

From THE DEPARTMENT OF CLINICAL NEUROSCIENCE  
Karolinska Institutet, Stockholm, Sweden

**GENETIC AND EPIGENETIC FACTORS SHAPING  
THE TRANSCRIPTOME IN MULTIPLE SCLEROSIS  
AND ITS ANIMAL MODEL**

Petra Bergman



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Department of Clinical Neuroscience

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AND ITS ANIMAL MODEL**

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By

**Petra Bergman**

*Principal Supervisor:*

Associate Professor Maja Jagodic  
Karolinska Institutet  
Department of Clinical Neuroscience

*Opponent:*

Dr. Esteban Ballestar  
Bellvitge Biomedical Research Institute  
(IDIBELL)  
Cancer Epigenetics and Biology Program

*Co-supervisors:*

Professor Tomas Ekström  
Karolinska Institutet  
Department of Clinical Neuroscience

*Examination Board:*

Professor Göran Andersson  
Swedish University of Agricultural Sciences  
Department of Animal Breeding and Genetics

Associate Professor Andor Pivarcsi  
Karolinska Institutet  
Department of Medicine

Professor Marie Öhman  
Stockholm University  
Department of Molecular Biology and  
Functional Genomics

Associate Professor Anna Fogdell-Hahn  
Karolinska Institutet  
Department of Clinical Neuroscience

Till Mormor och Farmor

Jag saknar er!

# ABSTRACT

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Multiple Sclerosis (MS) is a chronic inflammatory and demyelinating disease of the central nervous system. Susceptibility to develop MS is determined by both genetic and environmental factors. The epigenome resides in the interface of genetic and environmental factors thereby shaping the transcriptome. Furthermore, epigenetic mechanisms have been implicated in the etiology of MS. Epigenetics refers to changes in gene expression that are not a result of alterations in DNA sequence. Epigenetic mechanisms include histone modifications, DNA methylation and non-coding RNAs. MicroRNAs (miRNAs), small non-coding RNAs known to regulate gene expression, have been found dysregulated in most diseases, including MS.

This thesis utilizes an animal model of MS, experimental autoimmune encephalomyelitis (EAE), to investigate epigenetic mechanisms and miRNAs as mediators and modulators of autoimmunity. Through the use of a knockout mouse model we demonstrated that histone demethylase Kdm3a is not a ‘master regulator’ of EAE. However, we demonstrated that a genetic variant in the rat Kdm3a affects nucleotide secondary structure and potentially protein translation. The role of epigenetic mechanisms was demonstrated by the identification of parent-of-origin dependent effects (such as genomic imprinting) in the inheritance of EAE.

Using next generation sequencing we established a miRNA profile that associates with pathogenic immune activation in rat EAE, with potential miRNA-dependent regulation of several important functions in the development of autoimmunity. Furthermore, by investigating the potential of circulating miRNAs as biomarkers of MS, we identified miR-150 as a putative novel biomarker for MS in the cerebrospinal fluid.

Collectively, we show that modulation of the transcriptome by epigenetic mechanisms and miRNAs can explain more of the unknown underlying factors regulating susceptibility to autoimmune diseases and that dysregulated miRNAs can serve as markers of ongoing pathogenesis.

# LIST OF SCIENTIFIC PUBLICATIONS

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- I. **A silent exonic SNP in Kdm3a affects nucleic acids structure but does not regulate experimental autoimmune encephalomyelitis.**  
Gillett A, Bergman P, Parsa R, Bremges A, Giegerich R, Jagodic M.  
*PLoS One*. 2013 Dec 3;8(12)
  
- II. **Parent-of-origin effects implicate epigenetic regulation of experimental autoimmune encephalomyelitis and identify imprinted Dlk1 as a novel risk gene.**  
Stridh P\*, Ruhrmann S\*, Bergman P, Thessén Hedreul M, Flytzani S, Beyeen AD, Gillett A, Krivosija N, Öckinger J, Ferguson-Smith AC, Jagodic M.  
*PLoS Genetics*. 2014 Mar 27;10(3)  
\*Authors contributed equally
  
- III. **Next-generation sequencing identifies microRNAs that associate with pathogenic autoimmune neuroinflammation in rats.**  
Bergman P, James T, Kular L, Ruhrmann S, Kramarova T, Kvist A, Supic G, Gillett A, Pivarcsi A, Jagodic M.  
*Journal of Immunology*. 2013 Apr 15;190(8):4066-75.
  
- IV. **Profiling of circulating microRNAs in cerebrospinal fluid identifies miR-150 as a putative novel biomarker for Multiple Sclerosis**  
Bergman P, Piket E, Khademi M, James T, Lindén M, Kockum I, Brundin L, Piehl F, Olsson T, Jagodic M  
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# LIST OF ABBREVIATIONS

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APC	Antigen Presenting Cell
BBB	Blood Brain Barrier
CIS	Clinically Isolated Syndrome
cDNA	Complementary DNA
CNS	Central Nervous System
CpG	Cytosine-Guanine dinucleotide
CSF	Cerebrospinal Fluid
CXCL13	Chemokine (C-X-C motif) Ligand 13
DA	Dark Agouti
DC	Dendritic Cell
DMD	Disease-Modulating Drugs
DNA	Deoxyribonucleic Acid
EAE	Experimental Autoimmune Encephalomyelitis
EDSS	Expanded Disability Status Scale
GWAS	Genome-Wide Association Study
HLA	Human Leukocyte Antigen
iOND	Inflammatory OND
IFN	Interferon
ICR	Imprinting Control Region
Ig	Immunoglobulin
IL	Interleukin
MHC	Major Histocompatibility Complex
MOG	Myelin Oligodendrocyte Glycoprotein
MMP9	Matrix Metalloproteinase 9
MRI	Magnetic Resonance Image
miRNA	MicroRNA
mRNA	Messenger RNA
MS	Multiple Sclerosis
NFL	Neurofilament-light chain
NGS	Next Generation Sequencing
OCB	Oligoclonal Bands

OND	Other Neurological Disorders
PBMC	Peripheral Blood Mononuclear Cells
PCR	Polymerase Chain Reaction
qPCR	Quantitative Real-time Polymerase Chain Reaction
PPMS	Primary Progressive Multiple Sclerosis
ROC	Receiver Operating Characteristic (curve)
PVG	Piebald Virol Glaxo
QTL	Quantitative Trait Locus
RNA	Ribonucleic Acid
RRMS	Relapsing Remitting Multiple Sclerosis
SNP	Single Nucleotide Polymorphism
SPMS	Secondary Progressive Multiple Sclerosis
TH	T Helper
TLDA	TaqMan Low Density Array
TNF	Tumor Necrosis Factor
TLR	Toll-Like Receptor
UTR	Untranslated Region

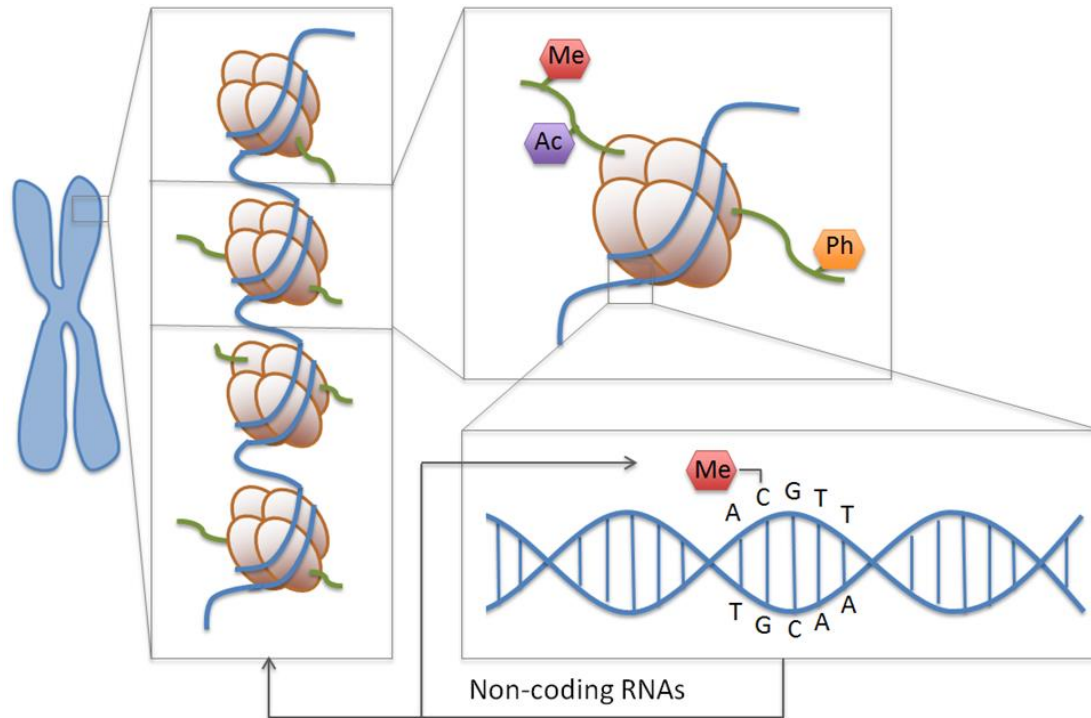
# 1 INTRODUCTION

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The central dogma of molecular biology, first described by Francis Crick in 1958<sup>1</sup>, suggests a unidirectional transfer of information whereby deoxyribonucleic acid (DNA) transcribes into ribonucleic acid (RNA), which then translates into proteins. However, it was not long until scientists discovered exceptions to this rule, such as viruses using RNAs as templates to make DNA<sup>2</sup>, a process now known as reverse transcription. Today we are starting to appreciate the complexity of the link between nucleic acids, protein products and phenotype<sup>3</sup>. This thesis touches upon a few of the mechanisms whereby epigenetics and microRNAs (miRNAs) can modulate and mediate the genetic information in the context of experimental neuroinflammation, and how some of these factors can be beneficial in the diagnosis of complex diseases such as Multiple Sclerosis (MS).

## 1.1 Epigenetics

Conrad Waddington coined the term *epigenetics* in 1942 in order to explain the process by which genes may interact with the environment to produce a phenotype. Epigenetics was later redefined by Arthur Riggs in 1996 as the study of ‘mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence<sup>4</sup>’. Epigenetic changes are presumed to be passed from parent to offspring through the germline and retained through consecutive cell divisions. However, epigenetic modifications are very flexible in a spatiotemporal manner as they undergo precise and dynamic changes during development that contribute to lineage- and cell-specific gene expression patterns<sup>5</sup>. Apart from developmental regulation of epigenetic changes there is considerable stochastic variation<sup>6-8</sup>, likely mediated by environmental factors. The term epigenetics refers to mechanisms underlying phenotype variations such as environmental influences, parent-of-origin effects, genomic imprinting and X-chromosome inactivation. Molecularly, epigenetics encompasses mechanisms of chromatin and histone modification, large nuclear complexes (e.g. polycomb proteins), DNA methylation and non-coding RNAs (ncRNA) (Figure 1).



**Figure 1:** Epigenetic mechanisms. DNA (blue) in the chromosome is organized into chromatin. DNA is tightly packed around a core of histone proteins, together forming the nucleosome. Accessibility of the DNA to the transcriptional machinery is regulated both by modifications of histone tails (green) and by methylation of cytosine bases of the DNA. In addition, non-coding RNAs can regulate chromatin and DNA methylation.

