliferation (*CDKN1C, CAMTA1)*, cell migration (*IGF2BP1, CDC42BPB*), iron sequestering (*FTL)* and xenobiotic metabolism (*UGT1A10*). Recently, joint analysis of the combined Norwegian and Australian data (Bos et al. 2015; Maltby et al. 2015, 2017) reported five significant DMRs in $CD4^+$ and $CD8^+$ T cells from MS patients compared to controls (Rhead et al. 2018). Two intragenic DMRs, that map *HLA-DRB1* and *SLFN12* genes, were found hypomethylated and hypermethylated, respectively in RRMS patients compared to controls in both $CD4^+$ and $CD8^+$ T cells, while an intergenic DMR located between *MOG* and *ZFP57* genes was specific to $CD4^+$ T cells. Additionally, two DMRs in *NINJ2* gene and downstream *SLFN12* locus were identified in $CD4^+$ T cells only when comparing treatment-naïve MS patients and controls. Methylation differences at *HLA-DRB1*, *NINJ2* and *SLFN12* genes associated with changes in expression in whole blood from MS patients compared to controls.

Despite disparities between studies (see explanations in Sect. 2.3), interesting findings point to two MS-related features. Predominant genome-wide hypermethylation could be observed in $CD8^+$ T cells, specifically (i.e. not $CD4^+$ T cells), from RRMS patients compared to controls (Bos et al. 2015; Rhead et al. 2018), in PBMCs from PPMS patients, specifically, compared to RRMS patients and healthy controls (Kulakova et al. 2016) and in $CD4^+$ T cell from MS patients after pharmacological treatment with the MS-drug dimethylfumarate (Maltby et al. 2018). One can speculate that dysregulation of DNA methylation machinery, such as DNMT and TET enzymes could have such a global impact on DNA methylation. Moreover, we and others have found striking hypomethylation of the *HLA-DRB1* gene (Graves et al. 2014; Maltby et al. 2017; Kular et al. 2018; Rhead et al. 2018), the relevance of such altered methylation at the major MS risk locus will be further described in Sect. 3.1 of this chapter.

a



Common genes: *HLA-DRB1, HLA-DRB6, ADARB2*



Common genes: *FTL, CDKN1C, MEGF10, LMO3, IGF2BP1, CDC42BPB, UGT1A10, ARHGAP22, BAI1, CRTAC1, DLGAP2,TNXB, PRDM8, HEATR2, CAMTA1, PLD5, ZC3H14*

b

CD4+ T cells

Chr.	probeID	Gene name	Studies 1	2	3
1	cg11733135	-		-	+
1	cg01533966	*LRRC8D*		-	+
1	ch.1.230734885R	-		+	+
2	cg10481584	-		-	+
3	cg08977311	*C3orf50*		-	+
3	cg22542451	-		-	-
5	cg17386240	*TGFBI*	+	+	
6	cg01341801	*HLA-DRB5*	+	+	
6	cg19383211	*HLA-DRB6*		+	+
6	cg08578320	*HLA-DRB1*	-	-	
6	cg09139047	*HLA-DRB1*	-	-	
6	cg15982117	*HLA-DRB1*	-	-	
6	cg10632894	*HLA-DRB1*	-	-	
6	cg17416722	*HLA-DRB1*	+	+	
6	cg13423887	*HLA-DQB1*	-	-	
6	cg11082635	*RPS6KA2*		-	-
8	cg08682625	*LOC727677*		+	-
10	cg16255663	*MGMT*		-	+
11	cg08822897	-		-	-
19	cg18624102	*FBXO27*		+	+
21	cg10296238	*C21orf56*	-	+	

CD8+ T cells

Chr.	probeID	Gene name	Studies 2	4
2	cg22509113	-	+	-

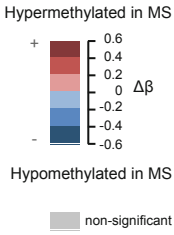


Fig. 1 Overlap between DNA methylation studies in immune cell type-specific in MS. (**a**) Venn-diagram illustrating the number of differentially methylated genes between MS patients and healthy controls in studies profiling $CD4^+$ (left panel) and $CD8^+$ (right panel) T cells. The names of overlapping genes between all cell type-specific studies appear below the diagram. (**b**) Heatmap of differentially methylated CpGs between MS patients and healthy controls reported in at least two studies profiling $CD4^+$ (left) and $CD8^+$ (right) T cells. The color gradient represents the direction of change ($\Delta\beta$-value), with blue and red being hypomethylated (−) and hypermethylated (+), respectively, in MS patients compared to controls. Studies 1, 2, 3 and 4 correspond to $CD4^+$ (Graves et al. 2014), $CD4^+$ and $CD8^+$ (Bos et al. 2015), $CD4^+$ (Maltby et al. 2017) and $CD8^+$ (Maltby et al. 2015), respectively. Note that comparisons were conducted on the reported DMPs with varying significance thresholds: genome-wide significance (False Discovery rate or Benjamini-Hochberg-adjusted p-value <0.05, $\Delta\beta \geq \pm\ 0.1$) in studies 1, 3 and 4 and nominal significance (p-value <0.05) for study 2

2.2 DNA Methylation in Post-Mortem Brain

Neuroimaging and histopathological studies of the CNS white matter in MS patients have distinguished a variety of lesion types differing in the degree of leukocytes infiltration, demyelination, remyelination and neuro-axonal injury. Importantly, areas from the seemingly unaffected normal appearing white matter (NAWM) frequently manifest diffuse pathology along with focal abnormalities even in the absence of infiltrating leukocytes (Barnett and Prineas 2004; Henderson et al. 2009; van Horssen et al. 2012; Burm et al. 2016). NAWM lack of integrity has been further associated with neurological disability (Dineen et al. 2009; Francis et al. 2014; Meijer et al. 2016). Additionally, recent evidence suggests that neurodegenerative processes might start earlier in life (Chard et al. 2002; Hagstrom et al. 2017; Tortorella et al. 2018), without prior demyelination (DeLuca et al. 2006) and in the grey matter as well (Geurts and Barkhof 2008; Calabrese et al. 2010). Overall, brain atrophy is the strongest predictor of disability, its impact becoming apparent later in life when the neurological reserves are likely exhausted from long-standing compensatory mechanisms. Due to the limited accessibility of the target organ in MS, the molecular mechanisms underlying the neuropathology of MS remain elusive. Given that DNA methylation is chemically stable, studies have so far relied on observation in post-mortem brain tissue, composed of mixed cell populations.

The first study examining DNA methylation in the MS brain was a candidate gene study of *PADI2* encoding a citrunillating enzyme. This study was motivated by the observation of elevated citrunillated myelin basic protein (MBP) in NAWM of MS patients, which is suggested to contribute to myelin destabilization in MS. The authors found hypomethylation of *PADI2* promoter, which associated with increased levels of PADI2 enzyme and citrunillated MBP in NAWM from 12 MS patients compared to white matter samples from 19 non-MS controls, i.e. non-neurological controls and patients with other neurological diseases (Mastronardi et al. 2007). Altogether, epigenetic dysregulation of PADI2 enzyme might participate, at least in part, in aberrant citrunillation of MBP and subsequent myelin breakdown. Interestingly, hypomethylation and upregulation of *PADI2* gene could also be observed in peripheral blood from MS patients compared to controls, suggesting that some changes occurring in the brain can be detected in blood (Calabrese et al. 2012).

