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THE ROLE OF EPIGENETICS IN MULTIPLE SCLEROSIS DEVELOPMENT, PROGRESSION AND TREATMENT

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The role of epigenetics in Multiple Sclerosis development, progression and treatment
THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my parents, for always supporting me in my endeavors.

ABSTRACT

The overall aim of this thesis was to determine epigenetic changes in peripheral immune cells from Multiple Sclerosis (MS) patients. MS is a chronic inflammatory neurodegenerative disease, which initially presents itself during young adulthood. Big consortia have identified over 230 different polymorphisms contributing to the risk of developing disease, with many of these polymorphisms located in immune genes. However, the odds ratios of these polymorphisms are small and many known environmental risk factors are contributing to the disease. This indicates that the risk may partially be conferred through epigenetic changes such as DNA methylation.

In this thesis, we investigate the role of DNA methylation in different peripheral immune cells using genome-wide DNA methylation arrays. We first characterized DNA methylation patterns in four different immune cell types form relapsing-remitting (RRMS), secondary-progressive (SPMS) patients and healthy controls (HC) and compared them with each other. Here we found a shared signature between all cells types, and in SPMS we found a specific neurodegenerative signal, while in MS patients, we saw lymphocyte signaling and T cell activation being affected. The top changes in CD4+ T cells indicate a change in the *VMP1/MIR21* locus. We functionally investigated this and found lower miR-21 expression and an increase of miR-21 target genes. Because the most numerous methylation changes were found in CD19+ B cells, we further investigated CD19+ cells in a second larger cohort. After meta-analysis, the changes in B cells indicate differences in metabolism and activation between RRMS and HC. To analyze the shared pathway data, we developed a method to cluster pathways, which we further developed into an R package called GeneSetCluster.

We investigated the effects of dimethyl fumarate (DMF) and rituximab treatment on DNA methylation in CD4+ and CD14+ cells. The different treatments had a different cell type specific signature as well as different kinetics. After DMF treatment, we found changes in reactive oxygen species (ROS) signaling and T cell subtype associated genes. Furthermore, we identified a polymorphism associated with treatment outcome and ROS production that does not associate with disease susceptibility. After rituximab treatment, we found differences in activation, metabolism and motility associated genes.

Our findings collectively underline the importance of investigating epigenetic changes in multiple cell types to identify novel, potentially modifiable, mechanisms involved in the etiology and pathogenesis of complex diseases like MS.

LIST OF SCIENTIFIC PAPERS

I. **Combining evidence from four immune cell types identifies DNA methylation patterns that implicate functionally distinct pathways during Multiple Sclerosis progression.**

Ewing E, Kular L, Fernandes SJ, Karathanasis N, Lagani V, Ruhrmann S, Tsamardinos I, Tegner J, Piehl F, Gomez-Cabrero D, Jagodic M.

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II. **Hypermethylation of MIR21 in CD4+ T cells from patients with relapsing-remitting multiple sclerosis associates with lower miRNA-21 levels and concomitant up-regulation of its target genes.**

Ruhrmann S, **Ewing E**, Piket E, Kular L, Cetrulo Lorenzi JC, Fernandes SJ, Morikawa H, Aeinehband S, Sayols-Baixeras S, Aslibekyan S, Absher DM, Arnett DK, Tegner J, Gomez-Cabrero D, Piehl F, Jagodic M.

Mult Scler. 2018 Sep;24(10):1288-1300. doi: 10.1177/1352458517721356. Epub 2017 Aug 2.. PMID: 28766461

III. **GeneSetCluster: a tool for summarizing and integrating gene-set analysis results.**
Ewing E, Planell-Picola N, Jagodic M, Gomez-Cabrero D

Manuscript

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Nat Commun. 2019 Jul 12;10(1):3081. doi: 10.1038/s41467-019-11139-3., PMID: 3130067

V. **Investigating a role of B cells and their depletion in relapsing-remitting Multiple Sclerosis using DNA methylation patterns**

Ewing E, Al Nimer F, Kular L, Stridh P, Olsson T, Khademi M, Gomez-Cabrero D, Piehl F, Jagodic M.

Manuscript

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I. **Anti-citrullinated protein antibodies acquire a pro-inflammatory Fc glycosylation phenotype prior to the onset of rheumatoid arthritis.**

Rombouts Y, **Ewing E**, van de Stadt LA, Selman MH, Trouw LA, Deelder AM, Huizinga TW, Wuhrer M, van Schaardenburg D, Toes RE, Scherer HU.

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III. **Epigenetic research in multiple sclerosis: progress, challenges, and opportunities.**

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Physiol Genomics. 2017 Sep 1;49(9):447-461. doi: 10.1152/physiolgenomics.00060.2017. Epub 2017 Jul 28. Review. PMID: 28754822

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Needhamsen M, **Ewing E**, Lund H, Gomez-Cabrero D, Harris RA, Kular L, Jagodic M.

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V. **Fatal demyelinating disease is induced by monocyte-derived macrophages in the absence of TGF-β signaling.**

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CONTENTS

LIST OF ABBREVIATIONS

MS	Multiple Sclerosis
CNS	Central nervous system
BBB	Blood brain barrier
EDSS	Expanded disability status scale
MRI	Magnetic resonance imaging
RRMS	Relapsing-remitting multiple sclerosis
CIS	Clinically isolated syndrome
SPMS	Secondary progressive multiple sclerosis
PPMS	Primary progressive multiple sclerosis
MZ	Monozygotic twins
HLA	Human leukocyte antigen
SNPs	Single nucleotide polymorphisms
APC	Antigen presenting cells
BMI	Body mass index
EBV	Eppstein-Barr virus
Tregs	Regulatory T helper cell
TCR	T cell receptor
EAE	Experimental autoimmune encephalomyelitis
MOG	Myelin oligodendrocyte glycoprotein
MAIT	Mucosal-assocaited invariant T cells
CSF	Cerebral spinal fluid
BAFF	B cell activating factor
DMF	Dimethyl fumarate
ROS	Reactive oxygen species
HDACs	Histone deacetylase
5mC	5-methylcytosine
DNMT	DNA methyltransferases
TET	Ten-eleven translocation
5hmC	5-hydroxymethylcytosine
DMP	Differentially methylated positions

DMR	Differentially methylated region
450K	Infinium Human Methylation 450K BeadChip
EPIC	Infinium Human Methylation EPIC BeadChip
WGBS	Whole genome bisulfite sequencing
RRBS	Reduced representation bisulfite sequencing
MBD-seq	Methyl-CpG binding domain sequencing
meDIP-seq	Methyl-CpG sequencing
β	Beta respresenting the ratio C / T
BMIQ	Beta-mixture in quantile normalization
PBMC	Peripheral blood mononuclear cells
PCA	Principal component analysis
LT-α	Lymphotoxin-alpha
TSS	Transcription start site
IFN	Interferon
NRF2	Nuclear factor erythoid 2-related factor 2
VDR	Vitamin D receptor
LCL	Lymphoblastoid cell lines
NAWM	Normal appearing white matter
HATs	Histone acetyltransferase
lncRNA	Long non-coding RNA
sncRNA	Small non-coding RNA
miRNA	mircoRNAs
Ago	Argonaute
KS	Kolmogorov-smirnov test
IPA	Ingenuity pathway analysis
HC	Healthy controls
gRNA	Guide RNA

1 MULTIPLE SCLEROSIS

1.1 DISEASE AND SYMPTOMS

Multiple Sclerosis (MS) is a chronic inflammatory disease, it is located in the central nervous system (CNS), and MS is defined by the autoimmune destruction of myelin, which results in the loss of neurons (1, 2). It is the second most common debilitating disease in young adults, and it affects around 2.5 million people worldwide. Patients are usually diagnosed between 20-40 years of age, with women being affected nearly three times as often as men (3). The disease is characterized by an immune response against myelin, which is a lipid-rich layer produced by oligodendrocytes. Myelin insulates axons to allow for fast transmission of electrical impulses within the nervous system as well as providing metabolic support for the axons. This immune response causes the breakdown of the blood brain barrier (BBB), infiltration of immune cells into the CNS and subsequent development of inflammatory and demyelinating lesions in both brain and spinal cord (4). Later in the disease course, there is notable neurodegeneration and severe brain atrophy, tough brain atrophy can be observed in the initial stages also.

MS symptoms can vary greatly depending on the localization of the lesions in the CNS and can include changes in sensation, muscle weakness, muscle spasms, difficulties with coordination and balance, problems in speech, visual problems, fatigue and acute or chronic pain syndromes, bladder and bowel difficulties, cognitive impairment, or depression. The patient disability is measured using the Expanded Disability Status Scale (EDSS) (5). Using modern magnetic resonance imaging (MRI) we can detect these lesions in the brain (Fig. 1), these are classified either with different time pulses or with contrast enhancing agents. Lesions detected using longer pulses are classified as T2 lesions, and three or more new T2 lesions in a 2 year period predicts unfavorable for patient outcome (6). The most commonly used contrast-enhancing agent in MS is Gadolinium-based, which normally is unable to pass through the BBB unless there is an active or chronic lesion in the brain (7).

The initial diagnosis of most MS patients (~85-90%) is the relapsing-remitting form of MS (RRMS). However, there is a preclinical phase where the disease is ongoing but does not present itself with symptoms yet (8). The initial clinical event is called a clinically isolated syndrome (CIS), but after the second relapse, patients are diagnosed with RRMS (Fig.1). The RRMS form of the disease presents itself with recurring episodes of acute neurological symptoms (relapses) followed by recovery (remission). While inflammation is present in all stages of MS, it is more pronounced in RRMS than in the progressive phase. The RRMS stage is currently quite successfully treated with several disease-modifying therapies, described below, which broadly target the immune system and treatments are sometimes accompanied by severe adverse effects (9). However, after roughly 15-20 years (10, 11)the majority of RRMS patients convert to a more progressive form of MS called secondary-progressive MS (SPMS). SPMS is noted for the accumulation of axonal damage, neuronal loss and the persistent increase in neurological disability. The remainder of the patients exert a different disease course, called primary-progressive MS (PPMS), which presents itself with a more severe disease course from onset without the complete or partial recovery phase and displays less inflammation while still causing a lot of nerve damage. Recently the first drugs

became available for disease stages other than RRMS, recently ocrelizumab was approved for PPMS and siponimod was approved for SPMS patients.