### 1.1.1 Chromatin and Histone modification

Fitting roughly 2 meters of double-stranded DNA into the cell nucleus requires efficient packaging. This is achieved through the formation of chromatin, where the DNA is wrapped around dimers of histone core proteins H2A, H2B, H3 and H4, together with linker histone H1, to form the nucleosome<sup>9,10</sup>. Modification of nucleosomal organization can be accomplished through variations in histone proteins, chromatin remodeling and post-translational modification of amino acids in the histone tails. Histone variants have been identified for histone H2A and H3 as well as for the linker histone H1, and these result in differences of chromatin accessibility and stability<sup>11</sup>. Chromatin remodeling, executed by the ATP-dependent chromatin remodeling complex, refers to the repositioning and sliding of nucleosomes for subsequent recruitment of transcription factors and transcriptional complexes to access the DNA<sup>12</sup>. Post-translational modifications of histone tails include acetylation, methylation, phosphorylation, citrullination, sumoylation and ubiquitination. Depending on the type of modification and histone tail residue that is modified the end result is either transcription activation or repression. For instance, acetylation of histone H3 lysines is generally associated with transcriptionally active chromatin<sup>13</sup>, whereas tri-methylation of

H3 lysines residues associates with both transcriptionally competent chromatin (H3K7, H3K36 and H3K79) and transcriptionally incompetent chromatin (H3K9 and H3K27)<sup>14,15</sup>. The addition and removal of histone marks is mediated by a number of specific enzymes. For instance, mono-, di- and tri- methylation of lysine residues is catalyzed by two different families of methyltransferases, the SET-domain containing proteins<sup>16</sup> and DOT1-like proteins<sup>17</sup>, respectively, and the removal of methyl-lysine marks is achieved by two families of demethylases, the amine oxidases<sup>18</sup> and jumonji C (JmjC)-domain- containing, iron-dependent dioxygenases<sup>19</sup>.

### **1.1.2 DNA methylation**

Methylation of DNA involves covalent addition of a methyl group to cytosine bases by DNA methyltransferases (DNMTs). DNMTs recognize cytosine bases of cytosine-guanine dinucleotides (CpG). Methylation of CpGs islands, i.e. CpG-rich regions, at transcription start sites is usually associated with transcriptional repression as the methyl group blocks the binding of transcription factors and complexes of the transcriptional machinery<sup>20</sup>. However, genome-wide mapping of methylation has demonstrated the occurrence of DNA methylation throughout the genome: in gene bodies, in gene-flanking regions and in intergenic areas<sup>21</sup>, thereby introducing a wide array of regulation by DNA methylation within different genetic contexts. DNA methylation is an important regulator of gene expression and is therefore a central component in numerous cellular processes such as embryonic development, genomic imprinting, X-chromosome inactivation and maintenance of chromosome stability<sup>22</sup>.

#### **1.1.2.1 Parent-of-origin effects and Genomic imprinting**

The term *parent-of-origin effects* refers to the phenomenon in which inheritance of genetic variation is dependent on parental transmission, i.e. inheritance from the mother or from the father. Parent-of-origin effects may be caused by genomic imprinting, the paternally inherited Y-chromosome, the maternally inherited mitochondrial genome and intrauterine effects. The extent to which parent-of-origin contributes to the heritability of complex traits is currently unknown.

Unequal mono-allelic gene expression, whereby the allele that is transcribed depends on parental transmission, is known as *genomic imprinting*. For example, an imprinted gene that

is active on a maternally inherited chromosome will be expressed from the maternal chromosome and silent on the paternal chromosome, in both male and female offspring. Imprinted genes primarily exist in clusters regulated by imprinting control regions (ICR). The ICR is differentially methylated to permit or to silence gene expression<sup>23,24</sup>. An imprinting mark is set in the germ cell and is stably inherited, on the same chromosome, during meiosis of the embryo and in the adult<sup>25</sup>. However, imprinting can be lost in adulthood and can be inconsistent across tissues of the same organism<sup>26</sup>. In addition to methylation of the ICR, long ncRNAs (lncRNA) and histone modifications can also provide regulation of mono-allelic expression. The underlying biological relevance for genomic imprinting is not clear, but it is hypothesized that there is likely a strong selective advantage for the evolution and maintenance of this phenomenon. Imprinting is conserved between humans and mice to a large extent and the importance of proper imprinting is evident from the observation of disturbed development and human disease when imprinting is lost<sup>24,27-29</sup>.

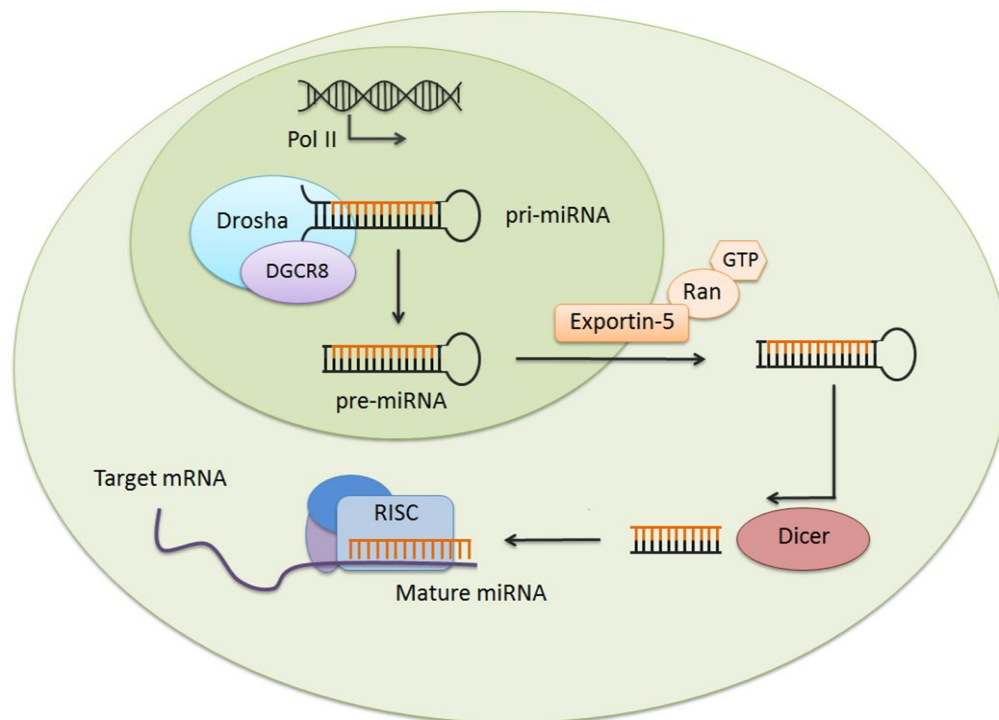
### **1.1.3 Non-coding RNA**

Coding genes only account for about 1% of the human genome<sup>30</sup>. For a long time the non-coding genome was believed to be unimportant, it was considered ‘junk DNA’. Thanks to the ENCODE project we now know that a broad spectrum of RNA molecules are being transcribed, ranging from long protein-coding messenger RNAs (mRNAs) to short non-coding transcripts<sup>31</sup>. However, compared to coding RNAs, only the functions of a small number of the ncRNAs are currently experimentally validated. A known feature of ncRNAs is that they can modulate epigenetic states via sequence homology. lncRNAs are known to regulate both genomic imprinting<sup>32</sup> and X chromosome inactivation<sup>33</sup>, and the shorter Piwi-Interacting RNAs (piRNAs) are known to be important for silencing of retrotransposons<sup>34</sup>. The most studied class of ncRNAs is the microRNAs (miRNAs), which are the focus of the next section of this thesis.

## **1.2 MicroRNA**

MiRNAs are small (20-23 nucleotides) single-stranded RNA molecules that regulate gene expression. They are generally transcribed by polymerase II, using independent promoters, into primary miRNA transcripts (pri-miRNAs)<sup>35</sup>. However, there are alternatives whereby

miRNAs are transcribed by polymerase III<sup>36</sup>, as well as miRNAs being transcribed together with coding genes, where they are present either in exons, introns or in close genomic proximity. MiRNAs encoded in clusters can be transcribed together as well as independently<sup>37</sup>. Pri-miRNAs are processed in the nucleus by an endonuclease complex comprising of the RNase III enzyme Drosha and the DGCR8 (DiGeorge critical region 8) protein, into smaller precursor miRNA (pre-miRNA) hairpin structures. After nuclear processing the pre-miRNA is exported to the cytoplasm by Exportin-5 coupled with Ran-GTP<sup>38,39</sup>. In the cytoplasm the pre-miRNA is cleaved by the endonuclease Dicer into a short double-stranded RNA molecule. The miRNA-processing factors Dicer, Dgcr8 and Drosha are all essential for viability in mice. Knockout mice lacking these key processing genes accumulate miRNA precursors and die during early gestation with severe developmental defects<sup>40-42</sup>. Following cleavage by Dicer, one of the strands of the miRNA duplex is subsequently loaded into the RNA induced silencing complex (RISC), and the other is degraded. The mature miRNA together with Argonaute proteins (Ago) of the RISC complex targets complementary sites of the target mRNAs. The biogenesis of miRNA is illustrated in Figure 2.



**Figure 2.** MiRNA biogenesis. MiRNAs are transcribed by polymerase II into pri-miRNAs. Drosha and DGCR8 process these into pre-miRNA before export, by Exportin-5, to the cytoplasm. In the cytoplasm Dicer cleaves pre-miRNA into double-stranded short RNA molecules. One of the strands gets incorporated into the RNA-induced silencing complex (RISC) to facilitate binding to target mRNA.

Interaction of miRNA and mRNA requires base-pairing of the miRNA ‘seed’ sequence with complementary nucleotides in the 3’ untranslated region (UTR) of the target mRNA<sup>43</sup>. The miRNA seed is composed of nucleotides 2-8 and is the only binding necessary for miRNA to exert post-transcriptional regulation of mRNAs. The binding generally results in a reduction of protein product, mainly due to mRNA decay but also through translational repression<sup>44,45</sup>. However, during certain circumstances miRNAs have also been demonstrated to up-regulate translation<sup>46</sup>.

### 1.2.1 MiRNAs in regulation of the immune system

The immune system is comprised of two sub-systems termed *innate* and *adaptive*. The former is the part of the fast-acting and relatively non-specific response against pathogens and is mediated by cells such as neutrophils and antigen presenting cells (APCs). The adaptive immune system is an acquired and specific response mediated in part by lymphocytes. When cells of the immune system encounter inflammatory cues, intracellular signals are sent to their nuclei to initiate changes in transcription, resulting in some genes being turned on and others being turned off. Similar to protein-coding genes miRNAs are also transcriptionally regulated during the immune response. MiRNAs are critical for fine-tuning immunity<sup>47,48</sup> and cells of the immune system express unique miRNA profiles contributing to their specific functions<sup>49</sup>. Furthermore, miRNAs are important regulators of hematopoiesis and lineage-specific cell commitment<sup>50-52</sup>.

A role for individual miRNAs in both innate and adaptive immune response has been demonstrated. One of these key miRNAs regulating the immune system is pro-inflammatory miR-155. Activation of toll-like receptors (TLR) and the interleukin (IL)-1 receptor induces expression of miR-155, which successively negatively regulates TAB2, an important molecule of the IL-1 signaling pathway<sup>53</sup>. This creates a negative feedback loop, thereby fine-tuning the microbial immune response. An opposite mode of action is apparent in viral infection as miR-155 has been shown to promote a positive feedback loop stimulating the production of type I interferon<sup>54</sup>. MiR-155 is also critical for T and B cell differentiation and function<sup>55</sup>, and it is therefore not surprising that miR-155 has been associated with most autoimmune disorders including rheumatoid arthritis<sup>56</sup>, systemic lupus erythematosus (SLE)<sup>57</sup> and MS<sup>58</sup>.