However, alteration of *PADI2* gene could not be identified in recent genome-wide DNA methylation studies comparing MS-NAWM versus (vs.) control white matter (Huynh et al. 2014) or demyelinated vs. myelinated hippocampi from MS patients (Chomyk et al. 2017) (Table 2). Epigenome-wide profiling of NAWM from MS patients instead revealed numerous, albeit subtle, changes clustering on 539 DMRs throughout the genome (Huynh et al. 2014). Interestingly, hypomethylated CpGs occurred predominantly within promoters, i.e. transcription starting sit (TSS) $\pm$ 2000 bp, and were depleted from gene bodies (intron, exon), the latter being enriched in hypermethylated CpGs. Gene ontology annotation of DMR-related genes further showed that hypomethylated DMRs affected genes associated with immune responses while hypermethylated DMRs are enriched in genes involved in general cell functions

Table 2 Genome-wide DNA methylation studies in *post-mortem* brain tissue from MS patients

Reference	Huynh et al. (2014)	Chomyk et al. (2017)
Brain tissue	Frontal lobe	Hippocampus
Analysis	MS-NAWM vs. NNC	MS-demyelinated vs. MS-myelinated
Cohort (F/M)	DC: 28 NAWM-MS (3 RRMS, 17 SPMS, 7 PPMS, 17 /11), 19 NNC (7/12); VC: 10 MS (SPMS, 7/3), 20 NNC (14/6)	8 myelinated MS (6 SPMS, 2 PPMS, 5/3), 7 demyelinated MS (6 SPMS, 1 PPMS, 5/2)
Method	450K, EpiTyper, RNA-seq	450K, ELISA, IHC, RT-qPCR
Findings	– 220 hypomethylated DMRs (1235 CpGs) – 319 hypermethylated DMRs (1292 CpGs) – at oligodendrocyte-specific genes (*BCL2L2, HAGHL, NDRG1, CTSZ, LGMN*). – correlation with expression change of a fraction of corresponding genes.	– 144 DMPs (75 genes) – 62 hypermethylated DMPs – 82 hypomethylated DMPs – at astrocytic and neuronal genes – Promoter (TSS)-DMPs at *AKNA, EBPL, HERC6, SFRP1, NHLH2, PLCH1, TMEM132B* and *WDR81* correlated with expression changes.

MS Multiple Sclerosis, *RRMS* relapsing-remitting MS, *SPMS* secondary progressive MS, *PPMS* primary progressive MS, *NNC* non-neurological disease control, *F/M* female/male number, *NAWM* normal appearing white matter, *DC* discovery cohort, *VC* validation cohort, *DMP* differentially methylated position, *DMR* differentially methylated region, *450K* Infinium HumanMethylation450 BeadChip, *TSS* transcription starting site, *IHC* immunohistochemistry, *TSS* transcription starting site, vs. versus

and oligodendrocyte-related processes. Some of the changes could be further associated with gene expression differences from RNA-seq data in MS NAWM compared to control samples. Of note, the transcriptional changes did not necessarily anti-correlate with DMR direction of change, probably due to differences in DMR location and cellular origin in bulk tissue.

A recent study has investigated DNA methylation changes following hippocampus demyelination in MS (Chomyk et al. 2017). Comparison of demyelinated vs. myelinated hippocampi from MS patients led to the identification of 144 hypomethylated and hypermethylated DMPs with large changes ($\Delta\beta > 20\%$). These changes coincide with differential expression of DNA methylation enzymatic machinery, with significant upregulation of methylating enzymes (DNMT1, DNMT3A/B) concomitant with downregulation of demethylating TET enzymes in the MS hippocampus following demyelination. This finding together with the predominant detection of DNMTs and TETs proteins in hippocampal neurons compared to other cell types, imply that methylation patterns likely differ in a locus- and, importantly, cell type-specific manner, which might not be reflected in bulk tissue analysis. In line with this, the 75 genes harboring DMPs had been previously reported to be expressed by multiple brain cell types, i.e. microglia, oligodendrocyte, astrocytes and neurons, with however, an overrepresentation of astrocytic- and neuronal-specific genes. Nevertheless, changes occurring at promoter-related sequences (TSS1500, 16 genes) could further

associate with anti-correlated transcriptional changes of the corresponding genes (Table 2), most of them being involved in immune or neuronal processes.

These genome-wide characterizations in blood immune cells and bulk post-mortem brain tissue of MS patients unravel DNA methylation changes at genes involved in immune and nervous processes and set the stage for future studies in larger and more homogenous cohorts.

2.3 *Methodological Considerations in Clinical DNA Methylation Studies*

We will here review several biases that might impair proper interpretation of DNA methylation changes, particularly in the clinical context.

2.3.1 Cohort and Sample Heterogeneity

The varying results between studies examining blood immune cells in MS underscores the context-dependent nature of epigenetic marks where DNA methylation is highly sensitive to sample and cohort heterogeneity. Therefore, cohort characteristics, such as genetic background, disease course and sub-type, age and sex, largely influence the outcome of the analyses. A variety of other confounders associated to treatment history, lifestyle habits or environmental exposures such as smoking likely affect the methylome and could account for some of the signal detected in DNA methylation studies as well.

Cell type-specificity of DNA methylation represents another challenge in data interpretation, as different cell proportions from mixed blood and brain tissue might drive the observed DNA methylation changes and therefore mask 'true' changes. Accordingly, DNA methylation changes in cell types sorted from the same case-control cohorts display little overlap (Graves et al. 2014; Bos et al. 2015; Maltby et al. 2015). Undoubtedly, cell type sorting prior to analysis offers the most optimal strategy to account for tissue heterogeneity. This approach is feasible in blood samples but it is confined only to the most abundant cell types and cell sorting is rather limited in *post-mortem* brain tissue, due to reliance on only a restricted number of cell type-specific nuclear antibodies (Yeung et al. 2014). Therefore, in most brain studies, spatial and cellular heterogeneity is therefore lost. In that context, progress in emerging fields such as single-cell methylomics (Smallwood et al. 2014) together with in situ *DNA* methylation analysis (Shiura et al. 2014) and spatial 'omics' (Stahl et al. 2016) will eventually provide useful tools to complement bulk methylome by mapping a molecular atlas at a single-cell level. Alternatively, analytical strategies correcting for confounders could aid in deciphering biologically relevant DNA methylation signals, as exemplified in two aforementioned studies in which accounting for age and blood cell proportions strengthened or even enabled the identification

of the *MIR21* and *IL2RA* loci, respectively, in $CD4^+$ T cells from MS patients (Field et al. 2017; Ruhrmann et al. 2018). It is noteworthy that the current lack of reference methylomes from distinct human brain cell types hinders the use of reference-based cellular deconvolution from brain tissue-generated data, contrasting with its common use in blood DNA methylation analysis (Titus et al. 2017). This challenge can be partly overcome by the use of unsupervised methods base on reference-free algorithms (Titus et al. 2017). Additionally, further work is needed in order to characterize changes in other immune cell types than $CD4^+$ and $CD8^+$ T cells, e.g. APCs such as monocytes and B cells which are believed to play pivotal roles in MS. This is supported by the considerable benefit RRMS patients gained after treatment with monoclonal antibody depleting B cells (Hauser et al. 2008). Finally, additional heterogeneity come from sub-cell types from immune (e.g. Th1, Th17, Th2, Treg $CD4^+$ T cells) and nervous (e.g. excitatory glutamatergic vs. inhibitory GABAergic neurons) cell populations, which are known to exert different functions. Optimization of genome-wide methylation profiling for sample with low input will undeniably aid in capturing this variety of changes occurring in MS. Altogether, these issues highlight the need to take into account cell and cohort heterogeneity in EWAS design prior to and during downstream analysis.