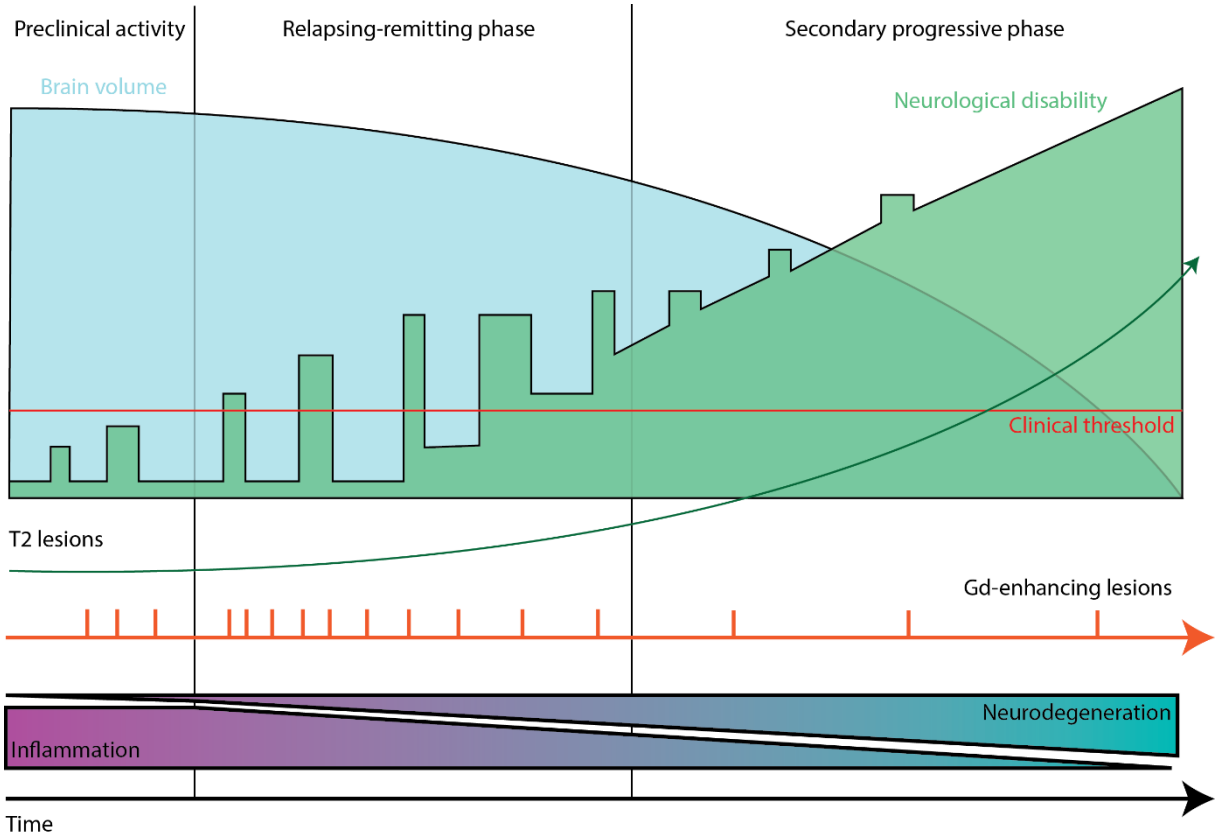

Figure 1. Multiple Sclerosis (MS) disease course. This figure displays the disease course of the average MS patient starting with relapsing-remitting phase and later on progressing to the secondary progressive phase of the disease. A relative contribution of inflammatory and neurodegenerative mechanisms throughout the disease course is depicted schematically.

2 EPIDEMIOLOGY

2.1 GENETICS

While the exact cause of MS remain unknown, current epidemiological data establish MS as a complex disease with a strong genetic and environmental component. Family members of MS patients have a higher chance of developing MS, with monozygotic twins (MZ) displaying a concordance rate of ~15-25% compared to 2-5% in dizygotic twins, indicating the importance of genetic as well as environmental factors (12, 13). The first discovered and still the strongest genetic risk factor lies within the Human Leukocyte Antigen (HLA) locus(14), specifically a class II allele of the *HLA-DRB1* gene, *HLA-DRB1*15:01*. Large international consortia (15, 16) conducted genome-wide association studies and custom-designed arrays, which have identified over 230 non-HLA single nucleotide polymorphisms (SNPs) which predispose for MS. Many of these lie in genes associated with T-cell related signaling pathways, as well as multiple variants and alleles of the HLA locus itself (17). The class II HLA molecules are expressed on antigen presenting cells (APCs) and present peptide antigens to $CD4^+$ T cells, thus playing a crucial role in the antigen-specific immune response.

2.2 ENVIRONMENTAL FACTORS

In MS, the relatively low concordance rate in MZ twins indicates a possible role for the stochastic nature of the adaptive immune system regarding molecular mimicry, but also the environmental exposure as crucial to disease development. Epidemiological studies have identified environment risk factors such as lack of vitamin D/sun exposure, smoking, elevated Body Mass Index (BMI) in early life and Epstein-Barr virus (EBV) infection (18). Interestingly, many of these environmental cues seem to act during a susceptible window encompassing childhood and adolescence (4, 19). This has been observed in migration studies, where a reduced risk in migrants moving from a high- to a low-risk area was seen if they moved during the first two decades of life (19). Conversely, the migration from a low- to a high-risk area did not alter the risk of MS in the migrants, however, the increased risk was seen in their children. Even though these environmental risk factors are well studied in their association with MS, other environmental factors such as the gut microbiome, including the brain-gut axis are attracting more attention recently (20, 21). Furthermore melatonin levels (22) also appear to influence MS disease.

3 THE ROLE OF IMMUNE CELLS IN MS PATHOLOGY

The autoimmune response in MS is a result of the interaction of many cell types. Although multiple immune cells have been implicated in the immunopathology of MS, this thesis focuses on T and B cells and monocytes, and thus their role in MS is briefly described in the following sections.

3.1 ROLE OF T CELLS

The $CD4^+$ T cells, also known as T helper cells, help regulate or suppress immune cells by releasing cytokines, which assist in activating cytotoxic T cells, B cell antibody class switching and macrophages. In MS, relevant subtypes are the pro-inflammatory Th1 and Th17 cells and the repressive regulatory T cells (Tregs). Activation of $CD4^+$ T cells occurs by binding of the T cell receptor (TCR) that recognizes a peptide antigen presented by the HLA molecule on a professional APCs, though a second and third activation signals are needed as well. The $CD4^+$ cells then express cytokines such as IFNγ and IL17A, which in turn create a positive feedback loop leading to the activation of more $CD4^+$ T cells. MS is associated with an unbalanced ratio of Tregs vs Th1 and Th17 (23). In addition, Tregs function has also been shown to be impaired in MS (24, 25). Tregs play a critical role in maintaining peripheral immune tolerance by suppressing autoimmune responses. Interesting is that in the MS GWAS several Treg related genes were found including IL-2 signaling, CD25, and CD127 (26). $CD4^+$ T cells help in disrupting the BBB and the production of cytokines attracting monocytes and neutrophils, together which form MS lesions (27). The role of $CD4^+$ T cells in MS is well established in part due to the animal model of M-like disease experimental autoimmune encephalomyelitis (EAE), where transfer of activated myelin oligodendrocyte glycoprotein (MOG)-specific $CD4^+$ T cells is enough to cause demyelinating disease (28).

$CD8^+$ T cells are much more predominant in active MS lesions than $CD4^+$ T cells, and they are the primary cytotoxic effector cells that drive myelin destruction. Measuring TCR variability in brain lesions indicate a limited range of TCRs, the cause for this could be local T cell expansion at the site of inflammation (29). Higher frequencies of $CD8^+$ T cells correlate with axonal damage (30). In MS lesions, 25% of the $CD8^+$ fraction produce IL17, a characteristic of mucosal-associated invariant T (MAIT) cells (31), MAIT cells are reported to be important for regulating Th1 responses and the number of MAIT cells is reduced in the peripheral blood of MS patients, indicating that these cells might have several anti-inflammatory properties (32).

3.2 ROLE OF B CELLS

B cells have been getting more interest in the immunopathology of MS over the last few years. B cells have many functions, these include antibody production, where differentiated B cells undergo affinity maturation and start to produce antigen-binding proteins. B cells are also APCs, and have roles in antigen presentation, where soluble autoantigens bound on the B cell surface are presented to T cells. Part of this APC function is autoantigen transport, where B cells capture specific antigens and transport them to the lymph nodes. Finally, B cells also produce several pro and anti-inflammatory cytokines (33). In some autoimmune diseases, B cells generate pathogenic autoantibodies, however, there is no conclusive evidence of such autoantibodies playing a pathogenic role in MS (34), and antibodies alone are also not capable of inducing EAE, indicating that in MS the role of B cells is related to cytokine production and APC function. Despite this, the IgG oligoclonal bands in the cerebral spinal fluid (CSF) of MS patient are a useful clinical biomarker for MS diagnosis (35). CXCR13, a chemokine specific for B cells is found in the CSF, and has a positive correlation with relapse rate (36).

Similar to T cells, there are many subtypes of B cells, with the memory B cells gaining increasing evidence in the context of MS. These are long-lived B cells, which remain after the initial inflammation, and that can evoke an enhanced response to reinfection, particularly in response to viruses (37). Only treatment with drugs which target memory B cells appear to be beneficial in MS. Tabalumab, which inhibits B cell activating factor (BAFF), depletes mature, but not memory B cells, and has no clinical effect in MS (38). Meanwhile, rituximab treatment is a highly successful MS drug and depletes both mature B cells and memory B cells (39). The number of memory B cells in the blood of MS patients has also been reported to be reduced after dimethyl fumarate (DMF) treatment (40) and fingolimod treatment results in a reduction of memory B cell migration into the CNS (41), where the memory B cells are the most numerous B cells (42). Furthermore, it is in the CNS where the EBV transformed B memory cells reside (43). Memory B cells are also reported to activate brain-homing autoreactive $CD4^+$ T cells (44), via interacting with HLA-DR on the $CD4^+$ T cell surface.

3.3 ROLE OF MONOCYTES/MACROPHAGES

Monocytes and macrophages are both APCs and part of the innate immune system, and their main functions are phagocytosis, antigen presentation and cytokine production. T cells recruit monocytes and macrophages to the CNS following BBB disruption. Preventing the accumulation of macrophages delays the initiation of EAE and results in a less severe disease course (45). Their contribution to MS could be due to the production of reactive oxygen species (ROS), which are potent immune regulators. With the different ROS being integral to many

processes in the immune system, including T cell activation, proliferation and apoptosis (46). DMF has been reported to dampen the monocyte response by altering its ROS production (47). Furthermore, active lesions are characterized by myelin-filled macrophages, which phagocytose myelin debris following T cell mediated destruction (48).

4 TREATMENT

Currently several treatment options exist for patients with RRMS, some are broadly targeting drugs such as interferons, others are more specific, such as natalizumab, which stops lymphocytes from passing the BBB by which it prevents CNS inflammation (49). Fingolimod (50) and siponimod (51) both target the sphingosine-1-phosphate receptor to trap lymphocytes in the lymph nodes, preventing their contribution to the autoimmune response, fingolimod also inhibits histone deacetylases (HDACs) in the brain. Glatiramer acetate is a mixture of several synthetic polypeptides that are believed to inhibit the binding of myelin proteins to HLA (52). DMF has several effects, though the exact beneficial means are unknown but seems to be involved in modulation of ROS signaling, which is essential in BBB, macrophage and monocyte functioning (53). Rituximab, ocrelizumab, ublituximab and ofatumumab are monoclonal antibodies against B cell marker CD20 (54). B cell depletion with anti-CD20 leaves the antibody-producing plasma cells remain unaffected, highlighting the importance of the antigen presenting role and cytokine production of the B cells as possible drivers of MS. B cell depletion seems to work by modulating T cell responses, reducing inflammatory cytokines and depleting the EBV reservoir (55). Despite the numerous available treatments for RRMS, most patients end up switching from first line treatment to a second or later choice, indicating that proper selection of initial treatment is difficult due to heterogonous nature of MS (56).