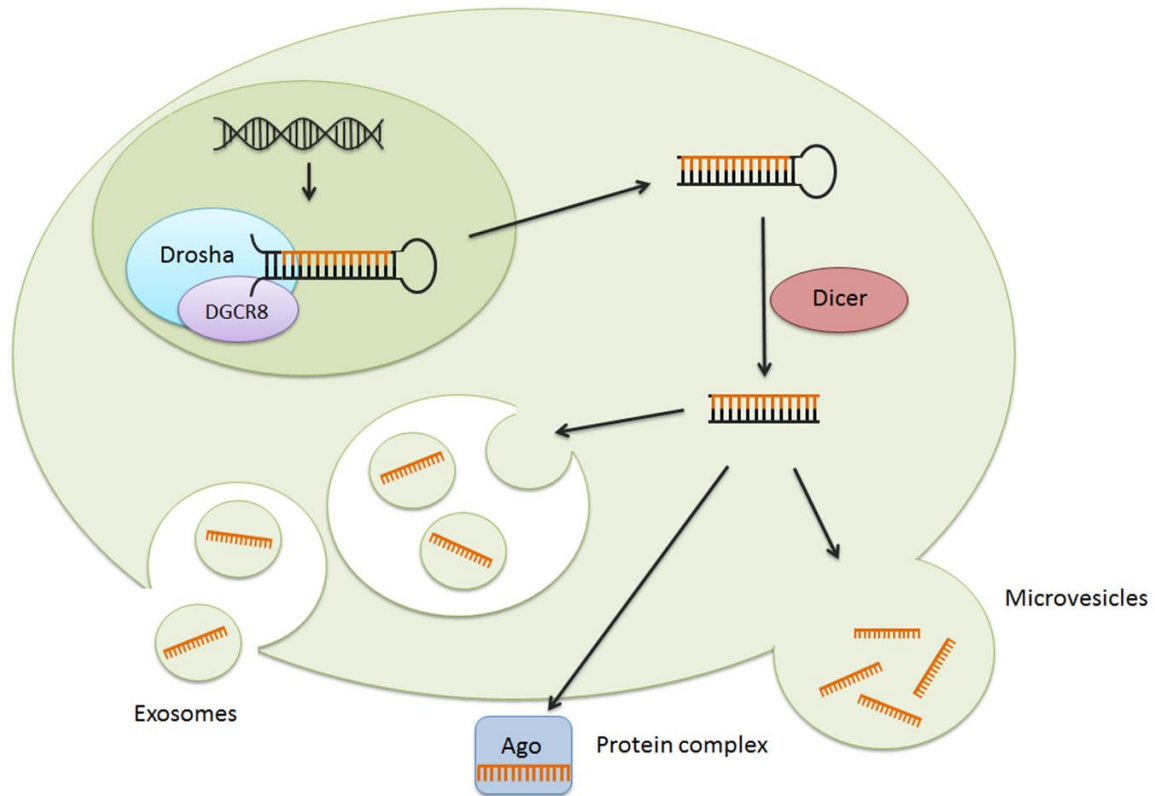


Other central miRNAs in the immune system are miR-146a, miR-181a and miR-150. MiR-146a was first identified as a negative regulator of NF- $\kappa$ B signaling upon TLR signaling through targeting the downstream molecules, tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) and IL-1 receptor-associated kinase 1 (IRAK1)<sup>59</sup>. It has since then continually considered as an important regulator of the innate immune responses, although evidence of miR-146a regulating T cell differentiation and activation has also been demonstrated<sup>60,61</sup>. MiR-181a is known to be important for T cell differentiation<sup>50,51,62</sup> and for regulating sensitivity of the T cell receptor<sup>63</sup>. MiR-150 is also a regulator of immune cell differentiation<sup>52,64,65</sup>, but has additional roles such as regulation of B cell receptor signaling<sup>66,67</sup>.

### **1.2.2 Circulating miRNAs**

In addition to identification in cells and tissues, miRNAs can also be detected in extracellular body fluids including plasma, serum, cerebrospinal fluid (CSF), urine and saliva<sup>68</sup>. They are released from cells, actively or passively, in membrane-bound vesicle particles or in protein complexes (Figure 3). The vesicles can either be formed inside the cells followed by exocytosis out into the extracellular space (exosomes), or by evagination and budding from the plasma membrane (microvesicle)<sup>69</sup> (Figure 3). Various cell types including neuronal cells, epithelial cells, immune cells and tumor cells are known to release extracellular miRNAs. Furthermore, miRNAs in exosomes have been shown to be transferred to other cells, thereby possibly mediating cell-to-cell communication. There are now a few studies demonstrating such exosome-transfer of miRNAs between cells of the immune system. For instance, exosomes from regulatory T cells containing Let-7d suppress pathogenic T cells<sup>70</sup>. Another example is T cell-mediated transfer of miRNAs to dendritic cells (DCs)<sup>71</sup>. The mechanism underlying exosome-mediated miRNA transfer and the loading of miRNA in extracellular vesicles remains to be elucidated.

During the last couple of years, circulating miRNAs have become a new and promising form of biomarker. There are several advantages in using circulating miRNAs instead of conventional disease markers, although there are also a few disadvantages (listed in Box 1). Circulating miRNAs have been proposed as putative biomarkers for a broad range of pathologies including different types of cancers, cardiovascular diseases, diabetes, neuro-



**Figure 3.** Circulating miRNAs. MiRNAs can be released into the circulation encapsulated in exosomes or microvesicles, or are protected from nucleases by protein complexes.

degenerative disorders and autoimmune diseases. In the case of cancers, different bodily fluids can be used to address the relevance of miRNAs as biomarkers of the specific type of malignancy, and for diseases of the cardiovascular system such as atherosclerosis, myocardial infections and stroke, the blood compartment presents the most obvious body fluid to study. However, circulating biomarkers in the blood are not so specific and can be a consequence of diseases (or organismal conditions) other than that studied. For neurodegenerative diseases and other diseases of the CNS, the CSF, lying at the interface between the CNS and blood, might better reflect the activities in the target organ. Unlike circulating miRNAs in the blood, miRNA biomarkers in the CSF are still in early stages of development, probably due to the lower accessibility of CSF compared to blood. However, recent progress in high-throughput sequencing of the extremely low levels of circulating miRNAs present in the CSF has proven fruitful and will undoubtedly aid in future discoveries of novel miRNA biomarkers<sup>72</sup>.

**Box 1. Advantages and disadvantages of circulating miRNAs as biomarkers**

<b>Advantages</b>	<b>Disadvantages</b>
High stability in extracellular environments	A single miRNA may not reflect the complex pathogenic events
Easy detection using qPCR	Detection of low amounts is technically challenging
Several miRNAs can be analyzed from the same isolation procedure	Data normalization (lack of endogenous controls)
Mirror events of the target organ (CSF)	miRNAs may be involved in diseases of other organs (blood)
Non-invasive sampling procedure	

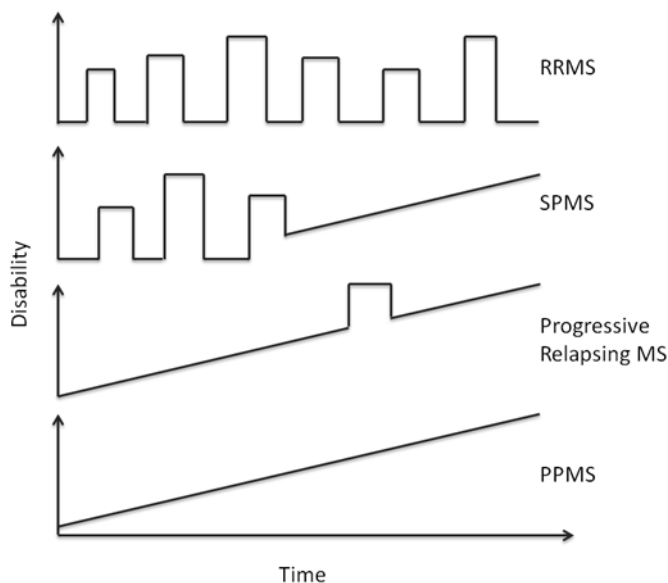
### **1.3 Multiple Sclerosis**

MS is a chronic inflammatory and neurodegenerative disease of the CNS first comprehensively described by Jean-Martin Charcot in 1868. MS affects millions of people worldwide and in Sweden has a prevalence of 0,2% of the population<sup>73</sup>. It is the major cause of non-traumatic neurological disability in young adults with a large associated economic burden on society. Onset of disease occurs around 20-40 years of age, with women being affected more than twice as often as men<sup>73</sup>. MS associates with a marked reduction in life quality of affected patients, with a majority of patients having to end their working career prematurely<sup>74</sup>.

MS is characterized by the breakdown of blood brain barrier (BBB) integrity, thereby allowing significant infiltration of immune cells into the CNS and subsequent development of inflammatory lesions, demyelination and ultimately neuronal degeneration and glial scar formation<sup>75</sup>. Symptoms of neurological defects in MS include disturbances of motor function, vision, sensation and autonomic systems, all depending on where in the CNS lesions occur. However, symptoms relating to disturbances of higher functions, such as various aspects of cognition, are also common<sup>76</sup>. Diagnosis of MS, according to the McDonald criteria, requires two episodes of demyelinating attacks separated in time and in space<sup>77</sup>. A first event

suggestive of MS is called *Clinically Isolated Syndrome* (CIS). In the most recent update of the McDonald<sup>78</sup> criteria, a second clinical event to confirm a diagnosis of MS, can be substituted with evidence of spread in time with magnetic resonance imaging (MRI). The procedure for MS diagnosis is primarily executed by examination of medical history and neurological symptoms. Measurement of functional states of MS patients is usually performed using the expanded disability status scale (EDSS)<sup>79</sup> and MRI is used to visualize presence of inflammatory/demyelinating lesions. Other paraclinical measurements include presence of oligoclonal immunoglobulin (Ig) G bands (OCB) and elevated levels of IgG in the CSF<sup>80,81</sup>. These two are present in about 70% and 90% of all patients, respectively.

MS has a heterogeneous disease course and clinical presentations. Half of the patients with CIS will suffer from a secondary attack of demyelination within two years, and will therefore fulfill the criteria for MS and within 20 years over 80% of all CIS patients will develop MS<sup>82</sup>. Four main clinical subtypes of MS can be categorized (Figure 4). Most MS patients (80-90%) present with the relapsing remitting form of MS (RRMS) which is characterized by recurrent episodes of acute neurological destruction (relapses) followed by partial or complete recovery (remission). However, most of these patients will later develop a more progressive form of MS called secondary progressive MS (SPMS), with accumulating neurological disability. About 10% of the patients experience progressive accumulation of disability from disease onset, termed primary progressive MS (PPMS)<sup>75</sup>.



**Figure 4.** MS can be divided into four subtypes. A majority in diagnosed with a relapsing-remitting form of MS (RRMS), which in most cases convert into a secondary progressive phase (SPMS). Some patients displays a progressive disease course from onset, with or without overlaid relapses; primary progressive and progressive-relapsing MS, respectively.

### 1.3.1 Immunopathology

Immunologically, MS is considered to be a CD4<sup>+</sup> T cell-mediated disease, where autoreactive T cells are activated in the periphery, through as yet unclear mechanisms<sup>83</sup>, which result in upregulation of adhesion molecules that facilitate migration across the BBB<sup>84</sup>. Once inside the CNS the infiltrating autoreactive cells become reactivated upon encounter of their target antigen presented by APCs. Following reactivation the effector T cells, infiltrating APCs and resident CNS microglia start to produce pro-inflammatory cytokines and chemokines that attracts additional immune cells. Although CD4<sup>+</sup> T cell are considered to mediate disease, CD8<sup>+</sup> T cells are the prominent T cell population within MS lesions<sup>85,86</sup>, and are suggested to mediate neuronal damage. The created milieu of immune cells and effector molecules results in the destruction of myelin, persistent axonal damage and eventually neurodegeneration<sup>87</sup>.

Although we can only speculate as to the nature of the events initiating MS pathogenesis, there is a strong evidence for autoimmune mechanisms, mainly supported by findings in animal models of MS. Autoimmunity refers to a break of immune tolerance and the resulting immune activation being directed towards self-antigens. The features of MS that support its definition as an autoimmune disease are: i) the presence of autoantibodies and autoreactive T cells directed towards CNS antigens; ii) infiltration of immune cells into the 'immuneprivileged' CNS; iii) a strong genetic association to the human leukocyte antigen (HLA) locus (which is associated with most autoimmune diseases); and iv) an animal model can be induced with self-antigens or transfer of autoreactive T cells<sup>88</sup>. However, the presence of autoreactive T cells does not have to imply autoimmune disease *per se*. Autoreactive cells are also present in healthy individuals<sup>89-91</sup>, but are probably kept under control by regulatory mechanisms, such as those provided by regulatory T cells (Treg)<sup>92</sup>. In MS, Tregs have reduced suppressive functions, which may further explain the evident breakdown of immunological tolerance<sup>93</sup>

There is accumulating evidence for an important role of B cells in MS. These have been determined to persist and expand within the CNS and can also be detected in the CSF<sup>94-96</sup>. The intrathecal production of IgG and OCB is likely to derive from B cells and plasma cells<sup>97</sup>. Elevated levels of chemokines, important for attracting B cells, are used as a marker of MS<sup>98,99</sup> and their levels in the CSF correlate with numbers of CSF cells and levels of IgG<sup>100,101</sup>. Furthermore, the therapeutic effect of B cell depleting antibodies (anti-CD20<sup>+</sup>) in MS further supports the perception of B cells as mediators of MS pathogenesis<sup>102</sup>.