2.3.2 Methodological Challenges

Genome-wide technologies have facilitated EWAS analysis by promising unbiased 'hypothesis-free' approach to comprehensively characterize variations associated to complex diseases. Among the technologies available, the utilization of cost-effective array platforms such as Infinium HumanMethylation BeadChips represents the best compromise for DNA methylation analysis in clinical samples. Despite their extensive use, methylation arrays pose several limitations (Barker et al. 2018), some being inherent to the technology itself, other to the commonly employed bisulfite (BS) treatment of DNA prior to sample hybridization. Indeed, even though these so-called "genome-wide" Illumina 450K and EPIC arrays cover ~99% of the Refseq genes together with some well-known intergenic regulatory regions, they target each gene with few probes and overall annotate only a fraction, i.e. 1.7% and 3%, respectively, of the total CpGs present in the genome. An additional bias in array-based methods is the role of SNPs in the pre-design probe locations (Chen et al. 2013), causing differences in binding to certain alleles for a given gene. Moreover, the most widely-used DNA methylation studies (array included) rely on the bisulfite (BS) treatment of genomic DNA, which converts unmethylated cytosines to thymine, leaving methylated cytosine unchanged. Signals from each base are subsequently used to estimate the proportion of methylated vs. unmethylated CpGs. Yet, by changing most cytosines at non-CpG sites, BS conversion drastically reduces the complexity of the genome to three bases, therefore hampering proper exploration of highly polymorphic loci. This is of particular importance in the HLA region since it is a highly polymorphic region with a complex pattern of linkage disequilibrium and the high similarity between the proximal *HLA* class II genes. The challenge to study such locus can be illustrated by our effort to validate BS array-generated DMR at

HLA-DRB1 using multiple methods (detailed in Sect. 3.1.1 of this chapter) (Kular et al. 2018). Additionally, conventional BS-based arrays do not allow distinction of true CpG methylation (5mC) from its antagonist CpG hydroxymethylation (5hmC), nor do they cover sufficient probes targeting non-CpGs, both non-canonical modifications being highly prevalent in the human brain, especially neurons. The impact of mixed 5mC/5hmC signals in *post-mortem* brain samples could be technically circumvented by the use of BS and oxidative BS side-by-side treatments prior to hybridization on the array (Stewart et al. 2015). Besides technical limitations, heterogeneity in analytical approaches, computational pipelines and statistical approaches play an evident role in the reported outcome. As seen earlier, studies have invariably favored either DMP or DMR analysis, reporting significant changes at nominal or adjusted P-value (with or without $\Delta\beta$ cutoff) and using different normalization strategies, thus making comparison between studies challenging.

2.3.3 Biological Relevance

Inferring the functional consequence of methylation changes is still very demanding given our partial knowledge of the impact of such changes. Data interpretation likely builds on an integrative model incorporating a variety of parameters such as the location (promoter, intragenic, intergenic), the nature (single vs. contiguous CpGs) and the amplitude of change. Importantly, DNA methylation acts in concert with histone posttranslational modifications and chromatin conformational regulators, these interactions shaping gene regulation in responses to internal (genetic) and external (environmental) influences. Therefore superimposing information from additional molecular layers, ideally transcriptional and organizational, appears crucial for proper data interpretation. These can be derived from publicly available databases integrating genetic architecture of the human epigenome and transcriptome, such as ENCODE, Roadmap and Blueprint epigenomes (Bujold et al. 2016), Genotype-Tissue Expression (GTEx) (Gamazon et al. 2018) or the brain-specific xQTL (Ng et al. 2017) platforms.

The biological relevance of epigenetics in complex human diseases such as MS is further complicated by the difficulty to infer causality between epigenetic marks and pathological processes. Further work is necessary to define the nature of the interactions between the genome, methylome and exposome. Indeed, DNA methylation can be triggered by genetic, environmental and stochastic cues and impact proper functioning of virtually all immune or CNS cell types implicated in MS. As such, DNA methylation could be a cause or consequence of disease, act independently or in mediation of risk factors. To address this issue, the use of analytical strategies, namely causal inference testing and Mendelian randomization (described in the next section) or methodological approaches such as longitudinal cohorts, could undeniably aid in elucidating the epigenetic contribution in MS disease. At the tissue and cellular level, the use of emerging methodologies such as CRISPR-dCas9-based epigenome-editing (Pulecio et al. 2017) (described in Sect. 3.3.2 of this chapter) in combination with adequate experimental design in animal and cellular models will certainly assist in the quest for biological relevance of identified epigenetic changes.

3 Exploiting DNA Methylation for a Better Understanding and Treatment of MS Patients

GWAS have revealed that the genetic architecture of MS is polygenic and related to more than 200 common variants. The identification of various environmental risk factors further increases the complexity of the risk for developing MS. Similarly, EWAS are continuously increasing the catalog of putatively relevant candidate loci associating with the disease. A key challenge now is to place the identified variants, exposures and methylation alterations in the context of pathological mechanisms. This further underscores the need for investigation of causal alleles in the relevant tissue/cell type and under specific environmental conditions. In that context, since DNA methylation integrates signals from both genetic and environmental influences, it can be regarded to act at different levels of genetic predisposition to disease: in an additive manner, in synergy or as a mediator of genetic risk (Fig. 2). In this section, we will describe the potential roles of DNA methylation in mediating risk for MS.