5 EPIGENETICS

The British embryologist C.H. Waddington coined the term epigenetics in 1942 as pertaining to epigenesis or the epigenotype (57). Currently, it is defined as the study of heritable changes in gene function that do not involve changes in DNA sequence. This means it covers mechanisms that make up the phenotypical state of a cell despite the same genetic background (Fig. 2A). The best known epigenetic mechanisms include DNA methylation, histone modifications and non-coding RNAs. Dysregulation of these regulators at any level can influence disease risk and severity. Despite over 230 SNPs currently known to affect MS risk, the odds ratio of these SNPs is small, indicating that there is a role for environmental risk factors. As epigenetics lies at the molecular interface between the genetic background and environmental exposure, it might provide further insights into MS risks and pathogenesis.

5.1 DNA METHYLATION

DNA methylation is the covalent addition of a methyl group to the 5th carbon of cytosine, known as 5-methylcytosine (5mC), and is usually occurs in a CpG dinucleotide context (Fig.

2B). CpG indicates that it is a cytosine followed by a phosphate followed by guanine that is methylated. Non-CpG methylation also occurs but is restricted to specific cell types such a pluripotent stem cells and CNS resident cells (58). A single CpG within a cell appears either methylated, hemi-methylated or de-methylated and estimations indicate that within the human genome there are roughly 28 million CpGs (59).

The chemical addition and removal of a methyl group to cytosine is performed by several different enzymes. DNA methyltransferases (DNMTs) act in the establishment of de novo methylation (DNMT3A and DNMT3B) or preservation of the methylation status during cell division (DNMT1). DNA methylation within CpG rich promotors of genes is associated with transcriptional repression (Fig. 2C/D) (60), while higher methylation in gene bodies positively correlates with expression, likely due to blocking the binding sites of alternative promotors and altering the kinetics of transcription. Furthermore, studies suggest that methylation can also influence splice variants.

Meanwhile, in addition to passive mechanisms, DNA de-methylation can occur by the ten-eleven translocation family of proteins (TETs), which oxidize 5mC to 5-hydroxymethyl cytosine (5hmC), followed by further downstream metabolization. 5hmC has been demonstrated to be stable and to functionally regulate the genome with higher abundance in certain tissues such as lungs, testes and CNS [41]. Higher levels of 5hmC in the body of genes correlate positively with transcription. The role of DNA methylation is highly complex and depends on genomic context, specific tissue and time-dependent events.

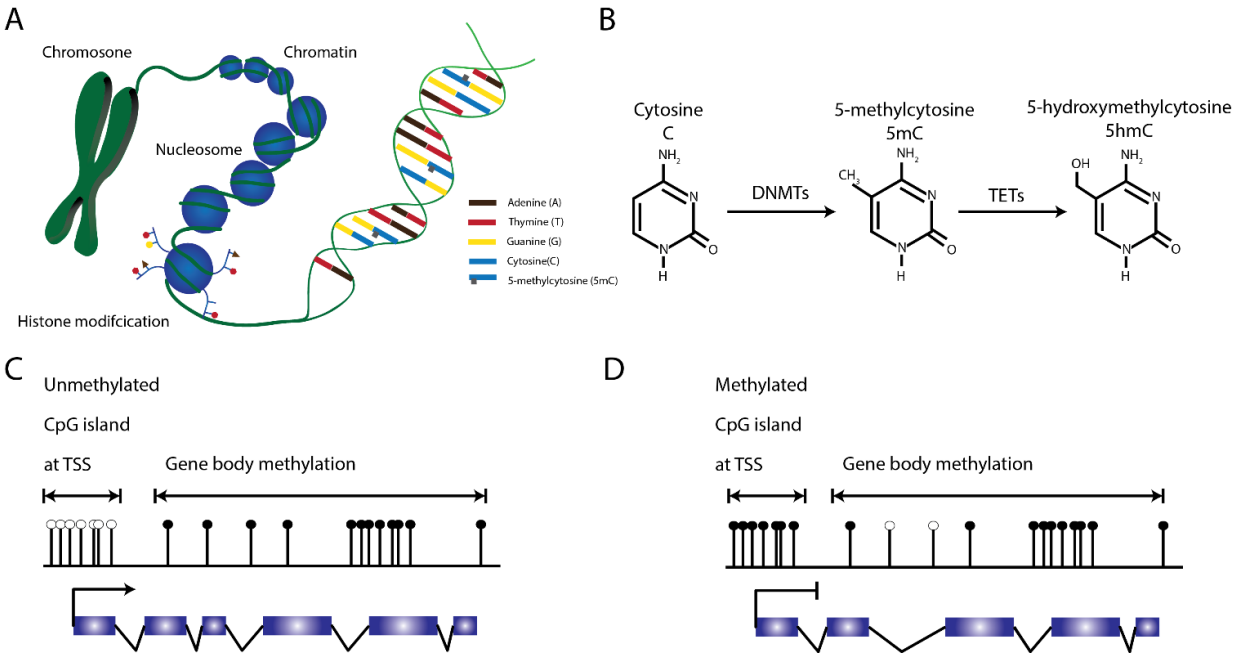

Figure 2. Chromosomal organization and DNA methylation. **A)** The organization of a chromosome, with the chromatin, nucleosome, with histone modifications, down to the DNA sequence where the chemical modification of cytosine takes place. **B)** The different bases with the different methyl groups attached. **C)** An unmethylated promoter with methylated gene body CpGs that results in expression. **D)** A methylated promoter of a gene that is not expressed.

5.2 EPIGENETIC METHODS

The methyl group can be distinguished either by methylation-sensitive enzymes or by chemical treatments such as bisulfite conversion, which chemically alters un-methylated cytosines to uracil, further amplified to thymine, with the ratio of C / T in the post-bisulfite downstream analysis representing the methylation level. Here it is important to realize that the level of methylation in a single cell is usually binary, but samples represent a mixture of many cells. Therefore this methylation measurement represents the average of the cells measured, and this also means that the difference in methylation levels found might represent a difference between cell mixtures.

Current high-throughput methods allow for genome-wide profiling of DNA methylation with a relatively small amount of DNA required. As a result, high throughput methods are well suited for clinical studies where MS immune or CNS cells are compared with healthy controls to identify differentially methylated positions (DMPs) and regions (DMRs). The most commonly used genome-wide methods include microarray approaches such as Illumina 450K (450K) and Illumina EPIC (EPIC). Common sequencing-based methods, including whole-genome bisulfite sequencing (WGBS) and reduced representation bisulfite sequencing (RRBS). Other available techniques include enrichment approaches like the Agilent Sureselect Methyl-seq or immunoprecipitation approaches such as methyl-CpG sequencing (meDIP-seq) or methyl-CpG binding domain sequencing (MBD-seq). Locus specific techniques include pyrosequencing, qPCR and methylation-specific PCR.

The ratio of C / T following bisulfite treatment in methylation data is calculated for every CpG as a beta (β) using the following formula:

$$Beta_i = \frac{max(Y_i, methy, 0)}{max(Y_i, unmethy, 0) + max(Y_i, methy, 0) + \alpha}$$

This beta can be transformed in to log2 space as a M value, which is calculated as:

$$M_i = Log_2\left(\frac{max(Y_i, methy, 0) + \alpha}{max(Y_i, unmethy, 0) + \alpha}\right) = Log_2\left(\frac{Beta_i}{1 - Beta_i}\right)$$

where $Y_i, methy$ and $Y_i, unmethy$ are the intensities measured by the i^{th} methylated and unmethylated probes (61). In the formula, the α is a constant offset added to the intensity value of methylation arrays to regularize the beta value when intensities are low. While the beta value is attractive due to the direct biological interpretation, it has a bounded range, and this creates problems with the assumption of a Gaussian distribution of many statistical tests, on the other hand, M value is more statistically valid in differential analysis. However, the M value is more challenging for biological interpretation.

5.3 ANALYSIS PIPELINES

The platform of choice for most human studies used the 450K array while recent ones have updated to the EPIC array. Illumina arrays are used due to the low costs and low amount of input DNA required. The array design started in 2008 with the Illumina 27K which had

~27 000 probes with a type I probe chemistry. This was followed up in 2011 with the 450K array, which has ~480 000 probes, covering ~99% of all refseq genes and was mostly focused on CpG islands. Due to the large number of additional probes required, the additional probes were designed with new chemistry for specific for type II probes. In 2016 Illumina released the Illumina EPIC array, which removed ~50 000 probes from the 450K design due to the proximity to SNPs, while the EPIC array has ~400 000 additional probes. These additional probes are designed to mostly cover enhancers regions, as identified by FANTOM5 (62). We recently demonstrated that the EPIC array could also be used in mice, with over 19 000 probes mapping to the mouse genome (63).

As mentioned above, there are two types of probe chemistry in the Illumina design, the type I probe design has one bead for the methylated state and another for the unmethylated state (Fig. 3). The type II probe design has a single bead, which determines the methylation state by using fluorophore coupled single base extension after hybridization (64). The methylation levels are then measured using red and green fluorescence. The type II probes have a higher variance, less accuracy and are less sensitive to extreme methylation signals (65). Due to this, several specialized normalization pipelines have been established using peak-based correction. A commonly used pipeline includes quantile normalization, followed by beta mixture in-quantile normalization (BMIQ) (66). The remaining batch effects, such as slide or position on the slide, can be identified with a data reduction method such as principal component analysis (PCA). The identified effects should then be corrected for using batch correction tools such as combat (67).

While running a differential analysis, we aim to compute p-value to address the statistical significance of the differences. When comparing, for example, MS cases with healthy individuals to identify statistically significant differences, several potential independent variables have to be considered and controlled for. These covariates are elements that can affect the methylation level that could increase the heterogeneity of the clinical signal. These can include age, gender, and treatment history, among others, but this depends on the exact statistical question and cohort design and the power to detect interactions. A proper way to statistically control for this is to set up a linear model to regress out the effect of the covariate so that only the dependent variable remains.

The output of a statistical test is a p-value, and the p-value represents the probability of a type 1 error, a false rejection of the null hypothesis, meaning that there is no difference between the groups tested. Therefore, if the p-value is smaller than 0.05 than there is a less than 1 in 20 probability that the null hypothesis is true, meaning there is a high probability that it needs to be rejected because there is a difference between the tested groups. Usually, the significance cutoff is set for 0.05, however, if we preform 450 000 tests on the 450K array, this would mean that there could be up to 22 500 type 1 errors if there is no adjustment for multiple tests. Adjusting for multiple testing refers to the re-calculating of the probabilities obtained for the number of times the test was performed. There are several methods for this, but the common methods include Bejamini Hochberg for False-Discovery Rate or Bonferroni for Family Wise Error rate correction (68). We usually select CpG sites with an adjusted p-value < 0.05.