### **1.3.2 Risk factors of MS**

Although the cause of MS remains unknown, several factors contribute to MS susceptibility. The disease is thus thought to arise from a complex interplay between genetic, environmental and probably epigenetic factors.

#### **1.3.2.1 Genetics factors**

MS is a polygenic disease with multiple genes affecting disease susceptibility. The genetic component was first demonstrated from studies of familial aggregation<sup>103,104</sup>. In addition, studies of MS discordance in twin pairs have highlighted an increased risk in monozygotic twins compared to dizygotic twins<sup>105,106</sup>. Conversely, non-biological siblings do not have an increased risk of developing MS if other family members are affected<sup>103</sup>.

The first identified, and still the strongest, genetic risk locus is the region harboring the HLA genes on chromosome 6. The strongest association is to the HLA class II allele variant HLA-DRB1\*15:01<sup>107</sup>, which increases MS risk by about 3-fold, whereas the HLA class I gene variant HLA-A\*02 is associated with a protective effect<sup>108,109</sup>. HLA has been considered for a long time as the only reproducible genetic risk factor in MS. Nevertheless, recent technical advancements and large international efforts have now identified more than a hundred non-HLA risk variants<sup>107,110</sup>. Most of these genes are implicated in immune system regulation, further supporting a role of inflammation in MS pathogenesis.

#### **1.3.2.2 Environmental factors**

A growing body of evidence suggests that exposure to environmental factors can influence MS etiology. There is a clear latitude gradient, whereby MS risk increases with distance from the equator<sup>111,112</sup>. Furthermore, migration studies have demonstrated that re-locating from a low-risk area to a high-risk area prior to adolescence results in an acquired risk similar to that of the new geographical location<sup>113</sup>. Proposed environmental contributors to this effect include influence of the ‘hygiene hypothesis’ and levels of sun exposure. The latter may provide direct effects<sup>114</sup> or may be mediated through levels of vitamin D. Low levels of circulating vitamin D have been reported in MS patients and are predicted to affect disease activity<sup>115-117</sup>. There is also a seasonal effect, as MS prevalence is lower for children born during the autumn compared to those born during spring months<sup>118</sup>.

Other environmental risk factors include infection<sup>119</sup>, smoking<sup>120</sup> and obesity<sup>121</sup>. Smoking has further been shown to strongly interact with the main MS HLA risk haplotype, HLA-DRB1\*15:01, and this demonstrates how genetic and environmental factors might interact with each other<sup>122</sup>. There are a number of viral infections implicated in MS including herpes simplex virus 6 and Epstein Barr virus. The exact role of viruses in contributing to MS pathogenesis is still unclear, but mechanisms likely involve molecular mimicry and bystander activation<sup>123</sup>.

### **1.3.2.3 Epigenetic factors**

A role for epigenetic mechanisms in MS has been previously suggested and is proposed as a plausible explanation for some of the ‘missing heritability’ observed in MS. There is a low concordance rate for MS in monozygotic twins (maximum 30%)<sup>105,106,124-126</sup>, further indicating that non-genetic factors are involved in disease susceptibility. These are most likely environmentally-induced epigenetic changes. However, investigation of CD4<sup>+</sup> T cells from three discordant MS twin pairs did not reveal any shared differences in DNA methylation and no evidence of genomic or transcriptomic changes that could explain the discordance<sup>127</sup>. Other studies have revealed altered epigenetic states in brain tissues of MS patients, identifying changes in both DNA methylation<sup>128,129</sup> and histone acetylation<sup>130</sup>. There is also evidence of epigenetic regulation of immune cell function in MS<sup>131-133</sup>.

Parent-of-origin effects have been implicated in MS susceptibility. There is a maternally favored transmission of disease, as demonstrated by maternal half-siblings of MS affected individuals having an increased risk of developing MS compared to paternal half-siblings<sup>134-136</sup>. The HLA risk haplotype, which is carried more often by women than men<sup>137</sup>, also displays preferential maternal transmission<sup>138</sup>. With the use of whole-genome DNA methylation analysis, hypomethylation of several sites in the HLA-DRB1 was associated with MS<sup>139</sup>, a finding that could explain the higher expression of DRB1 observed in HLA-DRB1\*15:01 carriers<sup>140</sup>.

### **1.3.3 MiRNAs in MS**

Study of miRNAs in MS is a relatively new field of research. The first study of miRNAs in MS, performed by David Otaegui and colleagues in 2009, analyzed the expression patterns of 364 miRNAs in peripheral blood mononuclear cells (PBMCs) of MS patients and healthy

controls. Since then, several studies have been conducted, profiling miRNAs in various cellular compartments and clinical settings. So far, little overlap in identified and validated miRNAs has been observed, further demonstrating the complexity of the disease and the intricate regulation performed by miRNAs.

Most of the studies of miRNAs in MS have focused on identifying miRNA dysregulation in the immune cell compartment. MiRNAs have been profiled in whole blood, PBMCs, in T cell subsets and in B cells<sup>141</sup>. The consensus for all these studies is that there is an altered expression of miRNAs during MS and that miRNAs are likely involved in crucial inflammatory processes, such as the induction and function of the pathogenic T-cell subsets, T helper (Th) 1 and Th17 cells<sup>142-144</sup>. Moreover, MS therapeutics have been reported to modulate miRNA expression<sup>145-147</sup>, but whether this is a functional mechanism or a secondary consequence of drug treatment requires further investigation.

In addition to the identification of immune regulatory miRNAs in the periphery, studies of miRNAs in post-mortem brains have revealed different miRNA expression patterns within inflammatory, active and chronic inactive lesions<sup>148,149</sup>. MiRNAs have also been shown to negatively affect BBB function in MS<sup>150</sup>. Finally, characterization of miRNAs that are important for oligodendrocyte function and differentiation could provide new miRNA-targeted therapies in order to increase remyelination<sup>151</sup>. The currently hottest area of miRNA research in MS is that of circulating miRNAs, either to identify new biomarkers<sup>152-155</sup> or to further understand mechanisms underlying both pathogenic inflammation and the regulation of remyelination<sup>156,157</sup>.

#### **1.3.4 Therapeutics**

MS used to be a disease with very limited treatment options, but during the last two decades there has been a large increase in development of novel disease-modulating drugs (DMDs). These now provide a dynamic range of treatment alternatives, with several novel drugs currently at various stages of clinical testing<sup>158</sup>. However, treatments are not yet effective in the progressive forms of MS. The first-line of treatment relies on the administration of recombinant interferon-beta (IFN- $\beta$ ) through subcutaneous or intramuscular injections. The exact mechanisms of action of IFN- $\beta$  treatment are not fully understood. Although IFN- $\beta$  reduces clinical relapse rate, disease activity and progression<sup>159,160</sup>, it is not effective in about



20% of the treated patients, and many treated individuals develop neutralizing antibodies to the cytokine<sup>159</sup>. Second- and third-line therapeutics include Natalizumab (Tysabri), and Fingolimod. Natalizumab is a humanized monoclonal antibody that binds to the  $\alpha 4$ -integrin of very late antigen-4 (VLA-4), a surface marker present on immune cells, hindering them from migrating across the BBB<sup>161</sup>. Natalizumab reduces both relapses and lesions<sup>162,163</sup>. However, this treatment is associated with an increased the risk of developing progressive multifocal leukoencephalopathy (PML), a serious and potentially lethal brain infection caused by the JC virus<sup>164</sup>. Fingolimod is one of the newest drugs on the market and the first oral drug. It targets sphingosine-1-phosphate receptors and results in reduced number of T lymphocytes in the peripheral circulation<sup>165</sup>. A common aspect of current DMDs is that they do not halt disease, repair tissue damage or revert disease progression, and thus are limited to reducing the disease activity.

### 1.3.5 Biomarkers of MS

Given the complexity of MS there is a great need for biomarkers in order to capture different aspects of disease heterogeneity. The establishment of good biomarkers may provide a better understanding of disease etiology, facilitate diagnosis, permit estimate of disease activity, permit prognosis prediction, and evaluate treatment response. While the field of biomarker discovery is highly active in MS, few of the prospective markers make it into clinical practice. Those that are routinely used in the clinic are the previously mentioned diagnostic markers, IgG and OCBs<sup>80,81</sup> as well as measurement of neutralizing antibodies to the common MS treatments (recombinant IFN- $\beta$  and Natalizumab). High titers of neutralizing antibodies to recombinant IFN- $\beta$  treatment result in reduced therapeutic efficacy as the antibodies bind to the IFN- $\beta$  receptor and thereby block expression of interferon-stimulated genes<sup>166,167</sup>. Conversely, antibodies to Natalizumab are only developed in a few percent of treated patients<sup>168</sup>, but can, in addition to reducing treatment efficacy, also result in humoral-mediated (infusion-related) adverse side-effects<sup>169</sup>.

Several other molecules that can be measured in the CSF have been validated as biomarkers of disease activity in MS. Some correlate with immune activity while others relate to ongoing axonal injury. An example of the latter is neurofilament-light chain (NFL), which is released into the CSF upon neuronal damage and their levels are elevated in the CSF of MS patients, especially during clinical relapses<sup>170,171</sup>. Inflammatory mediators include chemokine (C-X-C

motif) ligand 13 (CXCL13) and metalloproteinase 9 (MMP9). CXCL13 is a chemokine that binds to its corresponding receptor present on B cells. Levels of CXCL13 are increased in the CSF of MS patients<sup>98,99</sup>, correlate with an unfavorable disease prognosis of RRMS<sup>172</sup>, and further predict conversion of CIS patients to definite MS<sup>173</sup>. MMP9 is also elevated in the CSF of MS patients<sup>174</sup>. It is expressed by activated leukocytes to modulate the extracellular matrix, thereby facilitating entry to the CNS, but can also directly contribute to myelin damage<sup>175</sup>. None of these markers can alone be used to diagnose and monitor disease. Instead it has become evident that a larger profile of markers is necessary to serve this purpose.

## 1.4 Experimental Autoimmune Encephalomyelitis

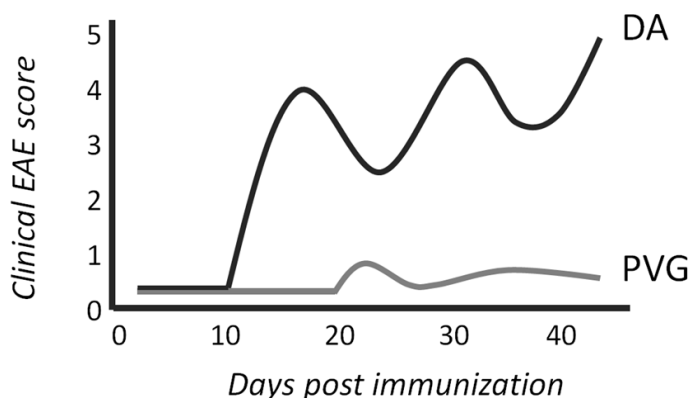
Animal models are widely used to study inheritance, disease transmission and the underlying disease mechanisms of complex diseases such as MS. Animal models provide a system with minimized genetic heterogeneity, controlled environmental conditions and a relatively unlimited sample size. Induction of disease also allows for investigation of initial events prior to disease onset, a time-point that cannot be studied in human MS. Furthermore, the use of animal models provides access to all types of cells and tissues, including the CNS. This is crucial as the CNS is difficult to access in human disease, and immune responses in this tissue are difficult to monitor. Although identified genes may not be identical in rodents and humans they may give insight into dysregulated functions and pathways important for disease development and activity<sup>107,176</sup>. Various techniques aimed at modifying the genome have also provided systems to study the effects of specific genes in isolation.