3.1 DNA Methylation as a Mediator of Genetic Risk in MS

3.1.1 DNA Methylation Mediates Risk from the Major MS Risk *HLA-DRB1*15:01*

Integrated approaches combining the multiple layers of the interplay between genetic and epigenetic factors in gene regulation have shown that, overall, a substantial fraction of the methylome is controlled by the DNA sequence (Liu et al. 2014; Chen et al. 2016). This dependence, referred to as methylation quantitative trait locus (meQTL), can occur by local SNPs disrupting the CpG site or by proximal or distal SNPs affecting epigenetic status in *cis* or *trans*, respectively, through long-range physical and functional interactions. Therefore, a genetic-epigenetic paradigm appears instrumental to understand how risk variants could shape individuals into susceptibility for MS. Importantly, DNA methylation changes at the major MS risk gene, *HLA-DRB1,* have been consistently identified in blood immune cells and seem to partially dependent on carriage of the risk *HLA-DRB1*15:01* variant (Graves et al. 2014). We have investigated whether DNA methylation mediates effect of genetic variation in MS by integrating genome-wide genotype data with epigenome-wide data in case-control cohorts (Kular et al. 2018). A summary of the results is depicted in Fig. 3. Using Illumina 450K arrays for methylation profiling in $CD14^+$ monocytes sorted from blood of MS patients and healthy controls, we found that monocytes of the risk *HLA-DRB1*15:01* carriers display a considerably lower methylation at 19 CpGs of a DMR encompassing the exon 2 of the *HLA-DRB1* gene (Fig. 3a). Validation of *HLA-DRB1*-specific DNA methylation changes using BS-pyrosequencing confirmed hypomethylation in *HLA-DRB1*15:01* carriers,

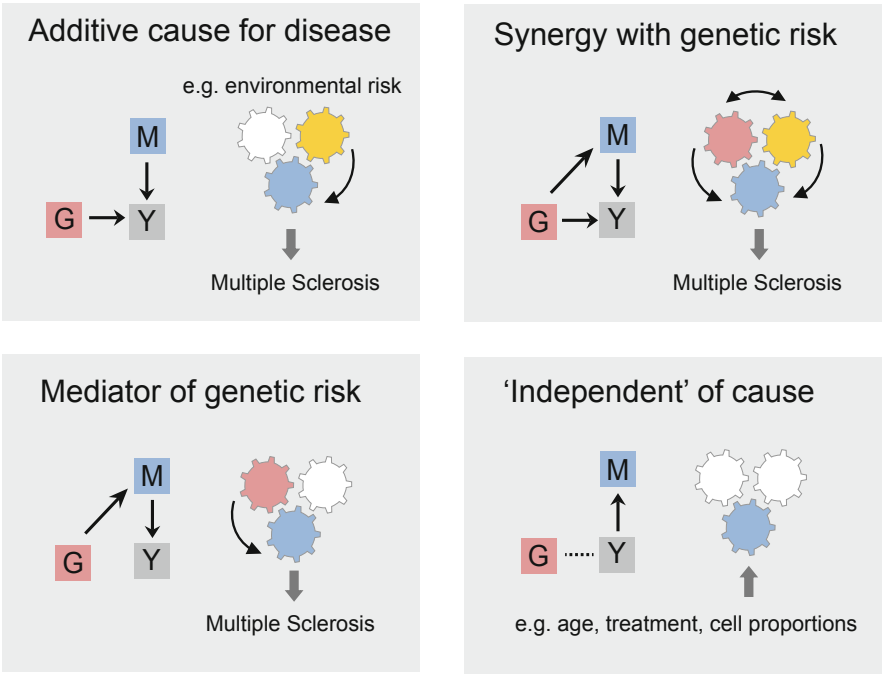


Fig. 2 Potential roles of DNA methylation in MS. Interplay between DNA methylation (M), genetic risk (G) and environmental risk exposure in phenotypic outcome (Y), depicted by blue, red and yellow wheels, respectively. DNA methylation can act in addition, in synergy, as a mediator of genetic risk or be seemingly 'independent' of any cause. The latter occur for example if DNA methylation changes are driven by cohort or sample confounders (e.g. age, sex or treatment history, cell proportions)

which could be further correlated with higher *HLA-DRB1* expression in monocytes from risk carriers compared to heterozygotes and non-carriers. We next tested whether the *HLA-DRB1*15:01* variant specifically, i.e. compared to other *HLA-DRB1* alleles, drives the observed differences using allele-specific DNA methylation and expression analyses. Single-strand BS-DNA cloning and sequencing of a fragment encompassing the DMR sequence in homozygous *HLA-DRB1*15:01* individuals confirmed the unmethylated status of more than 52 CpGs harboring exon 2 of the gene in *HLA-DRB1*15:01*. The use of methyl-sensitive restriction enzyme followed by allele-specific qPCR, which alleviates biases induced by BS conversion and pre-designed probes from commercial arrays, established *HLA-DRB1**15:01 as the sole hypomethylated variant compared to the most common *HLA-DRB1* alleles. We next functionally tested whether intragenic methylation change at *HLA-DRB1* can actively impact gene expression or rather be consequence of transcriptional activity in the locus, as reported for other genes (Mendizabal et al. 2017; Neri et al. 2017). Results revealed that PBMCs treated with a demethylating agent exhibit increased *HLA-DRB1* expression and the *HLA-DRB1* DMR sequence displays methylation-sensitive enhancer properties using an in-vitro reporter system. Taken

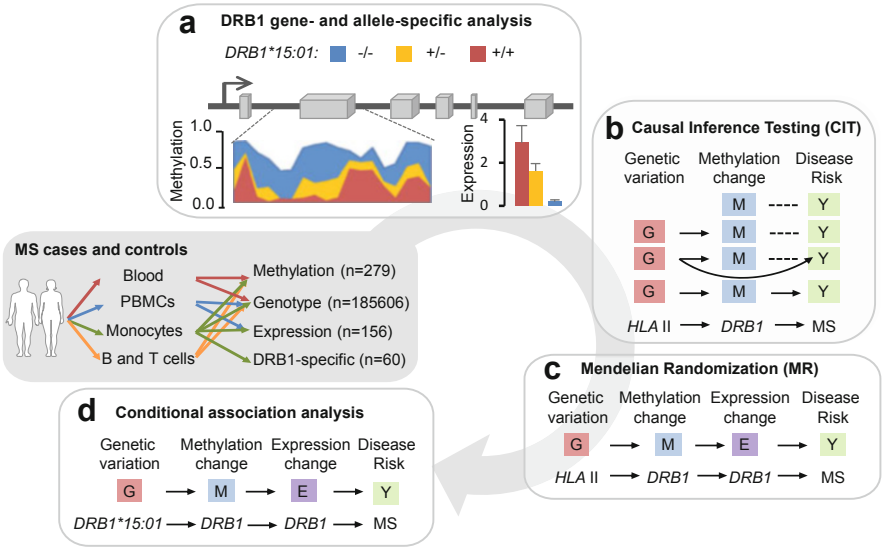


Fig. 3 DNA methylation as a mediator of genetic risk in MS. (**a**) Genome-wide, locus-specific, functional methylation analyses in monocytes revealed that *HLA-DRB1*15:01* is unmethylated and pre-dominantly expressed allele. Causal inference methods such as causal inference testing (CIT) with the different analytical steps described in (**b**) and Mendelian Randomization (MR) with Egger's regression (**c**) established significant causation between *HLA-DRB1* methylation, expression and MS risk. (**d**) Genetic association identified *HLA-DRB1*15:01* and a novel protective *HLA* variant as interacting variants acting through the changes in *HLA-DRB1* DNA methylation and gene expression to modulate risk of MS

together, these findings strongly suggest that DNA methylation in *HLA-DRB1* can mediate the risk of MS. To formally test this hypothesis we performed genome-wide mediation analysis using a large case-control cohort with genotype and methylation data and applied Causal Inference Test (CIT) to establish the mediation (Fig. 3b). CIT analysis identified 50 genetic variants that predispose for MS through methylation changes, primarily in the same *HLA-DRB1* exon 2 region identified in monocytes. We then addressed the functional impact of genetically-controlled methylation at exon 2 of *HLA-DRB1* on transcription by carrying out two-sample Mendelian Randomization (MR) and MR-Egger's regression. Findings corroborated a causal relationship between methylation at the *HLA-DRB1* DMR and *HLA-DRB1* gene expression, with MR-Steiger test validating this directionality (Fig. 3c). Finally, association analyses in four large cohorts (14,259 cases and 171,347 controls), conditioning for all known MS variants in the *HLA* locus, confirmed that the main effect comes from *HLA-DRB1*15:01* (Fig. 3d), as observed in monocytes, but also identified a novel protective MS variant (rs9267649) counteracting the effect of *HLA-DRB1*15:01* on methylation and expression.