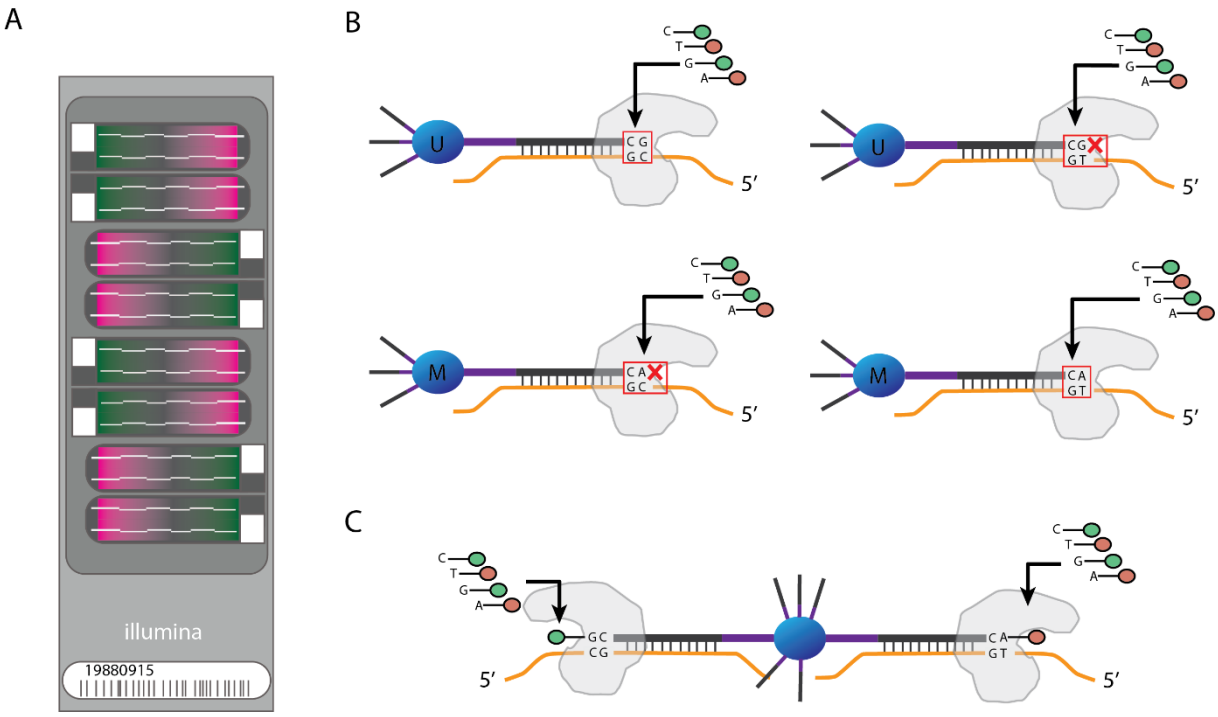

Figure 3. Illumina methylation array probe design (64). Blue is a single bead, the red box is the CpG locus and the orange line is the bisulfite converted DNA. **A)** The design of the Illumina EPIC slide. **B)** The design of the type I probes, which has one bead for the methylated strand and a separate bead for an unmethylated strand. **C)** The design of the type II probes, where there are both methylated and unmethylated signals on the same bead.

6 EPIGENETIC STUDIES IN MS

6.1 IMMUNE CELL STUDIES

The first genome-wide DNA methylation study in MS from Baranzini *et al.* used RRBS covering > 1.7 million CpGs in $CD4^+$ T cells of three MS-discordant MZ twin pairs (69). The study found very few DMPs but these had large variations within twin pairs, none of which passed genome-wide significance or were shared between the pairs. However, due to small sample size, heterogeneity of the individuals, and low genome coverage of their sequencing data, as well as their focus on large differences, several important sites may have been missed. Handel *et al.* (70) investigated the role of DNA methylation of the *HLA-DRB5* and *HLA-DRB1* locus in peripheral blood mononuclear cells (PBMCs). They compared patients classified as either benign or malignant based on EDSS, but none of the CpGs investigated showed any significant effects between the two groups. There was a non-significant trend towards more *HLA-DRB5* DNA methylation in the malignant than the benign group.

Graves *et al* (71) used Illumina 450K to analyze $CD4^+$ T cells from 30 RRMS patients and compared them to 28 healthy controls. Notably, contrary to Handel *et al.* (70) the authors found strong methylation differences in the HLA locus and especially the *HLA-DRB1* gene. In $CD4^+$ T cells the *HLA-DRB1* locus displayed strong hypomethylation, and in addition, 55 non-HLA differences were reported. Interestingly, a large portion (54%) of the non-HLA differences map to genes that have previously been reported in the context of MS. In the

follow-up study in $CD8^+$ cells from the same cohort, Maltby *et al.* (72) could not confirm the changes in *HLA-DRB1*, and none of the $CD8^+$ differences mapped to MS reported genes. Only one gene overlapped between the studies, the *MORN1* gene but with different CpGs in the two studies, indicating different epigenetic MS signatures between $CD4^+$ and $CD8^+$ T cells. Maltby *et al.* also studied $CD19^+$ cells and found a larger number of DMPs compared to $CD4^+$ cells or $CD8^+$ cells (73), with over 7000 sites having a nominal p-value < 0.05 and $\Delta\beta > 0.1$. They found a large DMR in the *LTA* gene, which encodes the Lymphotoxin-alpha (LT-α) protein. LT-α is a TNF family member produced by lymphocytes that promotes lymphocyte proliferation and immune activation via NF-κB (74). Increased LT-α has been implicated in MS before (75). Other reported genes belong to the NF-κB cascade, as well as IL21R, a receptor involved in B cell proliferation (76).

Bos *et al.* (77) analyzed $CD4^+$ and $CD8^+$ T cells sorted from peripheral blood, as well as matching whole blood of MS patients and healthy controls using the Illumina 450K array. This study also highlighted the importance of separating different immune cell subpopulations in DNA methylation studies, with only a few overlapping CpGs between the cell types. The biggest differences were seen in the *TMEM48* transcription start site (TSS), a gene involved in a nuclear pore complex. Nominally significant CpGs in the first exon of *APC2* were found, a gene involved in microtubule and beta-catenin binding. Additionally, the *DNHD1*, also involved in microtubule movement, displayed several CpGs throughout the gene. Interestingly, this study revealed a strong hypomethylation of $CD8^+$ cells in patients with duration over 7 years or lower, compared to those with a duration above 8 years. Rhead et al. (78) combined the data from Bos *et al.* with those of Maltby *et al.* and saw significant differential methylation in 4 different loci, namely those in the *MOG/ZFP57*, *HLA-DRB1*, *NINJ2/LOC100049716*, and *SLFN12* genes.

Neven *et al.* (79) investigated methylation of the Alu, LINE-1 and SAT-α, which are known as estimators of global DNA methylation levels, and together form around 33-35% of the entire genome. In total, 51 MS patients and 137 matched healthy controls were compared and showed significant hypermethylation of these repetitive elements. Lower levels of Line-1 methylation was associated with a lower EDSS while higher levels of Alu methylation associated with a lower EDSS.

Kulakova *et al.* (80) investigated DNA methylation using 450K in PBMCs from 14 RRMS and 8 PPMS patients compared with 8 healthy controls. More than half of the differential CpGs found had higher levels of methylation found in RRMS and PPMS when compared with controls, but PPMS genes also had higher levels of methylation than RRMS. Interestingly the *ESRRG* gene, a potential transcription factor for DMNT1, was found differentially methylated in RRMS. PPMS which is thought not to have a sizeable immune component, still had differential methylation of the immunological genes *HLA-F*, *MTPN*, *VIPR2* as well as autophagy gene *ATG16L2*. These data indicate that the methylation profiles of PBMCs from PPMS are distinctly different from that of RRMS.

Souren *et al.* looked in PBMCs from 45 monozygotic twin pairs discordant for MS using the EPIC array (81). After adjusting for cell-type composition, no genome-wide significant changes were detected, but the top genes included *ZBTB16*, *TMEM232*, *SEMA3C*, *YWHAG*, and *MRI1*. Epigenetic changes in *ZBTB16* and *TMEM232* were validated in a cohort of four

CD4+ samples analyzed with WGBS. *ZBTB16* is a GC response gene, which is not covered by the 450K array and while the *TMEM232* gene had a large DMR the function of this transmembrane gene is currently not known. In the CD4+ cohort, they also identified a prominent DMR in the intronic CTCF/YY1 bound regulatory region in *FIRRE*. In the specific setup of this study, they could also identify a signature associated with interferon (IFN) treatment, one of the most commonly used MS modifying treatments. The IFN analysis showed 212 genes associated with the top DMP located in the *RSAD2, MX1, IFI44L* and *PLSCR1* genes, which are reported to be higher expressed in the blood cells of IFN-treated MS patients.

The strongest genetic risk factor in MS is the HLA-DRB1*15:01 haplotype, which confers a 3-fold increase in MS risk and in the paper of Kular et al. we investigated the role of DNA methylation in the HLA-DRB1*15:01 gene (82). In the analysis of MS compared with healthy controls in CD14+ monocytes we identified a DMR in exon 2 of the HLA-DRB1 gene, where HLA-DRB1*15:01 carriers had lower methylation and a higher expression of the HLA-DRB1 gene. We used causal inference testing to establish a causal link between genetic background, DNA methylation levels and HLA-DRB1 expression. Causal inference testing identified seven DMRs that could mediate the genetic risk, six of which we could confirm using CD4+ T cells, CD8+ T cells and CD19+ B cells. We further used two-sample Mendelian randomization to determine the causal link between genetic variation in the HLA-DRB1 gene, its methylation and expression levels. This is the first study in MS demonstrating that DNA methylation can mediate the risk of developing the disease.

Few studies up to date investigated changes in the epigenome after treatment in MS patients. MS treatment is centered on the dampening of the immune response and reducing the inflammation in the CNS. DMF has several functions which are still being investigated, but a central effect is that it releases nuclear factor erythoid 2-related factor 2 (NRF2) from KEAP1 and results in the upregulation of ROS related genes and influences the polarization of T cell subtypes (83). Matlby *et al.* looked for the changes in CD4+ T cells between baseline and 6 months of treating with DMF, and identified four genes, *SNORD1A, SHTN1, MZB1* and *TNF (84).* Ntranos *et al.* compared DMF treated patients with both glatiramer acetate patients as well as treatment naïve patients (85). Here they found hypermethylation of *MIR21* locus in CD4+ T cells from the DMF treated patients. They also show that the hypermethylation of *MIR21* also occurs in cells *in vitro* in a dose-dependent manner.

6.2 EPIGENETIC INTERACTION WITH ENVIRONMENT

Several studies have looked at the influence of the environment on the epigenome in the context of MS.