Experimental Autoimmune Encephalomyelitis (EAE) is the most widely used animal model to study MS-like disease. It has proven extremely valuable for understanding of fundamental (neuro) immunological processes as well as adaptive inflammatory responses in general. EAE can be induced in multiple rodent species, but also in non-human primates. Disease induction is either *active*, using whole spinal cord homogenate, purified myelin, myelin proteins or peptides thereof, or *passive* through adoptive transfer of T cells reactive against CNS antigens. In addition, EAE develops spontaneously in a mouse model with genetically-engineered, myelin-specific T and B cells. Depending on species and induction protocols, different disease courses and pathologies can be modelled.

### 1.4.1 MOG-EAE

Myelin oligodendrocyte glycoprotein (MOG)-EAE can be induced both in mice and in rats<sup>177</sup>. In the C57BL/6 mouse model mice are injected with MOG<sub>35-55</sub> peptide or recombinant MOG, in emulsion with complete Freund's adjuvant containing *Mycobacterium tuberculosis*. The mice are also co-injected with pertussis toxin to further induce BBB permeability and to skew T effector cells to a pathogenic Th17 response<sup>178,179</sup>. This induction protocol results in a chronic form of EAE. We use mouse models of EAE to establish a role of specific targeted genes in the context of inflammation in general, and neuroinflammation in particular.

In the study of heritability of neuroinflammation and in order to identify miRNAs regulating disease we have employed a rat model of EAE. In rats, EAE is induced with recombinant MOG together with incomplete Freund's adjuvant (without *Mycobacterium*) and without pertussis toxin. In susceptible rats this induction protocol results in a chronic relapsing-remitting form of EAE, mimicking that of human MS<sup>180</sup>. We have used different inbred rat strains with varying susceptibility to MOG-EAE to identify genetic loci that regulate clinical EAE phenotypes and to further characterize functional implications of these responses. The two strains used in the studies are the EAE-susceptible Dark Agouti (DA) rat strain and the major histocompatibility complex (MHC) identical Piebald Virol Glaxo (PVG) rat strain that is resistant to the same immunization protocol (Figure 5). Following immunization both strains mount an immune response in the secondary lymphoid organs, but only in DA rats does the activation to the myelin antigen progress become pathogenic in nature. Onset of disease is around day 10-12 post-immunization, which corresponds to the initiation of demyelination in the CNS.



**Figure 5.** MOG-EAE in susceptible DA rats and resistant PVG rats. Animals are scored based on their accumulation of motor deficits. Onset of disease is usually around day 10.

Assessment of MOG-EAE is the same for both mice and rats. It consists of daily measurements of weight loss and visual inspection of clinical symptoms. The animals suffer from ascending paralysis beginning with paralysis of the tail. The symptoms are scored based on the following scale: 0 = no sign of disease, 1 = loss of tail tonus, 2 = hind limb paraparesis, 3 = hind limb paralysis, 4 = tetraplegia and 5 = death. Similarly to MS, the disease symptoms reflect the location of the lesions in the CNS. However, as opposed to MS, in which the majority of the lesions are found in the brain, EAE primarily affects the spinal cord in these rats and the C57BL/6 mouse model.

#### **1.4.2 EAE and the immune system**

Like MS, EAE is described as a T cell-mediated disease. With the use of different EAE models several key immunological mechanisms of MS pathogenesis have been revealed. IFN- $\gamma$ -producing Th1 cells were long thought to be the CD4<sup>+</sup> effector cells in EAE<sup>181</sup>. It was subsequently discovered that IL-23 was required for EAE induction<sup>182</sup>. This led to the identification of IL-23-induced Th17 cells that produce IL-17 as their major effector cytokine. This novel CD4 T cell subset has also been detected in MS, and have been the subject of several studies in MS and in EAE<sup>183</sup>. Both Th1 and Th17 cells can induce EAE<sup>184</sup>; however, neither of the effector cytokines is absolutely required for disease development in EAE<sup>185,186</sup>, indicating that they have redundant functions in disease initiation. Furthermore, EAE was used to establish the role of regulatory T<sup>187</sup> and B cells<sup>188</sup> in regulating autoimmune responses.

#### **1.4.3 Genetic regulation of EAE**

MS and EAE share many similarities when it comes to genetic regulation. They are both polygenic diseases, with the MHC locus (analog to the human HLA locus) as the major genetic risk factor<sup>189</sup> and an overlap of identified non-HLA genes<sup>190</sup>. In addition to the MHC locus, over 50 quantitative trait loci (QTL) have been identified to regulate EAE in rats and mice<sup>191</sup>. The identified QTLs harbor numerous genes and positional cloning of these has proven difficult and time consuming. Through continuous efforts in isolating congenic fragments and the use of gene targeting in mice we have been able to pinpoint some of

specific genes responsible for the observed phenotypes. Identified genes in both EAE and MS include the MHC class II transactivator (Ciita)<sup>192,193</sup> and IL22RA2<sup>110,194,195</sup> and Vav1<sup>196</sup>.

#### 1.4.4 MicroRNAs in EAE

The first evidence of an importance of miRNAs in EAE arose from the laboratory of Professor Pei in 2009, showing that miR-326 regulates differentiation of Th17 cells *in vitro* and *in vivo* through targeting of Ets1<sup>197</sup>. The differential expression and the regulatory function of miR-326 have been demonstrated in both MS and EAE. Since then several studies have defined a role for miRNAs in EAE pathogenesis, mainly as regulators of pathogenic Th1 and Th17 cells and the pro-inflammatory cytokines they produce<sup>58,198-200</sup>. A special interest has focused on the role played by miR-155. MiR-155 is a miRNA known to be important for the immune system<sup>201,202</sup> and it has been shown to be vital to EAE pathogenesis, as demonstrated by knock-out of miR-155 which results in markedly reduced EAE attributed to the role of miR-155 in regulating Th1 and Th17 responses<sup>203,204</sup>. EAE experiments have also shown the importance of miR-124 in microglia activation. Ponomarev *et al* showed that by increasing the levels of miR-124 they could switch microglia from an activated to a quiescent state and thereby inhibit onset of EAE<sup>205</sup>. Additionally, investigation of miRNA profiles of active lesions of primate and mouse EAE demonstrated high overlap in expression patterns<sup>206</sup>, further indicating conservation of miRNA function in neuroinflammatory processes.

## 2 THESIS AIMS

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The overall goal of this thesis was to investigate the contribution of genetic and epigenetic factors on gene regulation in the context of autoimmune neuroinflammation with the long-term goal to understand underlying mechanisms and to facilitate disease diagnosis.

The specific scientific goals for each study were:

Study I: to test the hypothesis that genetic variation in an enzyme that controls the epigenome and has a broad impact on transcription can affect immune system functions and susceptibility to autoimmunity.

Study II: to assess if there is an impact of epigenetic mechanisms on inheritance of autoimmunity.

Study III: to establish if miRNA-mediated pathways can lead to susceptibility to autoimmunity.

Study IV: to investigate the potential of using circulating miRNA as biomarkers in MS.

## 3 METHODOLOGICAL CONSIDERATIONS

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This thesis comprises a variety of materials and methods, all of which are described in detail in the included studies. Here I would like to take the opportunity to review some of the methodologies more thoroughly.

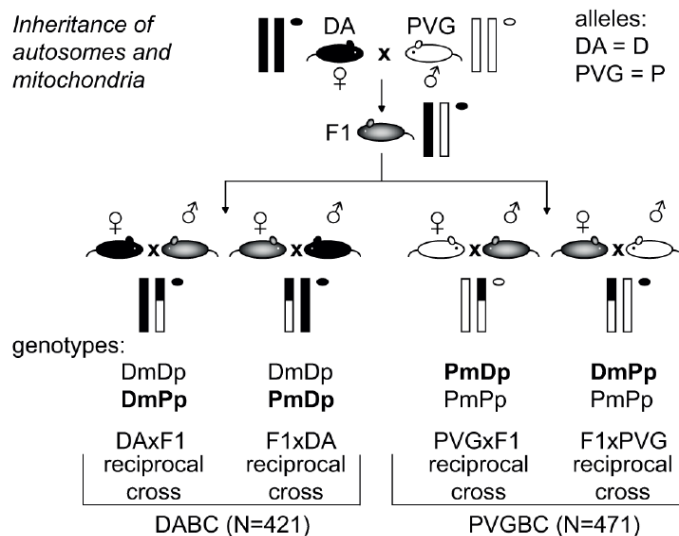
### 3.1 The era of the “Omics”

Technical advancements during the last decades have given rise to the era of ‘-omics’, e.g. genomics, epigenomics and transcriptomics to name a few. Genomics refers to the study of entire genomes, including sequence and structure, epigenomics to the study of the epigenome, i.e. the complete set of epigenetic modifications of a cell or a tissue, be it histone modifications or DNA methylation, and transcriptomics to the study of the transcriptome reflected by all transcribed RNAs. Studies in this thesis include methods to investigate the respective contributions of genomics, epigenomics and transcriptomics in the regulation of neuroinflammation.

#### 3.1.1 A genomic approach to identify epigenetically regulated disease-predisposing QTLs

The influence of epigenetic mechanisms (more specifically mechanisms that confer parent-of-origin effects) on predisposition to develop autoimmunity was investigated using genome-wide linkage analysis in rats (Study II). To enable mapping of the parental origin of disease-predisposing alleles we employed a reciprocal backcross breeding strategy of DA and PVG (Figure 6). In the DA backcross, (DAXPVG)xDA, DA mothers were bred with F1 fathers to create DAXF1, and F1 mothers were bred with DA fathers to create F1xDA. This result in PVG alleles that can be either exclusively paternally inherited (DAXF1) or exclusively maternally inherited (F1xDA). The same reciprocal breeding was performed for the PVG backcross, (DAXPVG)xPVG. This strategy creates a large cohort of genetically unique backcross animals of varying susceptibility to EAE, which enables genome-wide identification of genomic regions that are inherited together with disease. We identified different inherited alleles from the parental strains using microsatellite markers of di- or tri-

nucleotide repeats, where the number of repeats differs between the two strains. The objective of linkage mapping is to define markers that are linked to the measured phenotype in order to define QTLs regulating disease.



**Figure 6:** Schematic illustration of the reciprocal backcross design used to identify parent-of-origin dependent risk loci in Study II.

Employing a genome-wide approach enables simultaneous identification of all QTLs regulating the phenotype of interest. Linkage mapping is a fairly cost-effective and feasible way to identify all genomic regions underlying complex traits. However, QTLs are usually quite large and thus contain multiple genes, and further dissection of the QTLs is necessary to pinpoint the actual genetic variation that is responsible for the observed effect.

While using microsatellite markers to map the origin of the alleles is convenient and informative, we now have access to the sequenced genomes of DA and PVG rats<sup>207</sup>. This enables identification of all single nucleotide polymorphisms (SNPs) segregating between the two strains. As the bi-allelic SNPs occur with higher density in the genome they can be exploited to investigate genetic variations underlying QTLs. Using polymerase chain reaction (PCR) amplification and sequencing of SNPs in F1 hybrid animals we can investigate allele-specific expression and thereby identify imprinted genes. However, this approach has its limitations: i) SNPs are not present in all genes; ii) low sequence coverage can result in inaccurate SNP calling; iii) the gene must be expressed in the investigated tissue or cell-type; iv) the SNPs have to be present in the processed mRNA. Intronic SNPs could potentially be detected in unspliced transcripts, but if they are present they are detected at very low levels. We attempted to identify allele-specific expression of candidate genes underlying identified parent-of-origin loci, but we had very limited success due to the above-mentioned limitations.