This study highlights the importance of integrating multi-layered data to explore the molecular mechanisms underlying risk variants and to further identify new disease-modifying variants that might escape detection by conventional genetic studies. It also raises the novel hypothesis that methylation-mediated regulation of

expression of the HLA class II molecules, in addition to conformational changes of the protein, mediates the risk of MS. The HLA class II molecules present specific antigens, mainly derived from extracellular pathogens, on the surface of APCs. To do so, *HLA-DRB1*-encoded molecules are constantly being synthetized and remain ready to accept peptides, only translocation to the surface requiring peptide binding. In line with this, higher *HLA-DRB1* transcript levels observed in risk carriers would increase the probability of them binding to the peptide and presenting it in higher amounts on the surface. In the context of MS pathogenesis, this is most likely the case for specific APCs presenting MS autoantigenic peptide(s). The exposed peptides are then recognized by $CD4^+$ T lymphocytes, leading to a complex cascade of specific immune responses driving autoimmunity against CNS myelin. In this conceptual framework, it is tempting to speculate about the potential of DNA methylation as a mediator of cellular plasticity in MS.

3.1.2 DNA Methylation as a Mediator of Cellular Plasticity in MS?

Normal body functions during development and homeostasis and its aberrant expression in the case of disease involve changes in phenotypic plasticity. In this context, 'susceptibility' genes may in reality act as 'plasticity' genes, rendering some individuals more responsive than others to external (stochastic, environmental) factors (Belsky et al. 2009). This notion applied to development was already suggested by C. Waddington, pioneer in epigenetics, as for him, it should be possible to alter the degree of flexibility-inflexibility by selecting appropriate genotypes (Waddington 1959). Important work conducted in animal models of phenotypic plasticity in the context of genetic homogeneity has further evidenced the existence of a third source of phenotypic variability, neither genetic nor environmental, by demonstrating the contribution of DNA methylation changes in the varying phenotypes, even in parthenogenetic species upon environmental stability (Kucharski et al. 2008; Vogt et al. 2008). These studies are consistent with observations from MZ twin studies (Kaminsky et al. 2009) and collectively imply that epigenetic processes are likely pervasive guarantors of plasticity, from organismal to cellular and molecular levels. An altered epigenetic plasticity and chromatin dynamics could conceivably underpin pathogenic processes as well, as illustrated by altered DNA methylation variability in cancer (Hansen et al. 2011), aging (Cheung et al. 2018) or autoimmune diseases such as Rheumatoid Arthritis (Webster et al. 2018). Undoubtedly, low or high plasticity at specific genes are both likely to alter cellular phenotype in a locus-, cell type- and context-dependent manner, thus conferring adaptive or maladaptive response under specific circumstances. Whether genome-wide DNA methylation variability is affected in Multiple Sclerosis remains an open question warranting further investigation. Nevertheless, a shift from a risk model to a plasticity paradigm might aid in capturing the complexity underlying disease susceptibility at the individual level. Accordingly, genetic inheritance of risk from *HLA-DRB1*15:01* might rely not only on transmission of the impact of genetic variation on structural specificity of *HLA-DRB1* protein for MS autoantigen, but also on transmission of certain plasticity.

From an evolutionary perspective, exposure to pathogens is believed to be one of the strongest selective forces in human evolution, largely contributing to the remarkable genetic diversity found in the *HLA* locus. This phenomenon can be regarded as part of the classical genetic assimilation of an adaptive acquired trait. Interestingly, a recent study has found that, unlike other common *HLA* genes (e.g. *HLA-DQB1*), *HLA-DRB1* locus does not display any biologically meaningful pathogen group-specific bias (Pierini and Lenz 2018), indicating that specific *HLA-DRB1* alleles might have been selected by specific pathogens. Additionally, HLA-DR molecules have been associated to presentation of intracellular endogenous antigens as well, such as following autophagy of intracellular component (Munz 2016) or in the case of viral infection (Martin and Carrington 2005), including MS risk EBV infection (Paludan et al. 2005). Altogether, these findings suggest that the typical 'divergent allele advantage' in this locus (translating to a better chance for efficient immunosurveillance due to sequence diversity between alleles in heterozygous individuals) has probably not played a substantial role in *HLA-DRB1* allele selection, this locus has likely evolved under advantageous selection by specific pathogens.

In line with this, the remarkable sequence variation of *HLA-DRB1* gene mirroring the extended pathogen recognition repertoire maps to the HLA peptide-binding groove encoded by exon 2 of *HLA-DRB1* gene. It is noteworthy that the very same locus also harbors the identified hypomethylated DMR mediating risk via enhanced *HLA-DRB*15:01* gene expression. One can hypothesize that by buffering the molecular stochasticity and subsequent intrinsic noise at the DNA methylation (Smallwood et al. 2014) and transcriptional level (Elowitz et al. 2002; Chang et al. 2008; Kellogg and Tay 2015), *HLA-DRB1*15:01* variant is poised to a latent activation state. This suggests that the additional source of plasticity conferred by *HLA-DRB1*15:01* through DNA hypomethylation at exon 2 has been selectively favored over the years, probably due its potential to drive efficient immune response. At the cellular level in MS disease, because *HLA-DRB1*15:01* mean expression appears constitutively set at a high level, this plasticity might however elicit maladaptive responses upon stochastic events observed in MS-dependent context, such as autoantigen peptide encounter. This further supports the match-mismatch hypothesis underlying adaptive and maladaptive response in the case of a mismatch between one's predicted versus actual environment. Further work will be essential to decipher whether epigenetic-mediated cellular plasticity plays a role in autoimmune processes in MS.

3.2 DNA Methylation as a Mediator of Environmental Risk Factors

More than 10 environmental exposures or lifestyle habits have been suggested to increase susceptibility to MS. However, few studies have put focus on the mechanisms underlying these effects. Recent evidence indicates that DNA methylation could play a role in mediating the effect of two of the most established risk factors, cigarette smoking and lack of sun exposure/vitamin D, in disease pathogenesis. The main findings are illustrated in Fig. 4.