One of the top environmental risk factors is smoking. Smoking is a risk factor that has been associated with many diseases, and in MS it is associated not only with an increased risk of developing MS [32], but also with faster progression and clinical disability [33]. The odd ratios of several genetic risk factors increase significantly due to the interaction with smoking (86). Studies in the Nordic countries, such as Sweden, where oral tobacco is popular suggest that this effect might be independent of the nicotine and might be due to immune activation in the lungs. Marabita et al. investigated the effect of smoking on PBMC DNA methylation

in MS patients (87). Notably, although smoking in MS induced methylation changes on the same CpGs as in healthy individuals, the amount of smoking had significantly more pronounced impact on methylation changes in MS patients, as demonstrated, e.g. a CpG in the AHRR gene, among others. This implicates interaction between smoking and disease processes in impacting the epigenome. Interesting is that the Ahr pathway, where AHRR belongs to, is a known regulator of Th17 and Treg subsets (88).

Vitamin D, which has a strong associated with MS (89), is activated in the skin under exposure of UV from sunlight and has a protective role via dampening inflammatory actions of APCs, T and B cells. Vitamin D receptor (VDR) is a nuclear receptor, whose binding sites in lymphoblastoid cell lines are enriched near loci that associate with autoimmune diseases and with genes, which are differentially expressed between MS and controls (90). In EAE vitamin D supplements can dampen the severity of disease due to reduced proliferation of $CD4^+$ T cells (91). Using MBD-seq and ChARM arrays, it was demonstrated that vitamin D-supplementation in rats results in hypomethylation in $CD4^+$ cells and their reduced activation state and pro-inflammatory phenotype.

Previous studies have indicated that a risk factor for MS is a high BMI in early life. Castro *et al.* investigated the effect of BMI on the epigenome of peripheral monocytes (92). High BMI MS patients had hypermethylation of several genes, including *NRXN1*, *TP63* and *FZD7*, which indicates a negative regulation of anti-proliferative genes. Using lipidomic profiling of plasma samples identified the presence of increased ceramide species in MS patients with high BMI. Ceramide species are a precursor to sphingosine-1-phosphate, whose receptor is the target of fingolimod (50) and siponimod (51). Similar DNA hypermethylation changes in these genes could be detected after ceramide treatment in cell cultures, but only if the ceramide concentration was similar to those high BMI MS patients. Furthermore, a high BMI correlates with an increase in monocytes and a decrease in brain volume of MS patients.

A contribution of EBV to MS has been debated for a long time, as nearly all MS patients are EBV positive compared to 90-95% of the general population (93), though the odds ratio for developing MS increases significantly if the patient has a history of infectious mononucleosis (94). Furthermore, EBV infects memory B cells in the CNS (43). Interestingly, the EBV viral load is modulated by HLA alleles, with an increased presentation with *HLA-DRB1*1501* and lower load with *HLA-A*02*, which matches with *HLA-DRB1*1501* being a negative risk factor, while *HLA-A*02* has a protective effect (95). Ong *et al.* studied EBV transformation of lymphoblastoid cells lines (LCLs) to investigate the interaction between EBV infection and MS (96). They saw that despite that EBV transformation results in predominantly hypomethylation, the regions around the MS risk loci were less likely to be hypomethylated than randomly selected regions, though they argue that this could be in part due to the constitutive B cell utilization of these regions.

Several studies have tried to use DNA methylation to set up a clock that aims to capture the biological age of a tissue rather than the chronological age (97, 98). Theodoropoulou *et al.* looked at the effect of epigenetic aging as measured by DNA methylation in MS and found that epigenetic age is different between men and woman for different cell types (99). While the Age Acceleration Residual indicated a decelerated aging in MS woman, likely due to differences in cell frequencies, the Phenotypic Age Residual indicated accelerated

phenotypic aging in MS patients. Age is an important covariate in many diseases and in MS the risk for progression triples after the age of 50 (100). The potential for increased risk of progression after the age of 50 could be due to the accelerated aging of the immune system (inflamaging) caused by long-term chronic inflammation (101).

6.3 CNS STUDIES

Neurological diseases such as MS are challenging to study in a clinical setting due to the limited accessibility of the CNS. Nonetheless, several studies have investigated epigenetic changes of the CNS tissue from MS patients. The first study to do so by Mastronardi *et al* (102) studied citrullination of myelin basic protein (MBP), a major constituent of myelin in the CNS, which can display differential post-translational modifications. Citrulination of MBP by the peptidyl arginine deaminase 2 (PAD2) gene is increased in Normal Appearing White Matter (NAWM) of MS patients, and this is thought to contribute to loss of immune tolerance. In NAWM biopsies from MS patients, the *PAD2* promoter was hypomethylated, and this effect appears to be MS specific with no differences seen in the thymus gland or in white matter from other neurological diseases. The *PAD2* expression and promotor hypomethylation effect were also seen in PBMCs of MS patients by Calabrese *et al.* (103) though the role of PAD2 in PBMCs still has to be determined.

Huynh *et al.* (104) used 450K to investigate at genome-wide DNA methylation changes in pathology-free NAWM brain regions from MS patients compared to unaffected controls. In a cohort of 23 MS patients and 19 healthy controls, they found 539 significant DMRs in MS brains compared to controls, and the DMRs were preferentially found in genomic locations associated with enhancers. DNA was found to be hypomethylated at the TSS but was hypermethylated in gene body CpGs. Hypermethylated and lower expressed genes include *MBP*, *SOX8*, a gene associated with sex determination (105), *NDRG1*, a gene involved in oligodendrocyte response to stress and *BLCL2*, an anti-apoptotic BCL2 family member. Meanwhile, proteolytic processing related genes *LGMN* and *CTSZ* were hypomethylated and expressed at higher levels. An important note is that the used methodology cannot distinguish between 5mC and 5hmC, enriched in neurons, which might contribute to some of the differences seen in the NAWM. Nevertheless, this study still highlights the numerous epigenetic changes in the CNS of MS patients influencing its homeostasis and its ability to repair itself after damage.

We recently published a paper where we studied DNA methylation of neurons from *post-mortem* brain tissue from MS patients and non-disease controls (106). Due to the high proportion of 5hmC in neurons, we investigated both 5hmC and 5mC. We also deconvoluted for glutamate and GABA neuronal fractions, the two main types of neurons. Interestingly, MS neurons were characterized by a sustained hypo-5mC and hyper-5hmC methylation, clustering mainly in gene bodies and associating with decreased gene expression. Altered genes belong to processes involved in synaptic plasticity, axonal guidance and CREB signaling pathway. We identified a reduction of CREB activity in MS samples, which could be confirmed with immunofluorescence of brain slides.

6.4 OTHER EPIGENETIC REGULATION

There are abundant well-established methods to study DNA methylation, but 5mC is far from being the only level of epigenetic regulation. In the nucleus, the negatively charged DNA is tightly wrapped around a positively charged octamer complex of four dimer proteins called histones (Fig.2 A), and together they form a structure called a nucleosome, which controls the accessibility of the chromatin. The tails of the histones or the specific histones variants themselves can undergo changes, which can affect how accessible genetic loci are to the transcriptional machinery. This N-terminal tail can undergo a multitude of simultaneous post-translational modifications (PTM). The acetylation of histone H3 is associated with transcriptional activation and is acetylated by histone acetyltransferases (HATs) and HDACs. HDACs are not only blocked by the MS drug fingolimod, but it has also been demonstrated that EAE can be treated by the use of HDAC inhibitors (107, 108). Furthermore, SNPs in genes encoding HDACs have been suggested to predict brain volume in MS patients (109). Recent work by Roy *et al*. demonstrated that the methionine is absorbed by $CD4^+$ T cells, which serves as a donor for S-adenosyl-L-methionine (SAM) (110). Restriction of methionine in the diet of mice reduced H3K4 methylation in Th17 genes, and they demonstrate that restriction of methionine alters the expansion of pathogenic Th17 cells, which subsequently reduces EAE severity.

Another regulatory level sometimes included in the term epigenetics comprises non-coding RNAs, which can include long non-coding RNAs (lncRNA), some of which have shown to be deregulated in the serum of RRMS patients (111), as well as small non-coding RNAs (sncRNA). Among the most studied sncRNAs are microRNAs (miRNA). MiRNA are transcribed in the nucleus where they form a hairpin structure, which is transported to the cytosol, where Dicer cleaves it into single-stranded RNA. The 3p or the 5p strand are then incorporated together with the Argonaute (Ago) protein into a RISC complex. They are 20-25 bp long RNAs which use a 6-8 base seed sequence to identify and bind target mRNAs, with one miRNA being able to target several mRNAs, and control protein levels within the cell and tissues (112). MiRNA act intracellularly but can also be found in extracellular biofluids, such as plasma and CSF.

They are currently intensively investigated for their biomarker potential, *e.g.* miR-150 is increased in the CSF of MS patients and correlates with more active disease and clinical parameters such as CSF cell count, immunoglobulin G index, and presence of oligo clonal bands (113). As of 2019, the role of miRNA in MS has been studied in over 61 studies with over 500 miRNAs reported as dysregulated. Piket *et al.* reviewed these studies (114), and the results indicated that the top miRNA-affected pathways in MS are linked to TGF-β-, B cell receptor-, PTEN-, MERK/MAPK-, PI3/AKT- and NF-κB signaling. Furthermore, the data indicated that the circulating miRNAs have a promising potential as MS biomarkers. However, the data also demonstrated noticeable inconsistencies among the results, which could be due to the heterogeneity among the methodologies, samples and cohorts used.

7 CHALLENGES

A growing body of evidence suggests that epigenetics plays a role in the development and progression of MS but finding changes and markers, which are validated, has been a major issue. Several different studies have been performed, as described above, yet the studies have widely different results with minimal overlap. The disparity between the studies might have resulted from several different issues. The epigenetic marks found in different tissues and cell types are going to be highly diverse, and the potential low power of the study in question, the genetic variation, environmental factors as well as or on-going disease processes or therapy will further influence the different results.

7.1 COMPARING AMONG STUDIES

A large problem in comparing cohorts is the statistical analysis used to find differences, with every study using different pre-processing and normalization techniques for the data analysis, and, while the pipeline outlined above is common, several studies use different approaches. For instance, Graves *et al.* used a two-sided Kolmogorov–Smirnov (KS) test to find differences (71), which accompanied by a step-wise filtration process to remove probes associated with either gender or treatment in a subgroup of patients. Most of the other studies use a linear model that includes age and sex as covariates. Some studies use cell type deconvolution to remove the effects of different cell mixtures (115), but other studies do not. Furthermore, some studies with low power to detect changes do not reach statistical significance; therefore, they use a different cutoff to report results. The use of different linear models with corrections for different covariates and different statistical cutoffs for calling significant results poses difficulties with comparing the outcomes of different studies.

7.2 INTEGRATING STUDIES

An approach to integrate the efforts conducted in the different studies is to use meta-analysis (116), which combines the evidence from different independent studies. There are several ways to compare the evidence from different studies, either their p-value or the effects of the findings. A combination of the p-values can be done using several different methods, but a common one is to use the Fisher's combined probability test. An effects based meta-analysis can be conducted using either a fixed-effects or a random-effects model. A Fixed effect model considers that the effect of the covariates in the studies are comparable across all populations, while a random-effects means that the effect of covariates can vary between populations. As a result, if it possible to accept the assumptions, the fixed-effect model provides higher statistical power; however, considerable heterogeneity between studies, meaning inconsistent patterns between the compared cohorts, often requires the use of a random-effects model.