### 3.1.2 Transcriptomics

While the term *transcriptome* refers to the complete set of RNA molecules expressed by an organism, the term is more widely used to describe an array of RNA transcripts produced in a particular cell or tissue at a specific time-point. In contrast to the genome, which is characterized by stability, the transcriptome changes actively due to internal and external signals. Only about a decade ago the analysis of the transcriptome was restricted to the use of microarrays with a predetermined number of detectable transcripts. However, with the rapid technical advancement of massive parallel sequencing<sup>208,209</sup> and the development of bioinformatics tools to analyze the sequencing output, this situation has changed dramatically. The introduction of next-generation sequencing (NGS) not only provided whole-genome sequences, such as the case for our two rat strains of focus, DA and PVG<sup>207</sup>, but also the complete transcriptome of cells and tissues. As both the costs and time of sequencing continues to decrease, NGS is becoming an increasingly attractive option for transcription studies. NGS is now widely used in most aspects of RNA biology, with applications such as identification and quantification of both rare and common transcripts, analysis of splice junctions, detection of isoforms, novel transcripts and gene fusions, and detection of long and short non-coding RNAs.

In Study III we used NGS to detect and quantify all small RNAs present in the lymph nodes of DA and PVG. Small RNA sequencing allows detection of 17-35nt long RNA sequences and the sequence output is subsequently mapped to known miRNA of the miRBase repository<sup>210</sup>. In addition to all known miRNAs, NGS also allows for identification of novel miRNAs and other small non-coding RNAs. This is a clear advantage of NGS over microarray-based detection methods (such as the previously published affymetrix array<sup>211</sup> utilized in studies I and III) and methods using pre-spotted PCR plates and PCR-microfluid cards where predefined sets of probes and primers limit the detection possibility. Conversely, the latter produces a user-friendly output, which compared to NGS does not require bioinformatics expertise in data handling. In Study IV we used TaqMan low-density arrays (TLDA), a microfluid based system, to profile circulating miRNAs in the CSF. There TLDA cards have an advantage over NGS due to a requirement of far less input material, even though recent publications indicate that these ultra-low amounts of RNA can be sequenced<sup>72,212</sup>. Although not providing full small-RNA coverage the TLDA cards provided a decent level of detection, with 754 detectable miRNAs, in just a few hours, all in a straightforward analysis format.

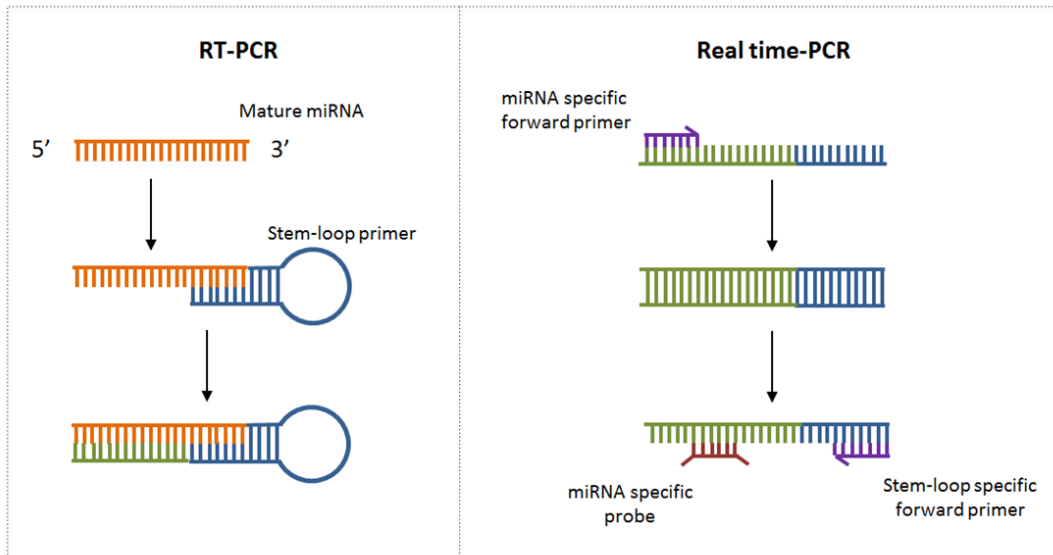
### 3.1.3 Using bioinformatics tools to decipher large data sets

The use of large-scale ‘omics’ experiments has introduced a need for bioinformatics tools to interpret data and to generate information on possible functionality. A multitude of databases has been developed to serve as repositories for diverse biological information such as basic nucleotide sequences (GeneBank)<sup>213</sup>, genomes (Ensembl)<sup>214</sup>, RNA families (RFam)<sup>215</sup>, miRNAs (miRBase)<sup>210</sup> and proteins (UniProt)<sup>216</sup>, but also for more special areas. Databases of known and predicted imprinted genes in humans and mice were used in Study II to predict possible genes underlying our identified parent-of-origin dependent QTLs. Several bioinformatics tools were utilized in Study III to identify miRNAs and miRNA-regulated functions and pathways. To identify the miRNAs detected in lymph nodes of DA and PVG rats we subjected all filtered sequences to the miRanalyzer online tool<sup>217</sup> which maps the sequenced reads to known miRNAs annotated in the miRBase registry. In addition, miRanalyzer also predicts novel miRNAs, a feature especially important for (mammalian) species other than human and mouse, in which fewer miRNAs are annotated (2558 human and 1915 mouse mature miRNAs compared to 765 mature miRNAs in the rat (miRBase v21)). As functional annotations of miRNA target genes are still in its infancy, numerous online computational tools have been developed to predict targets of known miRNAs based on seed sequence complementarity, sequence conservation, thermal stability of the miRNA/mRNA duplex and other parameters<sup>218</sup>. Each tool has its own algorithm and predicts slightly different, although to some degree overlapping, sets of target genes. With this in mind we chose to only consider overlapping targets predicted by two well-known target predictions tools, TargetScan<sup>43</sup> and miRanda<sup>219</sup>, to predict targets of differentially expressed miRNAs in rats. Despite overlooking some targets this improves the chance of identifying true target mRNAs. In order to interpret functionality and to reduce the complexity of differentially expressed gene sets they have to be put into context. Pathway analysis achieves this by integrating knowledge databases of biological processes in which genes and proteins are known to be involved, but also of where and how proteins interact with each other. Several tools, such as Ingenuity Pathway Analysis ([www.qiagen.com/ingenuity](http://www.qiagen.com/ingenuity)) which has been used in Study III, have been developed to facilitate this integration and to indicate downstream consequences of gene dysregulation. However, the classification of biological function and interactions is based on annotations, and so is restricted to what is known<sup>220</sup>. Furthermore, pathway analysis cannot display the dynamic nature of a biological system. Although there are limitations, the output of pathways and functions enriched in the data set enable biologists to focus on further relevant investigations.

## 3.2 Methods for RNA detection and quantification

In this thesis investigation of expression levels for individual transcripts relied on quantitative real-time polymerase chain reaction (qPCR), using either SYBR green or TaqMan-based methods. qPCR is a widely used and fairly simple fluorescence-based method that can measure small amounts of nucleic acids rather quickly and with relatively high sensitivity and specificity<sup>221</sup>. The latter is determined by the primers (SYBR) or by both primers and a probe (TaqMan). Although there are many advantages of qPCR, such as being robust and reproducible, there are disadvantages and limitations. qPCR is a highly sensitive method and it is therefore important to scrutinize all parameters of the qPCR reaction before making assumptions about gene expression. However, in Study I we demonstrate that although failed PCR amplifications cannot provide information on gene expression, it can help to discover interesting underlying biology, such as the implications of altered RNA secondary structure on protein translation.

Although miRNA profiling is beneficial to achieve an overview of the presence and regulation of miRNAs, findings of differential expression need to be confirmed with miRNA-specific methods in order to determine their accuracy. In Studies III and IV we used TaqMan miRNA assays to confirm and quantify mature miRNA levels. The short sequence length of mature miRNAs (approximately 22nt) and the fact that the mature miRNA sequence is also present in the pri- and pre-miRNA transcripts presented new challenges to qPCR technology. A couple of methods have since then been developed to circumvent this problem, all with the approach to add nucleotides to extend the miRNA length<sup>222</sup>. The extension is most commonly produced either by addition of a poly A tail<sup>223</sup> or, in the case of TaqMan microRNA assays by addition of a stem loop<sup>224</sup>, to the 3' end during the RT reaction (Figure 7). The TaqMan stem loop not only adds sequence length to the miRNA but also increases assay specificity by having a short single-stranded region that is complementary to the miRNA 3' end. The stem loop also improves the stability of the miRNA-DNA heteroduplex, thereby increasing the efficiency during the RT reaction<sup>225</sup>. For initial validation of the NGS results (Study III), most of the available methods for miRNAs quantification were evaluated. Although methods using polyA tailing (and universal reverse primer) are both time efficient and less expensive, TaqMan provided by far the most reliable results and was therefore used in all later experiments. In order to detect the ultra-low levels of miRNAs present in biofluids such as CSF, an additional pre-amplification step has to be introduced.



**Figure 7:** TaqMan miRNA assays add a miRNA-specific stem loop during RT reaction and utilize miRNA-specific forward primers and probe during real-time PCR.

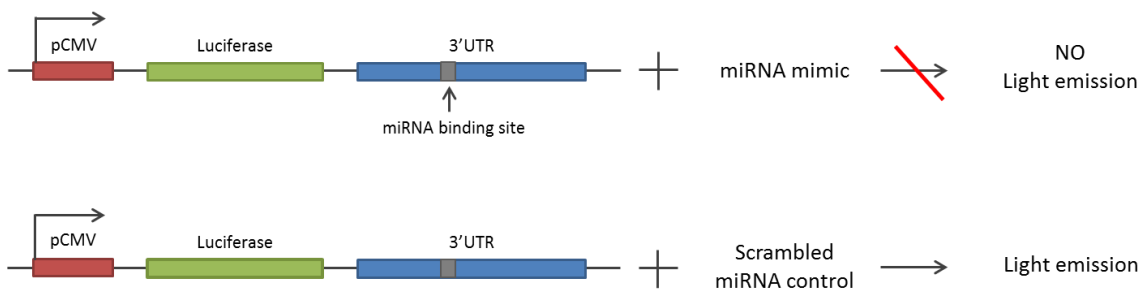
The most common way to normalize gene expression levels to the cellular RNA content is to compare the expression levels to an endogenous control RNA, i.e. a housekeeping gene. This approach was used in Studies I-III. The key characteristic of a housekeeping gene is that its expression does not change depending on experimental conditions and all housekeeping genes should therefore be tested accordingly. In the case of exogenous miRNAs in the circulation this method of normalization is not applicable, as the individual levels of miRNAs and other small ncRNAs in the circulation may not reflect the levels of the transcripts in the cells. Instead other methods of normalizing miRNA levels between samples have to be used. The most common method is to spike the samples prior to RNA isolation with one or more synthetic miRNAs that are absent in the studied species<sup>226</sup>, e.g. *C. elegans* or *Arabidopsis thaliana* (Study IV). The spike-in oligo is introduced after addition of a lysing solution that inhibits the activity of nucleases in the biological sample. Expression levels of the spike-in is used as control in the  $\Delta\Delta Ct$  method<sup>227</sup> and thus compensates for variability during RNA extraction and downstream detection procedures. Other normalization methods involve normalization to an abundant and least variable circulating miRNA<sup>228</sup>, or in case of large number of miRNAs to the average of all detected miRNAs<sup>229</sup>. In Study IV we also investigate the potential of using ‘miRNA pairs’<sup>230,231</sup>, i.e. the ratio of two miRNAs, as a way to discriminate patients from controls. This could be a very promising method when we are not necessary interested in measuring levels of specific miRNAs but rather deriving a biomarker that is a composite of several miRNAs.

### 3.3 Establishing functions and relationships *in vitro* and *in vivo*

Large-scale genomics and transcriptomics provide insight into the regulation that might occur during pathogenesis. However, correlation does not imply causality. Consequently a causative relationship must be established using *in vitro* and/or *in vivo* models. Such is the case for miRNA-mRNA interactions (Study III). As predictions of miRNA targets is based on computational algorithms, a direct binding of the miRNA to the mRNA 3'UTR should be established. This is commonly performed using Luciferase reporter systems where the 3'UTR sequence is coupled to Luciferase in a vector system. By co-transfecting the cells with the Luciferase vector and the miRNA mimics, binding of miRNA and subsequent loss of target gene expression can be measured as absent Luciferase activity. By mutating the miRNA binding site in the UTR, the direct binding will be disrupted and Luciferase will be produced (Figure 8). This Luciferase-based approach was also used to access the effect of the *Kdm3a* SNP on protein translation. By coupling sequences with either of the two SNP versions to Renilla Luciferase we could measure the translation efficiency of the SNPs, simply by measuring the levels of light emitted.