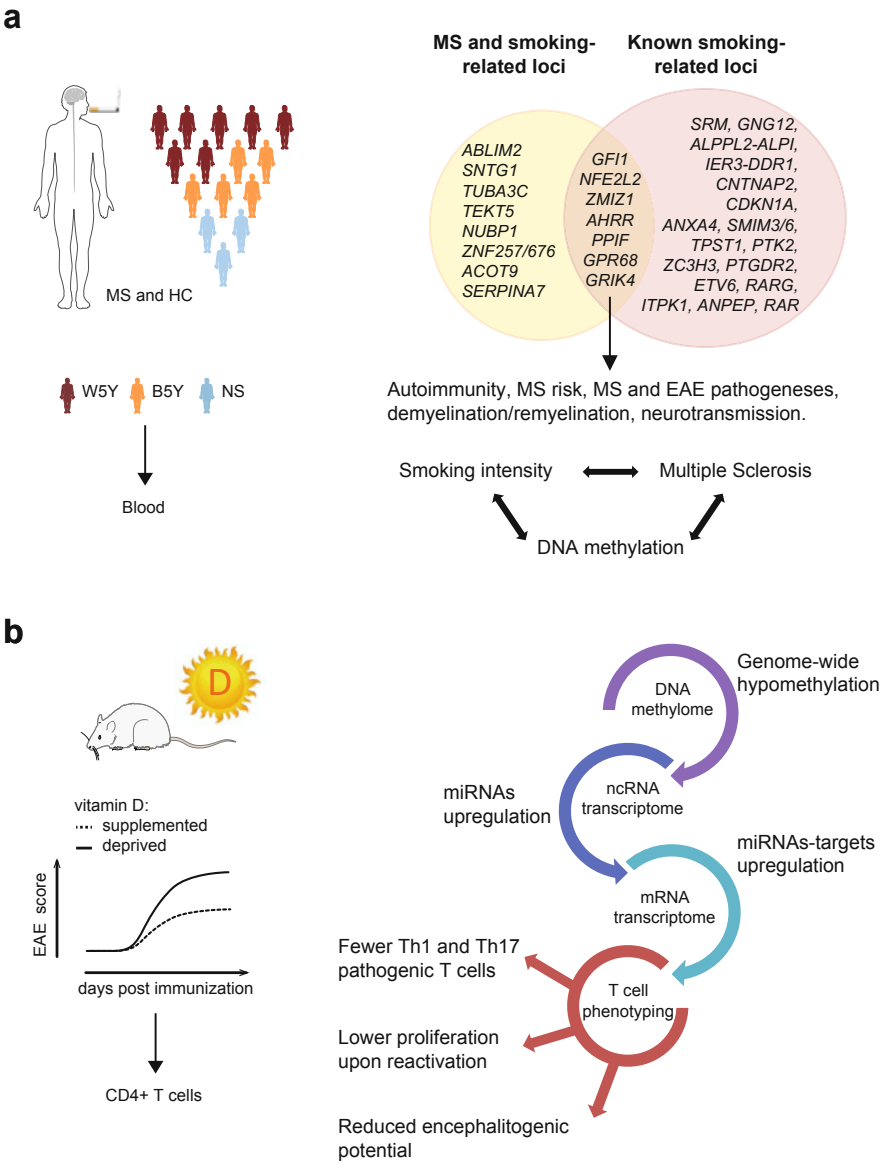


Fig. 4 DNA methylation as a mediator of environmental risk in MS. (**a**). DNA methylation changes underlying cigarette smoking in blood samples from case-control cohort with individuals categorized regarding the time post smoking cessation: within 5 years (W5Y) smoker (dark red), beyond 5 years (B5Y) smoker (orange) or never smoker (light blue). Altered CpGs implicate known smoking-related loci as well as novel loci in MS patients, with the smoking load having an enhanced impact in patients compared to controls. (**b**). DNA methylation changes mediating protective effect of vitamin D in $CD4^+$ T cells from juvenile rats vitamin D-supplemented or -deprived in an animal MS model, experimental autoimmune encephalomyelitis (EAE). Genome-wide changes correlate with dysregulation of microRNA (miRNAs) and their target genes, concomitant with differential phenotypes of $CD4^+$ T cells and protection against EAE in vitamin D-supplemented rats

3.2.1 DNA Methylation and Smoking in MS

Both active and passive smoking have been associated with increased risk, progression and disability in MS (Olsson et al. 2017). Pathogenic mechanisms underpinning smoke exposure likely involve lung irritation and inflammation rather than systemic nicotine exposure itself (Hedstrom et al. 2009, 2013a, b). In line with this, unspecific lung irritation due to other toxic compounds has been reported to increase disease severity (Bergamaschi et al. 2018; Jeanjean et al. 2018). Local immune responses resulting from lung irritation would trigger peripheral immune dysregulation and further promote autoimmune reactions. Both duration and intensity of smoking increase MS risk independently, and unlike other MS risk factor, the effect is not associated to a particular age of exposure (Hedstrom et al. 2009). Interestingly, the impact of smoking is reversible as it remains up to 5 years after cessation and is nullified a decade after cessation (Hedstrom et al. 2013a, b). Studies have examined interaction between the strongest genetic risk, i.e. carriage of the risk allele *HLA-DRB1*15:01* and absence of the protective *HLA-A*02* allele, and cigarette smoking. Results showed that both active and passive smokers, carrying both *HLA* risk factors, display considerably increased risk to develop MS compared to non-smokers, e.g. nearly 14-fold for active smoker compared to a fivefold increase in non-smokers (Hedstrom et al. 2011, 2014). More recently, *NAT1* gene involved in metabolism of smoke compounds emerged as a putative genetic modifier of tobacco smoke exposure in MS susceptibility (Briggs et al. 2014). However, even though several hypotheses implicate impact of pro-inflammatory processes occurring locally in the lungs, the underlying mechanisms supporting gene-environment interactions in MS immunopathogenesis are still elusive.

We recently explored the impact of smoking on blood DNA methylation profiles in cohorts of MS patients by comparing individuals among MS risk categories: less than 5 years after cessation (within 5 year (W5Y)-smokers), more than 5 years after cessation (beyond 5 years (B5Y)-past smokers) and never smokers (NS) (Marabita et al. 2017). Expectedly, comparison of methylome signatures in MS patients revealed that the majority of differences could be observed between W5Y-smokers and NS groups. A large fraction (84%) of the 58 identified DMPs (mapping 38 loci) were found hypomethylated after smoking, most of them corresponding to CpGs known to be affected by smoking in healthy individuals (Gao et al. 2015). In addition to these established loci, eight potentially novel smoking-associated DMPs were found in the context of MS. Overall, the identified DMPs locate in regulatory regions of genes that have been implicated in MS and/or EAE pathogenesis (Fig. 4a). Importantly, hypomethylation of CpGs of *AHRR* gene could be associated with increased *AHRR* expression in PBMCs from smoker compared to non-smoker MS patients. The aryl-hydrocarbon receptor (AHR) repressor *AHRR* gene was first described to encode a competitive repressor of AHR activity involved in xenobiotic detoxification, e.g. under smoke exposure, and was later implicated as a negative regulator of inflammation and aberrant proliferation. One possible interpretation would speculate that the observed hypomethylation and upregulation of *AHRR* gene in smokers could reflect compensatory mechanisms to AHR-mediated toxicity of compounds from cigarette smoke. Another interpretation links *AHRR*

dysregulation to a specific cell type involved in MS pathogenesis, where increased *AHRR* levels might contribute to impaired homeostasis in smokers. In support of this hypothesis, smoking-related DMPs found in MS patients were enriched in hematopoietic stem and progenitor cells (HSPC)-specific regulatory regions. Interestingly, a response to Natalizumab treatment in MS patients has been associated with HSPC mobilization (Mattoscio et al. 2015).