7.3 META-ANALYSIS OVER PAIRED DATA OMICS DATA

A recent addition to the meta-analysis toolbox comprises a method called OmicsNPC from the STATegra R package (117). This tool uses information from multiple omics layers and utilizes both permutations and non-parametric combinations to combine data with minimal assumptions. These layers could be paired data from several different cell types or the same samples analyzed with different technologies. The permutations are used to normalize between cohorts, making all the distributions on the same scale. When using the same samples in

multiple groups, it is essential to preserve local correlation by permuting the same way across all modalities. After permutations, a permuted p-value is generated based on the ranking of the statistic of interest with the permuted statistics. The permuted p-values are combined using one of several different non-parametric combination methods: Tippett, Fisher or Liptak. Each has its own specific assumptions and different rejections of the null hypothesis.

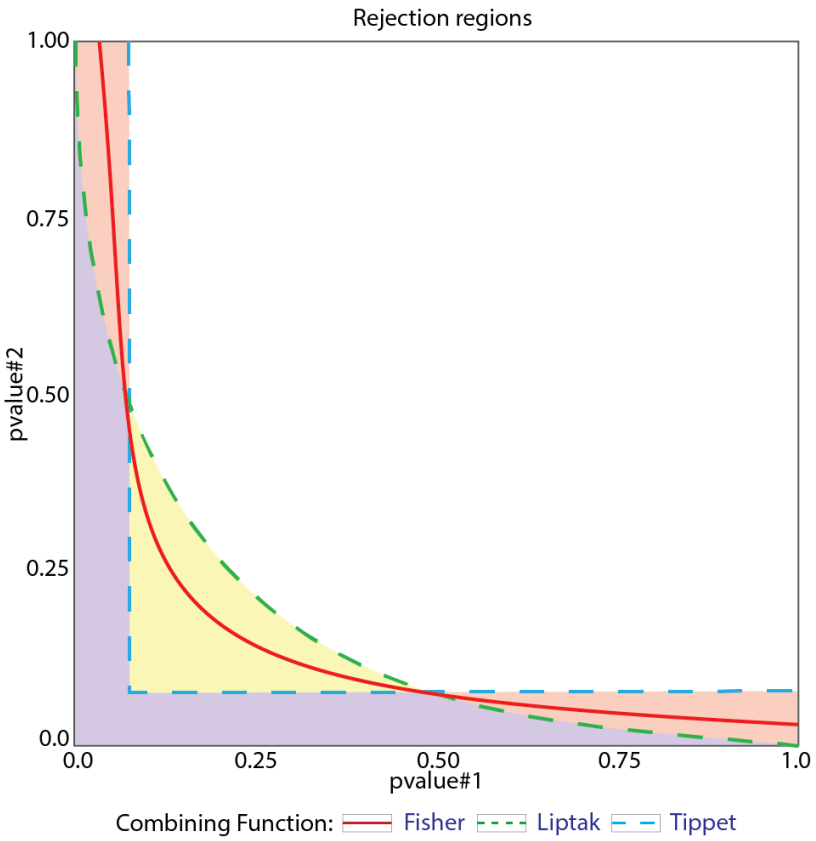

Figure 4. The different rejection limits of the different OmicsNPC tests. The green line indicates that if one of the Liptak p-value is too large, it will be rejected. Remade with permission from the original authors(117)

An interesting property of the Stouffer-Liptak method, $T_l = \sum \phi^{-1}(1-\lambda_b^i)$, is that there has to be minimal evidence for rejecting the null hypothesis in all studied combinations, or the test result would not be significant (Fig. 4). Practically, this means that if the studied change/gene is significant in all cohorts or modalities, it will be significant using the Stouffer-Liptak test. However, if the studied change/gene were not significant in one of the cohorts or modalities, then the null hypothesis would not be rejected even with overwhelming evidence from the other studied cohorts or modalities.

7.4 FUNCTIONAL RELEVANCE

Moreover, the functional relevance and causality of epigenetic changes need to be considered. Not only which genomic loci contain differences but also how sizeable are these differences, as MS typically exerts small changes in DNA methylation. The direction of the change can also mean different things depending on the affected genomic feature, e.g. promoter methylation correlates with transcriptional repression, gene body methylation correlates with transcriptional activity while methylation around splice sites can influence splice variants

(118). There is the question of whether studies should focus on DMPs or DMRs, as changes in DNA methylation are often correlated between the neighbouring CpGs, however here technology might limit what we can detect. The cross-talk with other regulatory mechanisms should also be taken into consideration when assessing the functional relevance, such as genetic background, histone modifications, ncRNAs and transcription. Because of potential variation in all these levels, it is challenging to assess the functional consequences of changes without proper *in vitro* or *in vivo* reporter systems.

To assess the functionality of the differences found, current studies use the closest gene as an identifier of functions. The Illumina arrays have a manifest which maps every CpG to a specific gene. While several CpGs are mapped to several genes, usually for interpretative purposes the closest is used. This could lead to problems where the gene affected by the differential epigenetic marks is not used in functional analysis. These genes are then typically used for gene set enrichment or pathway analysis tools, such as Ingenuity Pathway Analysis (IPA) (119), GREAT (120), GSEA (121), among others. These tools make use of curated collections of gene-sets such as Gene Ontology (122) or KEGG (123) to identify those relevant (statistically significant) gene-sets associated with the trait of interest. However, GSA outcomes may become challenging to interpret when the number of gene-sets identified is very large or if the results from different collections of gene-sets, i.e. different experiments, are combined. An additional challenge appears when identified gene-sets have a high gene content overlap, which could result in nearly identical gene-sets with different functional labels.

8 THESIS AIMS

The overall aim of this thesis was to characterize the changes in DNA methylation in immune cells from MS patients during disease development, disease progression and treatment, and from them to infer functional pathways of importance for MS pathogenesis:

Study I

To investigate DNA methylation patterns across four different peripheral immune cell types during the development and progression of MS.

Study II

To investigate changes in DNA methylation between MS cases and controls and characterize the role of DNA methylation in the dysregulation of miR-21 in $CD4^+$ T cells in RRMS patients.

Study III

To develop an R tool which offers a simple approach to gain biological insights from one or more gene-set analysis results or tools.

Study IV

To utilize epigenetic marks in peripheral immune cells following DMF treatment in RRMS patients to gain insights into the mechanisms of the treatment.

Study V

To characterize in depth the changes in DNA methylation of peripheral $CD19^+$ B cells between RRMS patients and healthy controls, as well as to investigate the effects B cell depletion on other peripheral immune cells.

9 RESULTS

9.1 STUDY I

In this study, we profiled DNA-methylation of four different peripheral immune cells ($CD4^+$ and $CD8^+$ T cells, $CD14^+$ monocytes and $CD19^+$ B cells) in RRMS and SPMS and matched healthy controls (HC) using the Illumina 450K array. The RRMS patients constituted of a mixture of newly diagnosed patients, and patients who were on a drug washout for a period of around 6 months, all who had non-active disease as confirmed with MRI. From every patient, we sorted all four immune cells, though due to the quality and quantity of the DNA, not every cell type was analyzed for every patient.

Differential methylation analysis detected 30, 666 and 1 511 significant DMPs in $CD8^+$, $CD14^+$ and $CD19^+$ samples between RRMS, SPMS and HC in a linear model that included age and sex as covariates. Because of the high overlap of the results between the different cell types, and in order to increase the detection power, we combined the evidence of the multiple cell types in a non-parametric way using OmicsNPC. We developed a permutation scheme that retained the local correlation between cell types and ran the pipeline with 10 000 permutations. This resulted in 1976 DMPs from all four cell types (Fig 4). We confirmed the shared nature of the probes by testing the direction change in HC vs RRMS, and RRMS vs SPMS and saw that the direction was shared more often than expected by chance, as well as a very high correlation of the t statistic between the different comparison for the 1976 shared probes. The additional discovery power was confirmed in a small independent cohort of HC and RRMS samples from $CD14^+$ samples run on the Illumina EPIC array. Furthermore, we saw an enrichment of the shared DMPs in MS susceptibility loci as identified by the latest MS GWAS.

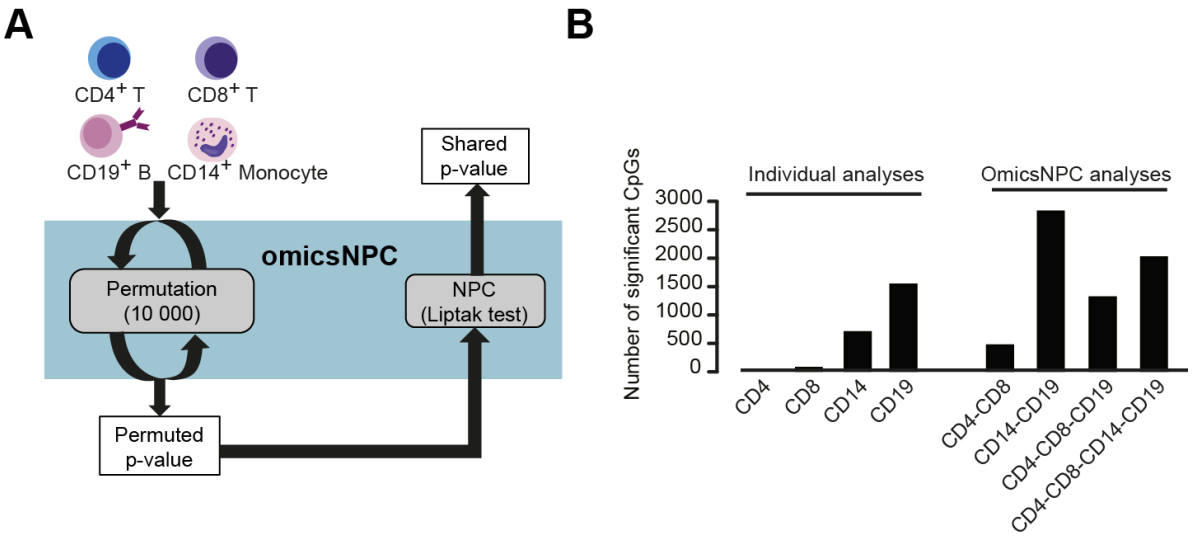

Figure 5. Overview of the omicsNPC function used in study 1. (124) **A)** Overview of the omicsNPC pipeline. Data was permuted 10,000 times using random labels that were consistent between individuals with the different cell types to maintain the correlation between the cell types. The permuted p-values were combined using the Liptak-Stouffer test, and the Liptak score was used for determining the shared p-value. **B)** Number of significant differentially methylated positions (DMPs) in individual and OmicsNPC analysis

Next, we clustered the shared DMPs into changes that were either specific for the MS samples in general (RRMS and SPMS), changes that were specific for SPMS samples or changes with no clear pattern between the samples or the different cell types. The functional annotation of the MS cluster implicated lymphocyte signaling, T cell activation and migration. The SPMS cluster implicated myeloid cell functions and metabolism, as well as an interesting signature coming from neurodegenerative genes, which we could confirm in a whole blood cohort of MS samples. In conclusion, OmicsNPC allowed for the detection of changes between cases and controls despite limited power of the study. Our findings provide new insights into the molecular mechanisms underlying MS pathogenesis and particularly disease progression.