To evaluate plausible candidate genes underlying identified QTLs we have adapted both knockout and transgenic mouse models. In Study I we utilize a murine knockout of *Kdm3a* to study its role as regulator of the immune system and in Study II we use a transgenic mouse in which the *Dlk1* gene is overexpressed. Gene targeting *in vivo* provides a great tool to study a gene in isolation, both in disease models and cellular assays *ex vivo*. Both models modulate levels of the gene of interest in all cells. This is advantageous as all cells can be studied for the role of the gene of interest in an *ex vivo* setting. However, complete knockout can be lethal, or could potentially result in initiation of compensatory mechanisms. This problem can be overcome by using conditional knockout/knock-in models, where the gene of interest is only deleted or introduced in specific cell types. Furthermore, genetic alteration can be induced at selected time points either during development or in adulthood. This is particularly important for genes involved in developmental processes and enables the mouse to develop normally before ablation of the gene of interest.

Traditional gene targeting is performed using homologous recombination in embryonic stem cells. Although it revolutionized our ways of studying gene function, this technique is both slow and expensive. Creating a single mutant mouse can take 2-3 years. Recently, new



**Figure 8.** A Luciferase reporter system can be used to determine direct binding of miRNA to target 3'UTR. Adequate binding of the miRNA to its binding sites will cause RNA degradation or translational inhibition resulting in absent light emission. However, when the binding sites are mutated the product gets translated, causing light emission.

methods of gene targeting have been developed to provide faster, cheaper and more efficient alternatives, such as the zinc finger proteins (ZNF) and the transcription activator-like effector nucleases (TALENs)<sup>232,233</sup>. These are genetically engineered sequence-specific DNA binding domains that are fused to a non-specific DNA cleavage module. The latest technique of genome editing is the CRISPR/Cas9 system. The system, which was originally discovered as a bacterial defense to recognize and eliminate foreign DNA, is based on the Cas endonuclease that is directed to cleave a specific target sequence using a guide RNA<sup>234</sup>. The benefits of these novel techniques are that they facilitate relatively fast and cost-effective genetic engineering in potentially all species, including rats. Moreover, in the case of the CRISPR/Cas9 method, the target design is fairly simple and multiple mutations can be introduced at the same time<sup>235</sup>. The limitations of these novel methods are the potential off-target effects in similar but not identical sites and that cellular mosaics can occur.

MiRNA levels *in vivo* can be altered either long-term using genetically modified animals or through transfection using viral constructs, or short-term using miRNA mimics and inhibitors. MiRNA manipulation *in vivo* is not part of this thesis but presents ways to study functions of miRNAs in developmental systems, physiological processes and in disease.

## 4 RESULTS

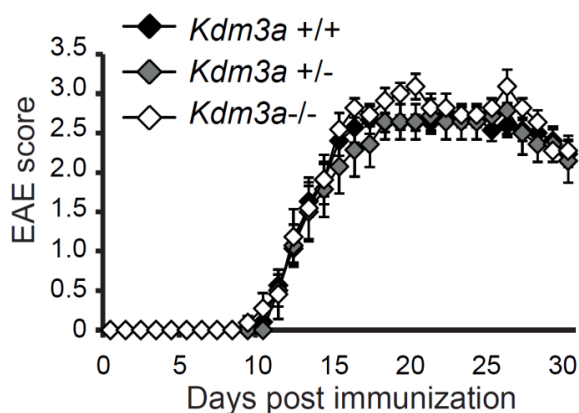
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The work presented in this thesis covers multiple areas of genetic, epigenetic and miRNA regulation of transcripts important for disease susceptibility and progression as well as the application of miRNAs as markers of disease.

### 4.1 Study I – Genetic regulation of an epigenetic mediator in EAE

Several previous studies have identified a QTL on rat chromosome 4 that regulates susceptibility to a variety of immune-mediated diseases<sup>236-238</sup>, including EAE<sup>239</sup>, but also TNF production by macrophages and multiple immune-associated genes<sup>240</sup>. By re-analyzing linkage using the latest rat genome assembly, we identified 15 known genes within the confidence interval of this QTL (Study I). Given the associated phenotype, we hypothesized that histone demethylase *Kdm3a* would be likely candidate gene due to its role in epigenetic regulation that could explain the regulation of expression of multiple immunological genes.

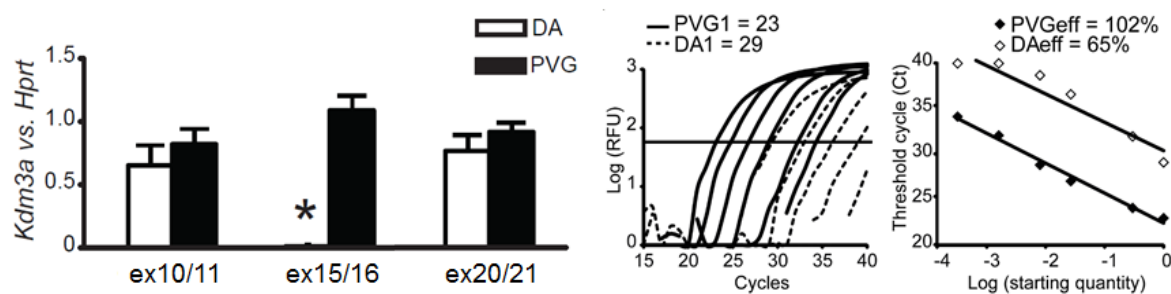
To investigate the hypothesis that *Kdm3a* is a master regulator of EAE pathogenesis we used a *Kdm3a* knockout mouse. Deletion of *Kdm3a* in mice had no effect on a number of cell subsets or activation of lymphocytes, macrophages and DCs, either at naïve state or during EAE. Consequently, it was not surprising that *Kdm3a* had no effect on EAE susceptibility or severity (Figure 9).



**Figure 9:** *Kdm3a* has no effect of EAE susceptibility or severity

#### 4.1.1 Implication of SNPs on nucleic acid secondary structure

Although we recognized that conventional deletion of *Kdm3a* did not regulate mouse EAE, we could not rule out the possibility that natural variations in *Kdm3a* could contribute to immune regulation and EAE in rats. We demonstrated the impact that silent genetic variation can have on the RNA structure. We observed that one of the synonymous SNPs in rat exon 15 of *Kdm3a* caused problems with RNA amplification in the DA strain. The difference, detected using qPCR, was not observed for other exons of the *Kdm3a* gene (Figure 10A). After establishing that this observation was not a result of primer annealing issues due to SNPs or alternative splicing, we identified a reduced amplification potential of the DA amplicon, where the PCR efficiency dropped 35% compared to PVG (Figure 10B).



**Figure 10:** A) Quantitative PCR of *Kdm3a* exons show drastic amplification differences in DA only for the primers surrounding the SNP in exon 15. B) The DA containing SNP sequence amplifies with higher Ct values and with lower PCR efficiency than the PVG containing SNP sequence.

Performing bioinformatics analysis looking at minimum free energy we detected differences in complementary DNA (cDNA) secondary structure, which in the DA competes with primer annealing. Using computational modeling of RNA shapes we could determine that the SNP had a significant potential to affect RNA folding. Examination of protein translation potential revealed a possible effect of the SNP on translation efficiency. Taken together, our data indicate that synonymous SNPs can have drastic effects on amplification efficiency (with a potential to impact levels of protein translation) due to alterations in secondary structure, making it important to consider seemingly ‘silent’ genetic variations.

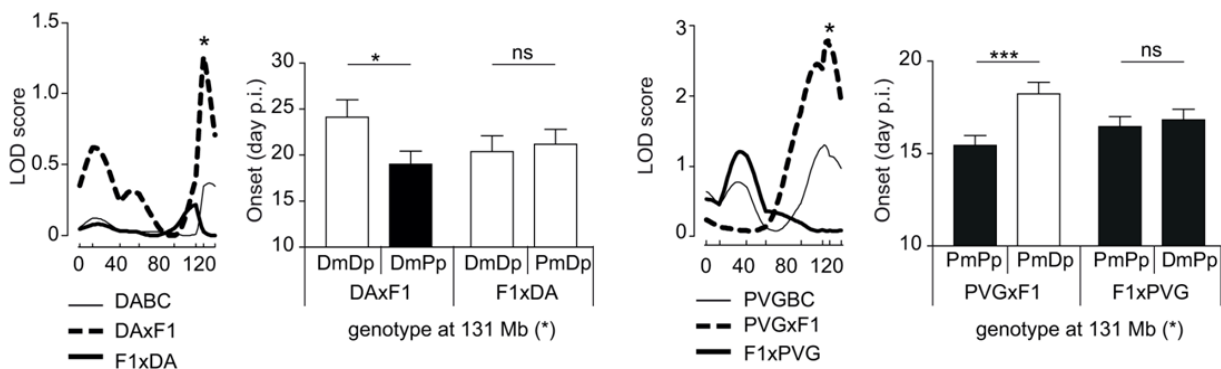


## 4.2 Study II - Contribution of parent-of-origin effects in EAE

Besides the well-documented (straightforward) genetic contribution to EAE, another level of genetic and epigenetic regulation speculated to be involved in EAE etiology concerns parent-of-origin effects. Parent-of-origin effects have been observed in MS in which a preferred maternal inheritance of the disease is demonstrated<sup>134-136</sup>, although less is known about parental transmission in the context of EAE. Factors contributing to parent-of-origin effects include genomic imprinting, Y-chromosome effects and mitochondria. In order to unambiguously address the contribution of such effects in EAE, we employed a strategy using two reciprocal backcross. The reciprocal backcross design enabled identification of parent-of-origin dependent regions that regulate susceptibility to EAE. Using this strategy we observed that at least one in three of all identified disease-predisposing loci depended on parental transmission, with a majority of the identified parent-of-origin QTLs being maternally inherited. Our breeding design also allowed us to investigate the influence of the paternally inherited Y chromosome and the maternally inherited mitochondria. We found that the males that inherited the Y chromosome from the DA developed more severe EAE than those males that inherited their Y chromosome from PVG. As opposed to the Y chromosome, we did not detect differences between rats with varying mitochondria.

The maternally transmitted disease-predisposing alleles were discovered on chromosomes 1, 2, 4, 5, 14 and 18. One example of such a QTL is the on chromosome 4 for which the maternal DA allele conferred earlier onset of EAE in the F1xDA and F1xPVG rats, but not in DAxF1 and PVGxF1. In contrast, the paternally inherited PVG allele at the QTL on chromosome 6 conferred more severe EAE in DAxF1 and PVGxF1 animals, but not in F1xDA and F1xPVG (figure 11A and B, respectively). Such paternally transmitted disease-predisposing QTLs were found on chromosome 6, 7 and 10. This pattern of parent-of-origin dependent transmission indicates underlying genomic imprinting. Several of the parent-of-origin dependent QTLs, both maternal and paternal, overlap with previously established imprinted genes identified in human and mouse. The paternally transmitted EAE QTL on chromosome 6, overlaps with a well-known cluster of imprinted genes, the *Dlk1-Dio3* cluster which has been implicated in embryogenesis and cancer<sup>241,242</sup>, but has also been found to be associated to type I diabetes<sup>243</sup>. In addition to previously established imprinted genes, we also attempted to identify novel imprinted genes underlying the parent-of-origin dependent QTLs. As previously discussed, we used the complete genome sequence of DA and PVG to find

SNPs that could facilitate investigation of allele specific expression<sup>207</sup>. However, despite large efforts we were unable to conclusively demonstrate allele-specific expression.