Of note, the identified DNA methylation changes appear more pronounced in a cohort considered to be at high risk, i.e. composed of only MS female patients with both MS genetic risk (*HLA-DRB1*15:01*$^{+}$, *HLA-A2*$^{-/-}$) and higher smoking load, suggesting a modifying effect of any of these variables at the DNA methylation level. A putative interaction between genetic risk and smoking-related DNA methylation change is consistent with the increased risk observed in *HLA-DRB1*15:01*$^{+}$ MS smokers mentioned above. This hypothesis is further supported by the significant effect that the intensity of smoking has on DNA methylation in W5Y-smoker as well. Moreover, the effect of smoking is reversible, as methylation levels reached the ones observed in MS patients who have quit smoking more than 5 years prior to blood sampling or who have never smoked. Jointly, these findings are in accordance with observations from epidemiological studies on the impact of smoking in MS. Interestingly, while the disease status (RRMS, SPMS) did not have a significant impact on DNA methylation, the presence of MS disease per se exacerbated the effect of smoking load, inflating the extent of hypomethylation. This novel finding about a modifier effect of disease on the impact of smoking intensity is of high relevance both for clinicians and MS patients.

3.2.2 Vitamin D and DNA Methylation in MS

The first evidence of the effect of environmental factor in MS susceptibility arose from the observation of latitude-gradient effect on MS prevalence (Koch-Henriksen and Sorensen 2010; Simpson et al. 2011). The effect was later attributed to lack of sun exposure, low vitamin D levels or a combination of both, as the major source of vitamin D originates from skin exposure to UV-radiation and to a lesser extent from dietary intake. Additionally, polymorphism in genes involved vitamin D metabolism such as the MS risk *CYP27B1* locus (Sundqvist et al. 2010) have been associated to vitamin D levels as well (Bahrami et al. 2018). The age of exposure was refined to childhood/adolescence, with migration studies showing that moving to country of high latitude before adolescence, and not at adulthood, is likely responsible for greater individual risk to develop MS (Gale and Martyn 1995). In line with this, low vitamin D levels detected before the age of 20 years old associate with increased MS risk (Munger et al. 2006) and vitamin D levels in adolescent, but not adult, rats affect EAE incidence and course via immunomodulatory actions (Adzemovic et al. 2013). These findings jointly support recommendation for prophylactic vitamin supplementation of adolescents in prevention of MS risk. Overall, lack of sun exposure and/or hypovitaminosis D were shown to affect MS susceptibility, disease activity, disability and progression (Olsson et al. 2017). Interestingly, a vitamin D-dependent regulation of *HLA-DRB1*15:01* variant involving functional

interaction at vitamin D response element (VDRE), has been reported in-vitro (Ramagopalan et al. 2009). Evidence of a long-term impact of vitamin D/lack of sun exposure, remaining after the period of exposure, further reinforces the hypothesis of a role of epigenetics in mediating the effect. Even though the interaction between vitamin D cognate receptor VDR and transcription factors and histone posttranslational modifiers is well established (Lu et al. 2018), the impact of vitamin D on DNA methylation is less documented.

Using several 'omic' approaches in rodents, we recently investigated the mechanisms underlying the protective effect of vitamin D supplementation, compared to deprivation, on EAE in juvenile rats (Zeitelhofer et al. 2017). Genome-wide DNA methylation profiling of $CD4^+$ T cells displayed subtle but widespread methylation changes upon vitamin D supplementation compared to deprivation. Notably, the vast majority of DMRs were found hypomethylated, probably due to reduced expression levels of all DNMTs in these cells. Moreover, a large fraction of the identified DMRs affected the expression of corresponding genes, which were suggested to be proximal mediators of VDR signaling. Among them, hypomethylation and concomitant upregulation of small non-coding RNAs was associated to subsequent modulation of their target genes (Fig. 4b). Expectedly, altered genes were enriched in pathways related to T cell activation and differentiation. Accordingly, $CD4^+$ T cells presented with reduced ability to differentiate into Th1 and Th17 cells, to proliferate and importantly, to exert encephalitogenic effect. This study thus provides functional evidence that vitamin D affects the pathogenic potential of $CD4^+$ T cells directly via DNA methylation changes. Results contrast with findings from another study where global methylation in $CD4^+$ T cells, assessed at LINE-1 sequence, was found increased in EAE adult mice following vitamin D treatment (Moore et al. 2018). This effect was associated with increased methyltransferase *BHMT1* gene involved in methionine epigenetic metabolism and with a transition from encephalitogenic $CD4^+$ T cell to regulatory Treg cell population. The differences between studies can be explained by the large disparities in experimental conditions or by differential mechanisms of vitamin D in euchromatin and heterochromatin compartments. Limited studies have reported association between DNA methylation changes and vitamin D status in humans (Bahrami et al. 2018) and future research is needed to confirm these findings in MS.

Altogether these data support the relevance of studying DNA methylation in understanding the interaction between the exposome and the genome, and how this interaction may thus affect risk to develop MS.

3.3 DNA Methylation as Biomarker and Putative Therapeutic Target

Despite advance in identifying the molecular mechanisms underpinning MS disease and progress in development of potent immunomodulatory drugs for early stages of disease, no treatment cure disease, leaving MS patients with constant progression of

disability. Moreover, valid biomarkers are still lacking for disease phenotyping and prediction of treatment response and disease progression. In this context, the remarkable properties of stability and reversibility of DNA methylation offer unprecedented perspective for improved biomarker and therapies.

3.3.1 DNA Methylation as Biomarker

Monitoring and prognostic methods in MS are primarily based on neuroimaging methods such as Magnetic Resonance Imaging (MRI) detecting brain burden, i.e. atrophy and lesional damage which correlate with long-term disability. On the other hand, the use of biomolecular biomarkers such as DNA methylation has the potential to reflect ongoing rather than delayed events occurring in the brain of MS patients. Correlation of some DNA methylation changes between post-mortem brain and peripheral blood, as observed for *PADI2* gene (Calabrese et al. 2012, 2014), advocates the use of DNA methylation as supplementary information compared to existing molecular biomarkers. In line with this, profiling of DNA methylation in cell-free blood samples has been shown to accurately capture cell type-specific signature of dying cells from peripheral organ, such as oligodendrocyte degeneration in RRMS patients (Lehmann-Werman et al. 2016). Additionally, the use of locus-specific DNA methylation patterns in $CD4^+$ T cells, such as *IL17A* and *FOXP3* genes, allows for accurate estimation of Th lineage commitment and imbalance, thus representing interesting phenotyping tool (Janson et al. 2011). Methylation patterns of cell-free plasma DNA have been shown to potentially serve as a discriminatory biomarker of relapse vs. remission for RRMS patients (Liggett et al. 2010). Finally, novel approaches based on droplet/digital assay (Yu et al. 2018) or the generation of methylation biosignatures from multiplexed DNA methylation profiles might represent new strategies for early, specific and quantitative detection of DNA methylation-based biomarkers in MS patients.