9.2 STUDY II

In this study, we investigated the functional changes of the top DMP found in the $CD4^+$ analysis of the DNA methylation data from study I. In $CD4^+$ T cells, we did not identify genome-wide significant DNA methylation changes; however, among the top most significant hits we identified a DMR in the *MIR21*/*VMP1* locus, where RRMS samples had higher methylation than HC or SPMS.

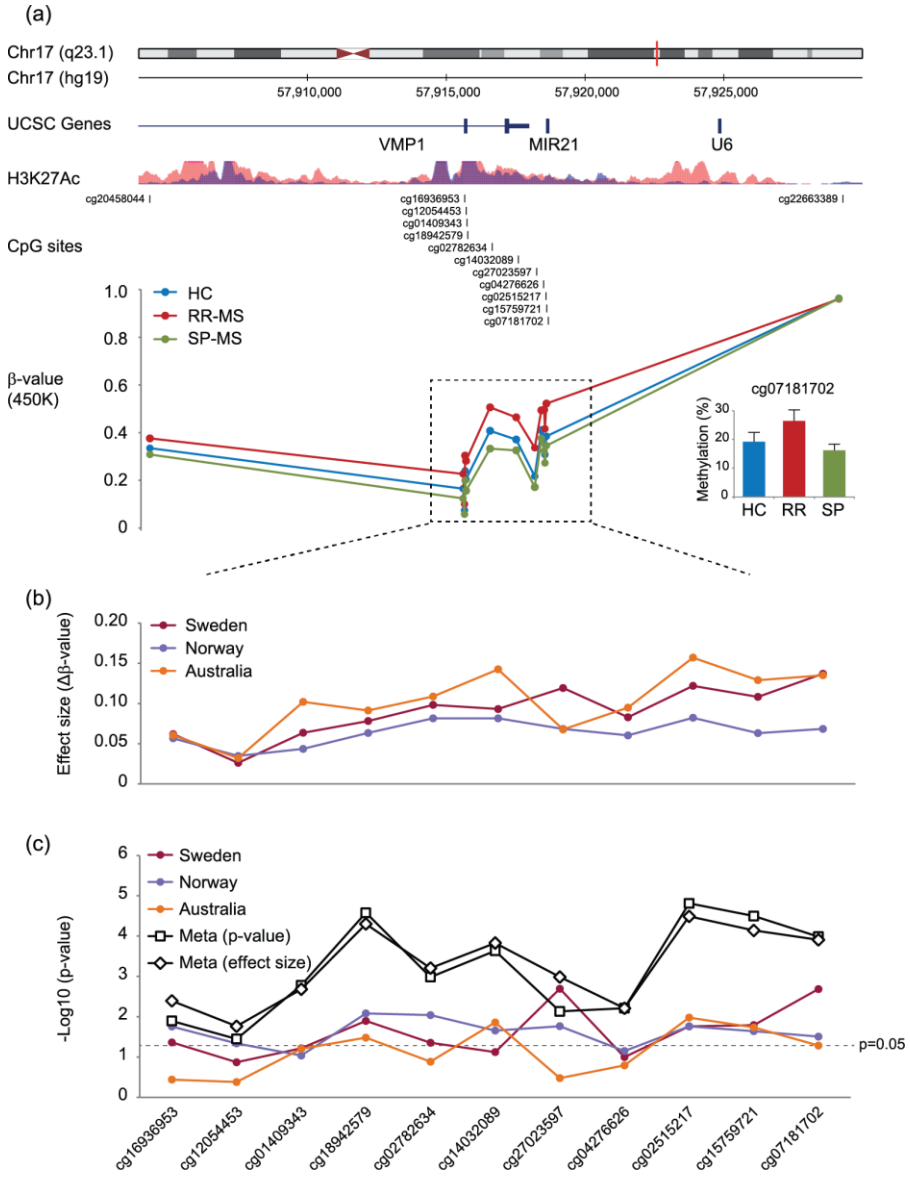

Figure 6. Overview of the DNA methylation changes surrounding the *VMP1/MIR21* locus. (125)

We replicated the findings using a meta-analysis between our data and that of the independent cohorts from Bos *et al.* (77) and Graves *et al.* (71). Here both a p-value based and an effects-based meta-analysis confirmed the higher methylation in RRMS. Using the $CD4^+$ T cell methylation data from the GOLDN cohort (126), we excluded a substantial effect of a genetic variation in this locus, known to be one of the MS risk loci, on DNA methylation only. The GOLDN data also confirmed that there was no effect of smoking on *MIR21/VMP1* DNA methylation, while DNA methylation levels did correlate with lymphocyte count and age. Using qPCR, we confirmed that the DMR only had an effect on miR21 levels but not on VMP1, with lower expression levels of miR21 in RRMS patients, which we could validate in an independent cohort. Using the data from the RNA sequencing of the same samples used for DNA methylation (127) we could confirm the higher expression of miR21 target genes. Thus, our findings suggest epigenetic silencing of mir-21 in $CD4^+$ T cells, leading to a reduced miR-21 mediated silencing of target mRNAs.

9.3 STUDY III

In this study, we further developed the tool used in studies I, IV and V. GeneSetCluser is an R package we developed to help in understanding the results from pathway analysis. When we ran IPA on the genes mapped to the DMPs from the OmicsNPC analysis, we struggled to interpret the results due to the same genes appearing repeatedly enriched in the multiple significant gene-sets. To aid in the interpretation, we used the overlap of genes in order to calculate a distance score between the different gene-sets to cluster them into more easily interpretable clusters. After the usefulness of this methodology in studies I and IV, we further developed this into an R-package which we called GeneSetCluster. GeneSetCluster can be used to cluster together data from a single gene set enrichment run as well as multiple experiments and conditions. The package was designed to be able to use information from all databases and tools and can convert between gene ID from multiple sources and species. Data can be visualized using a heatmap, an example of how these results look from study IV can be seen in figure 7B, and networks and can aid in the interpretation of gene-set enrichment results. Our method is currently available in an R-package from GitHub (https://github.com/TranslationalBioinformaticsUnit/GeneSetCluster).

9.4 STUDY IV

In this study, we investigated the molecular effects of DMF treatment on the monocyte response and changes in $CD4^+$ T cells. To this end, selected patients, which started DMF treatment at the MS clinic of the Karolinska University Hostpital and who met the 2010 McDonald criteria for RRMS. Patients were characterized at baseline, 3 months and 6 months after DMF treatment.

Nrf2 is a target of DMF but the precise effects remain elusive. We saw that there was an increase of intracellular ROS after 3 months, which increased further after 6 months. Changes in expression, measured using microarrays, showed enrichment of the GO term: Regulation of response to oxidative stress. DNA methylation was measured on the Illumina EPIC array and analysis of DMF treatment in $CD14^+$ cells after 3 and 6 months in RRMS patients indicated that the methylome changes occur around 3 months but go back to baseline after 6 months.

Conversely, the changes in $CD4^+$ T cells do not appear until 6 months after the start of treatment.

When stratifying the patients into DMF responders and non-responders, using 24 months of follow-up data, we identified a SNP in the ROS-generating NOX3 gene. This SNP is associated with both treatment response and ROS production, interestingly though this gene is not significantly associated with the risk of MS. The NOX3 gene displayed different expression based on the genotype of the SNP as well as different methylation levels.

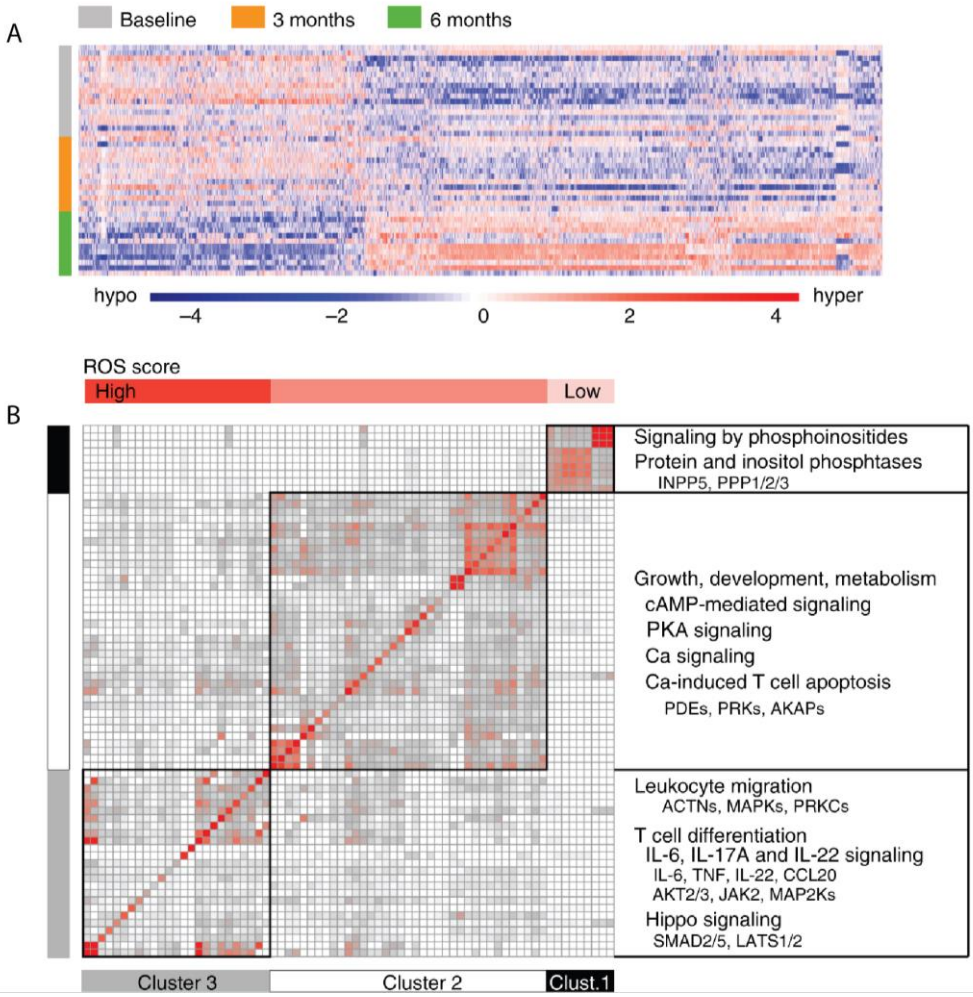

Figure 7. Effect of DMF treatment on DNA methylation in $CD4^+$ T cells. Figure adapted from (128)

The delayed DNA methylation changes in $CD4^+$ T cells around 6 months did coincide with the changes of naïve and effector memory T cells in DMF responders, even though the total number of T cells was down in DMF responders. The changes in $CD4^+$ T cell subtypes were supported by the identified differentially methylated genes, enriched in pathways such as HIPPO signaling and T cell differentiation, and the increase of Th17 associated plasma cytokines in DMF responders after 6 months. This data implicate monocyte-derived processes involved in DMF treatment and identify a NOX3 SNP that may indicate drug response and treatment outcome.

9.5 STUDY V

In study I we saw that the majority of DNA methylation changes between RRMS and HC occurred in CD19^{+} B cells. Due to this finding coupled with the increasing interest in the role of B cells in MS due to the highly effective B cell depleting drugs such as rituximab, we decided to further explore the epigenetic changes in CD19^{+} B cells. To that end, we analyzed a second larger cohort of CD19^{+} samples, which was collected from all patients treated in the local MS clinic. DNA methylation was measured using the Illumina EPIC array, which we compared with the results from study 1 using four different meta-analysiss approaches. These included two different fixed effects and one random-effects model as well as a combination of both data sets where batch effects were removed with ComBat. We identified 3 003 different DMPs with a significant adjusted p-value in all four different pipelines. The functional annotation of the epigenetic changes in CD19^{+} B cell indicates alterations of multiple processes in MS. These include changes associated to phosphoinositides, glucose and lipid metabolism, as well as changes associated with the regulation of cell cycle, apoptosis and differentiation of B cells, and changes in cell adhesion. Interestingly, the latter involved changes in multiple molecules, typically known as axon guiding genes.

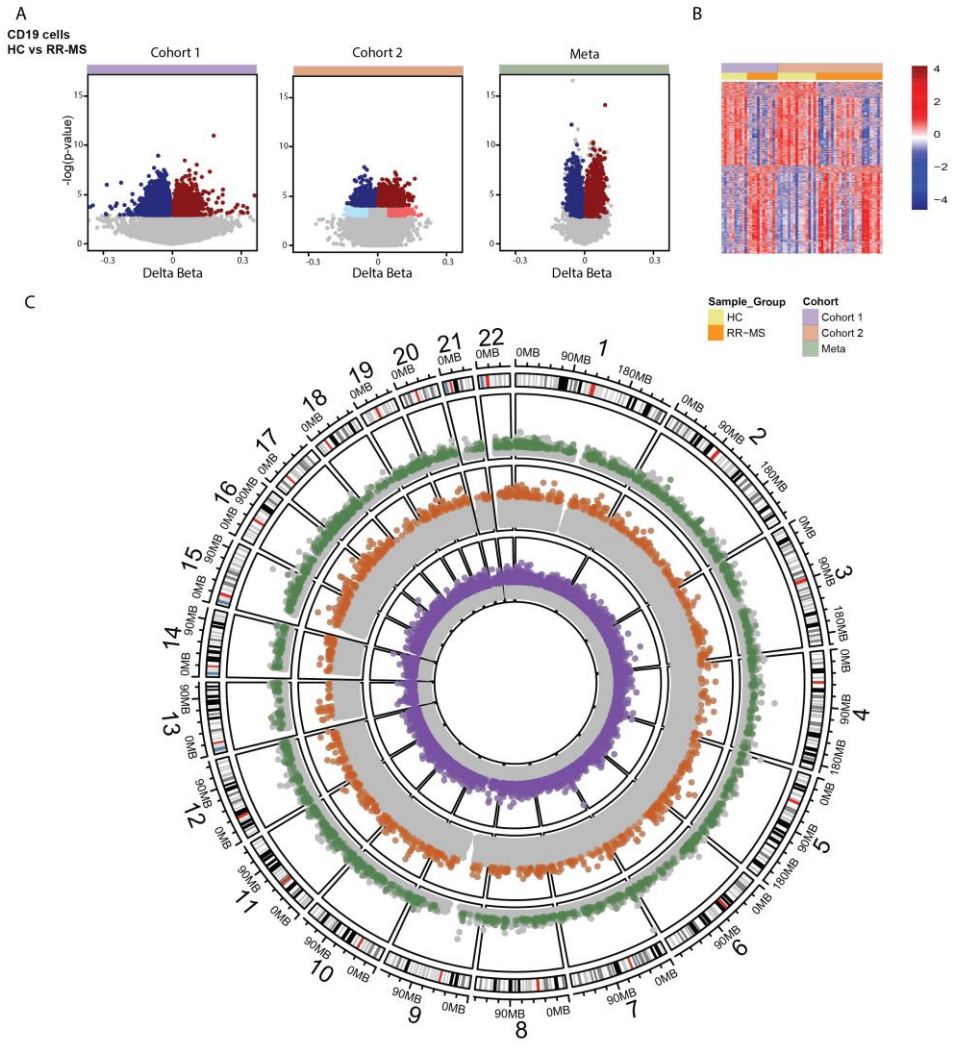

Figure 8. Methylation changes in CD19^{+} B cells sorted from peripheral blood of relapsing-remitting Multiple Sclerosis (RRMS) patients and healthy controls (HC). Cohort 1 (purple), measured using

Illumina 450K arrays, Cohort 2 (orange), measured using Illumina EPIC arrays, and Meta (dark green) **A)** Volcano plots illustrate differences in DNA methylation between RRMS and HC. Hyper- and hypo-methylated CpGs with p-value < 0.001 are indicated in light red and light blue, respectively, while darker red and darker blue indicate CpGs with adj. p-value < 0.05. **B)** Heatmap of the 3 003 probes with an adj. p-value < 0.05 (the scale represents Z-score). **C)** A circular Manhattan plot for Cohort 1 (inner circle), Cohort 2 (middle circle) and the meta-analysis (outermost circle).

In this study, we also investigated the effect of B cell depletion with rituximab on $CD4^+$ T cells and $CD14^+$ monocytes in 17 RRMS patients where we measured DNA methylation at baseline and after 6 months. The patients had significant depletion of B cells after 6 months, but there were no genome-wide significant DMPs in either $CD4^+$ or $CD14^+$ cells. Nonetheless, the top hits in both $CD4^+$ and $CD14^+$ were associated with activation of immune cells. Functional analysis of the changes in $CD4^+$ cells after rituximab treatment were associated with immune activation, and involved in, among other things, Th17 differentiation and IL17 signalling. The $CD14^+$ associated changes indicated changes in genes associated with the epigenetic switch between pro-inflammatory and anti-inflammatory macrophages as well as monocyte motility and activation.

This data confirm widespread DNA methylation changes in $CD19^+$ cells from RRMS patients compared with healthy individuals and inform about the functional consequences that associate with MS disease. Accordingly, B cell depletion results in a reduction of activation signals for T cells and monocytes in genes associated with activation, differentiation and motility.

10 CONCLUSIONS AND FUTURE PERSPECTIVES

In this thesis, we have used several cohorts and methodologies to investigate the role of DNA methylation in the development, progression and treatment of MS. We investigated both patients with RRMS and SPMS and compared those to healthy controls. We initially looked at the four major cell types from PBMC, namely $CD4^+$, $CD8^+$, $CD14^+$ and $CD19^+$ cells, where we could identify a DNA methylation signature shared between the different cell types, which we investigated more thoroughly using OmicsNPC. Interestingly, using OmicsNPC we identified DNA methylation changes in neurodegenerative genes in SPMS patients. In $CD4^+$ cells, the top hits indicated changes in the *VMP1/MIR21* locus, which we functionally investigated and found lower miR21 expression in RRMS patients, which resulted in a higher expression of miR21 target genes. Because of the numerous DNA methylation differences found in $CD19^+$ cells, we further explored changes in $CD19^+$ cells in a second larger cohort. We compared the two different cohorts using several different meta-analysis approaches and this resulted in identifying many processes altered in $CD19^+$ cells, including metabolism and activation. To analyze the pathway data from the different studies, we developed a method to cluster pathways based on the genes inside them, which proved very useful, therefore we have further developed this into an R package called GeneSetCluster.

While epigenetic alteration are stable, they could be affected by changes in the cellular environment, so we investigated changes of the epigenome in MS patients after disease-modifying treatments. The treatment with DMF identified that the changes happen in a cell type specific manner, where each cell type has its own specific kinetics. DMF treatment resulted in changes in ROS production in monocytes and led to changes in Th17 associated genes in $CD4^+$ T cells. Interestingly, we identified a SNP, which associated with ROS production and treatment outcome but not with MS susceptibility. Rituximab treatment, which unlike DMF, has an indirect effect on the cells studied, resulted in changes associated with a lack of B cell interaction. In $CD4^+$ T cells, we found epigenetic changes in Th1, Th17 and Treg associated genes. In $CD14^+$ monocytes, we identified changes in metabolism and motility as well as changes in epigenetic genes associated with switching between pro- and anti-inflammatory macrophages.

Epigenetic changes, such as the cell type specific DNA methylation changes detected above, might provide useful biomarkers for diagnostic, prognostic and therapeutic purposes. The reliable quantification of genome-wide DNA methylation levels, which are relatively stable and insensitive to handling, make it highly suitable for the clinical setting. For example, identification of neurodegenerative signals from in peripheral cells from SPMS patients, offers a great potential for insight into diseases, such as MS, where the target tissue is inaccessible. The identification of MS associated epigenetic marks will increase in the future when larger cohorts are studied with more varied cell types. Furthermore, the rise of new epigenetic technologies capable of measuring DNA methylation at a single-cell resolution will allow for even more detailed investigation of epigenetically distinct cell mixtures associated with disease (129).

Because changes in epigenetic marks are associated with both disease and treatment, this offers promising prospects in correcting dysregulation that can potentially alter the course of MS. Besides investigating the epigenetic changes after treatment, changing the epigenetic marks

themselves might be a viable strategy for treating MS as well. Epigenetic drugs are classified as either, globally altering the epigenome by targeting readers, writers and erasers (HATs, HDACs, DMNTs, TETs, etc.) or by altering epigenetic states of specific loci in the genome. Global drugs include e.g. the two drugs called azacitidine (AZA, 5-azacitidine) and decitabine (DAC, 2'-deoxy-5-azacitidine), which are cytosine analogues that block DMNTs during replication leading to demethylation. Two different studies have demonstrated an ameliorating effect of these drugs in EAE (130, 131).

Locus specific methylation changing drugs offer a more exciting approach as they would present less adverse effects and more specificity than global drugs. The recently discovered ability of CRISPR/dCAS9 to edit the genome in addition to the TALENs and zinc fingers proteins offers promising tools to edit the epigenome as well (132, 133). These tools, known as EpiEffectors, are fusions of DNA recognition elements fused with catalytic domains of DNA methylation related enzymes. The DNA recognition capabilities of guide RNAs (gRNAs) used in CRISPR/dCAS9 allow for easy targeting of specific sequences. In contrast, TALENs and zinc finger proteins require the design of specific proteins complexes, though CRISPR/dCAS9 has been noted for its potential number off-target effects, which seem less prevalent using the other tools. Current research is investigating the stability of the modified epigenetic marks, as well as methods to deliver epigenome-editing tools to target specific tissues and cells (134). While more research into epigenome-editing tools is necessary, it is easy to see the potential benefits of such technologies.

The epigenetic studies discussed here, demonstrate changes in epigenetic marks between MS patients and healthy controls, however, the interpretation of these epigenetic changes remains challenging. More investigations are required to gain a functional understanding of the dynamic chromatin changes, so that the causal link between genetic background and environment in the context of MS can be understood. Such studies will lead to a better understanding of risk factors and pathogenic mechanisms of MS and can lead to robust biomarkers and useful therapeutic targets.

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