3.3.2 DNA Methylation as Therapeutic Target

The plastic nature of DNA methylation marks makes them attractive target for pharmacological therapy. The impact of DNMT inhibitors such as 5-aza-2′deoxycytabine (5-aza, known as decitabine) has been investigated in the context of EAE. Decitabine, an FDA-approved hypomethylating agent, is a chemical analog of cytidine that incorporates into replicating DNA where it irreversibly blocks DNMT1 activity and leads to loss of methylation in a cell division-dependent manner. Several studies have demonstrated protective effect of 5-aza treatment in EAE and consistently associated protection with a direct effect on Th1 and Th17 pathogenic cells (Chan et al. 2014; Mangano et al. 2014; Wang et al. 2017). Both prophylactic and therapeutic administration of 5-aza resulted in amelioration of EAE clinical score and histological hallmarks, i.e. reduced lymphocyte infiltration in the CNS and demyelination. Interestingly, differential effects were observed depending on the dose and duration

of 5-aza treatment, with a chronically administered low 5-aza dose promoting polarization of T cells into a beneficial Treg phenotype (Chan et al. 2014; Mangano et al. 2014) while high acute 5-aza treatment acting primarily on T effector (Teff) cell proliferation (Wang et al. 2017). Indeed, low doses of 5-aza result in higher number of circulating and infiltrating Treg cells, concomitant with decrease of Th1 and Th17 pathogenic T cell populations. Thymic Treg display enhanced immunosuppressive activity, inhibiting proliferation and lowering the activation potential of Teff cells (Chan et al. 2014). These effects were attributed to predominant hypomethylation of Treg-specific loci such as the *FOXP3* gene (Mangano et al. 2014). In contrast, the infiltrating Treg compartment was unchanged when treating animal with higher 5-aza dose (Wang et al. 2017), this acute administration could instead inhibit activation of the CNS-resident macrophage/microglia and proliferation of encephalitogenic T cells. Unexpectedly, inhibition of T cell proliferation was further shown to be mediated by a TET2 action on cell cycle-related genes. While 5-aza treatment of $CD4^+$ T cells leads to increased TET2 and TET3 and reduced TET1 expression, knock-down of TET2, specifically, can partially abrogate 5-aza effect on proliferation by restoring the expression of key cell cycle inhibitors (p15, p16 and p21 genes) to basal level. TET2 mediation of 5-aza effect operated through direct binding of TET2 to the promoters of these genes (Wang et al. 2017). Thus, 5-aza can favor promoter demethylation not only by inhibiting DNMT1 but also by promoting TET expression in certain cell types. It seems however that the use of global epigenetic modifiers such as DNMT inhibitors for clinical purpose is limited, such global manipulation eliciting broad effects on the methylome. Additionally, as previously mentioned, 5-aza action relies on cell division, thereby restricting its impacts on proliferating cells only, as observed for immune cells, and can exert serious neurotoxic effect (Wang et al. 2013). These deleterious effects therefore hamper its potential for chronic neurodegenerative diseases such as MS.

Novel approaches based on targeted-epigenetic therapy are expected to overcome the global effect of DNMT inhibitors. Such epigenome-editing strategies aim at correcting deleterious DNA methylation changes while leaving homeostatic marks unaltered and would therefore set the stage toward precision and personalized medicine. Among the epigenome editing tools available in animal experimentation, the recently developed CRISPR-dCas9 system appears a promising strategy for targeted epigenetic therapy (Pulecio et al. 2017). CRISPR-dCas9 design builds on the ability of the CRISPR-based system to induce stable locus-specific changes in DNA methylation (Klann et al. 2017). A short single guide RNA (sgRNA) is used to deliver a catalytically inactive Cas9 (dCas9) fused to the catalytic domain of methyltransferase (Dnmt3a) or demethylase (Tet1) to modulate methylation at a specific locus without modifying the DNA sequence. To this aim, a chimera comprising catalytic domains of Dnmt3a and Dnmt3l has been shown to induce more robust degree of methylation compared to Dnmt3a alone (Stepper et al. 2017). Additionally, it has been recently proposed that fusing Dnmt3a-Dnmt3l with the Krüppel-associated box repressor domain (or other repressors) can achieve stable loss of expression that is resistant to external activation stimuli (Amabile et al. 2016). Conversely, CRISPR-dCas9 construct tethering Tet1 catalytic domain, successfully used for targeted demethylation of specific loci, can be utilized to demethylate genes and release them from repression, as

demonstrated in-vivo (Liu et al. 2016). Therefore, the attractiveness of targeted epigenetic therapy would rely on its locus-specific, long-lasting, albeit reversible, action, thereby possibly limiting continuous or repeated administration in patients. However, the therapeutic potential of CRISPR-dCas9 epimodifier system is still at early pre-clinical stage of development and additional concerns related to toxicity and safety, with possible off-targets and poor tissue-specificity, warrant further investigation. In that regards, novel molecular design and delivery approaches, e.g. using natural or synthetic carrier nanoparticles (Lu et al. 2014) to either pathogenic immune cells or nervous cell types, would provide complementary tools for specific delivery and efficiency of the epimodifier system.

4 Conclusion and Future Perspectives

Multiple Sclerosis is a clinically heterogeneous disease that affects young individuals and results in progressive debilitating disability. Neurologists face daily challenges in the care of MS patients, not the least with variable efficacy and side effects of MS medications and unpredictable disease progression. The MS paradigm proposes that environmental exposures operate on susceptible genetic background to cause disease. The potential of DNA methylation studies to aid in better understanding and treating disease relies on its remarkable properties of sensitivity, stability and reversibility. A growing body of evidence from EWAS supports alteration of DNA methylation levels at specific loci of the genome involved in immune and nervous processes in affected patients compared to controls. However, methylome characterization in MS is still in its infancy and thus warrant further investigation in order to overcome current challenges related to cohort and samples heterogeneity, methodological limitations and biological interpretation of the data. Nevertheless, emerging findings suggest that DNA methylation could mediate genetic and environmental risk in MS and further support DNA methylation as a mediator of phenotypic plasticity driving disease development and progression. In addition, DNA methylation may provide potential biomarkers for phenotypic profiling and prognosis of MS patients. Future perspectives also include the use of DNA methylation as therapeutic targets based on targeted-epigenetic therapy. Overall, DNA methylation could serve as a molecular substratum for precision medicine and personalized care of MS patients. Future research incorporating advanced epigenetic methods in large and homogeneous cohorts appears essential to further integrate encouraging scientific findings to a clinical perspective.

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