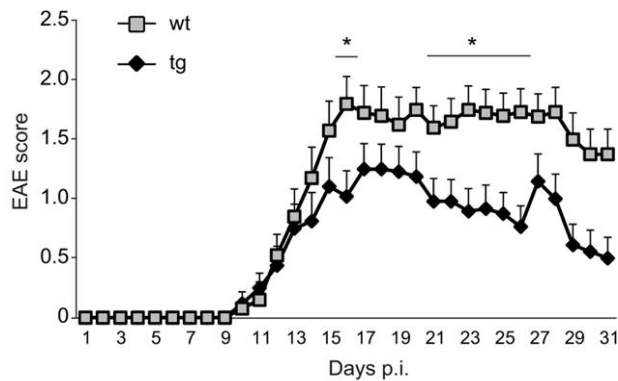


**Figure 11:** Paternal transmission of-disease predisposing PVG allele A) LOD plot of chromosome 6 shows linkage for disease onset in PVGxF1 but not in F1xPVG rats (peak at 131 Mb, indicated by \*) B) The same QTL shows linkage in DAxF1 but not in F1xDA rats.

Even though there were no coding SNPs between DA and PVG in the genes of the *Dlk-Dio3* cluster to establish allele-specific expression we decided to investigate the region for potential genomic imprinting. We found ~50% methylation at a putative ICR in spleen, which concurs with imprinting status. By investigating the expression levels of the three known paternally expressed genes, *Dlk1*, *Rtl1* and *Dio3* we found that the PVG allele predisposed for lower levels of *Dlk1*. The *Rtl1* and the *Dio3* genes conversely did not show any expression differences depending on parental transmission. To further explore the role of *Dlk1* in EAE we used a transgenic mouse overexpressing *Dlk1*. The lower expression of *Dlk1* in the wildtype controls resulted in a more severe EAE score (Figure 12), similar to what was observed when *Dlk1* was paternally inherited from the PVG. Characterization of the *Dlk1* transgenic mouse demonstrated a lower frequency of activated CD4<sup>+</sup> T cells and B cells compared to littermate controls and we observed less IFN- $\gamma$  producing Th1 cells in the *Dlk1* transgenic mice when differentiating naïve CD4<sup>+</sup> T cells into different effector subsets *in vitro*. Taken together this study demonstrates a role of the imprinted *Dlk1* gene on the adaptive immune response in EAE.

Apart from coding genes the *Dlk1-Dio3* locus contains a large cluster of more than 50 miRNA genes, all of which are maternally expressed<sup>241</sup>. Similarly, some of these miRNAs were also determined to be differently expressed between DA and PVG during EAE immune

activation (Study II), although with low expression. We decided to investigate potential allele-specific expression of five miRNAs, miR-127, miR-136, miR-431, miR-434 and miR-541 using reciprocal F1 hybrids. We observed some indication for parent-of-origin dependent expression, although not significant. Further exploration in different cells types and at different ages could provide more information as to where and when imprinting of these miRNAs is set.



**Figure 12:** *Dlk1* transgenic mice (tg), producing a double dose of *Dlk1*, presented with less severe EAE compared to wild type littermate controls (wt).

### 4.3 Study III - miRNAs as post-transcriptional regulators of EAE

In addition to genetic and epigenetic regulation of transcription, miRNAs have during the last 20 years been proven to have a great impact on shaping the transcriptome and ultimate phenotype. Prior to the onset of EAE, at day 7 post MOG-EAE induction, an immune activation is ongoing in the lymph nodes of both DA and PVG rats. However, this activation will only result in subsequent immune cell migration and infiltration into the CNS in the DA rats. There is no difference in immune cell populations at this early stage<sup>244</sup>; instead the difference between the two strains relies on the quality of the immune response. By determining all miRNAs that differ between the two strains at this time point we were able to identify a miRNA profile that could be responsible for the pathogenic immune activation. Using NGS (Illumina) we could quantify a total of 329 miRNAs in the lymph nodes of DA and PVG rats. Forty-three of these miRNAs were differentially expressed between the two strains. Most of the differentially expressed miRNAs were more highly expressed in the EAE-susceptible DA strain. Some of the miRNAs were highly abundant whereas others were expressed at very low levels. The latter are likely to be less important for the abundant cell types such as CD4<sup>+</sup> T cells<sup>244</sup>, but could be specific for less prominent cell types. We further investigated miRNA expression kinetics and the cellular source for some of the miRNAs to get a better understanding of how miRNAs could be regulated during EAE. We observed that

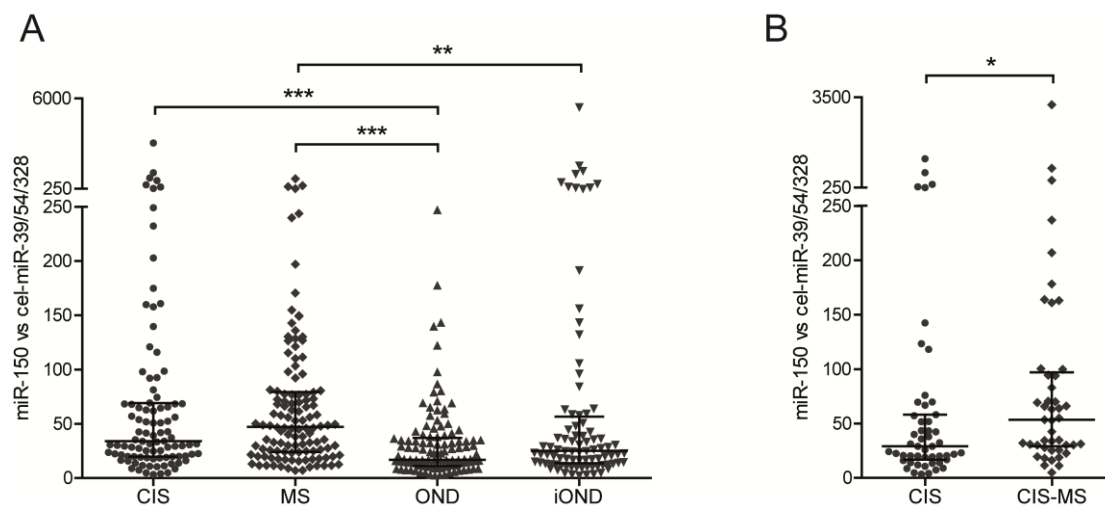
some miRNAs are consistently more highly expressed in DA, whereas other miRNAs display changes at day 7 only. The cellular source also differed between miRNAs as some (e.g. miR-181a and miR-128) were predominantly expressed in T cells, and others (e.g. miR-199a-3p and miR-223) were rather exclusively expressed in non-lymphocyte cell populations. We could thus illustrate genetically controlled and cell-specific miRNA profiles that regulate development of pathogenic immune responses.

To understand the functional implication of this miRNA profile one needs to identify the potential genes that the miRNAs regulate. For this purpose we used a combination of *in silico* target prediction and known mRNA expression levels in day 7 lymph nodes<sup>211</sup>. By only focusing on those genes that were differentially expressed in opposing manners in the lymph nodes we were more likely to find lymph node-specific mRNAs that are regulated during immune activation. Using this approach we identified 109 target genes potentially regulated by the miRNAs with higher expression in the DA strain, and 54 target genes of the miRNA with higher expression in the PVG strain. Three of the targets for miR-181a were confirmed using *in vitro* validation experiments, showing that the combination of target prediction tools and whole genome expression data can be used to establish targets of miRNAs. By performing pathway analysis we could further explain how differentially expressed miRNAs could potentially regulate different pathways of importance during immune activation in the EAE-susceptible and the EAE-resistant rats.

#### **4.4 Study IV - Establishing circulating miRNAs as biomarkers of MS**

The establishment of miRNAs as biomarkers of MS is currently focused on the identification of miRNA in the circulation<sup>152,154,155,245</sup>. Such circulating extracellular miRNAs are advantageous candidates for disease biomarkers as they have been found to be remarkably stable, even during harsh treatments such as variations in temperature and multiple freeze-thaw cycles<sup>246</sup>. In addition, they can be fairly easily detected in most biofluids<sup>68</sup>. In Study IV we aimed to profile miRNAs in the CSF of MS patients and controls with the prospect of identifying novel biomarkers of disease. Using TLDA cards we were able to detect a total of 88 miRNA in CSF pools of CIS, MS, other neurological disorder (OND) and inflammatory OND (iOND) patients.

Based on detection we selected 15 miRNAs for further validation in a larger independent sample cohort comprising 142 individuals (31-43 patients per group). We could detect significant differences in miRNA levels between MS and controls for two miRNAs, miR-145 and miR-150. MiR-145 was of particular interest as it has previously been identified as a biofluid marker for MS in both serum and plasma<sup>154,245</sup> as well as in circulating PBMC<sup>154,247</sup>. MiR-150 has also been associated with MS as a marker in T-cells and PBMCs<sup>248,249</sup>. The utilized patient cohort is similar in size to that of other miRNA biomarker studies, but given the rather uniquely large biobank established at the Karolinska University Hospital, we sought to further investigate miR-145 and miR-150 in a much larger cohort (n=430). In this large sample cohort we were able to confirm differential levels of miR-150, but not of miR-145, thus demonstrating a necessity for validation in large cohorts. In our study we determined miR-150 levels to be significantly different between MS and both control groups, as well as between CIS and OND (Figure 13A). When subdividing CIS patients we also observed higher levels of miR-150 in those individuals that later converted to MS compared to those not yet converted in follow-up (Figure 13B), indicating a potential use of miR-150 to facilitate earlier diagnosis.

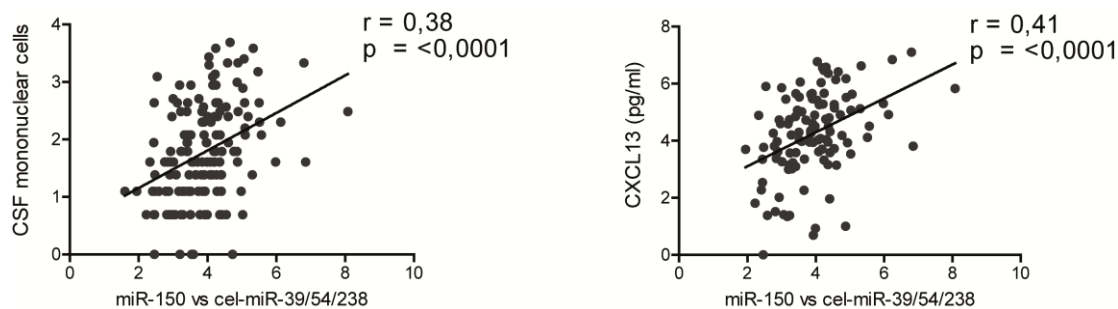


**Figure 13:** A) Levels of circulating miR-150 in CSF is significantly different between MS and controls. B) MiR-150 levels are higher in CIS patients that converted to MS (CIS-MS) compare to those not yet converted.

To evaluate the potential of miR-150 to act as a biomarker distinguishing MS and OND we constructed a Receiver Operating Characteristic (ROC) curve. An ROC curve is used to evaluate diagnostic accuracy of a biomarker, visualizing true-positive rate (sensitivity) and false-positive rate ( $1 - \text{specificity}$ ). Area under the ROC curve (AUC) can range from 0.5 (no

predictive ability) to 1 (perfect discrimination/accuracy)<sup>250</sup>. AUC for miR-150 was 0.74, indicating that miR-150 could differentiate MS and controls providing optimal cut-off at 2.84 with a specificity of 88% and sensitivity of 51%.

Circulating miRNAs in the CSF likely reflect events in the target organ, the CNS. In MS the pathogenic events are mainly represented by inflammation and demyelination. By investigating the relationship between miR-150 levels and established markers of disease we observed a positive correlation with several immune parameters. Levels of miR-150 correlated with the amount of cells in the CSF (Figure 14) and with the IgG index, both factors indicating immunopathology in the CNS. We also measured higher levels of miR-150 in OCB-positive patients. In further support of the immune-association of miR-150, we observed a positive correlation of miR-150 with the recognized immune biomarkers, CXCL13 (Figure 14) and MMP9. Conversely, we did not determine any relationship between levels of miR-150 and markers of neurodegeneration such as EDSS score, number of lesions or levels of NFL in the CSF.



**Figure 14:** Levels of circulating miR-150 in MS correlates with several immunological disease parameters such as A) the number of cells in the CSF and B) levels of biomarker CXCL13 in the CSF.

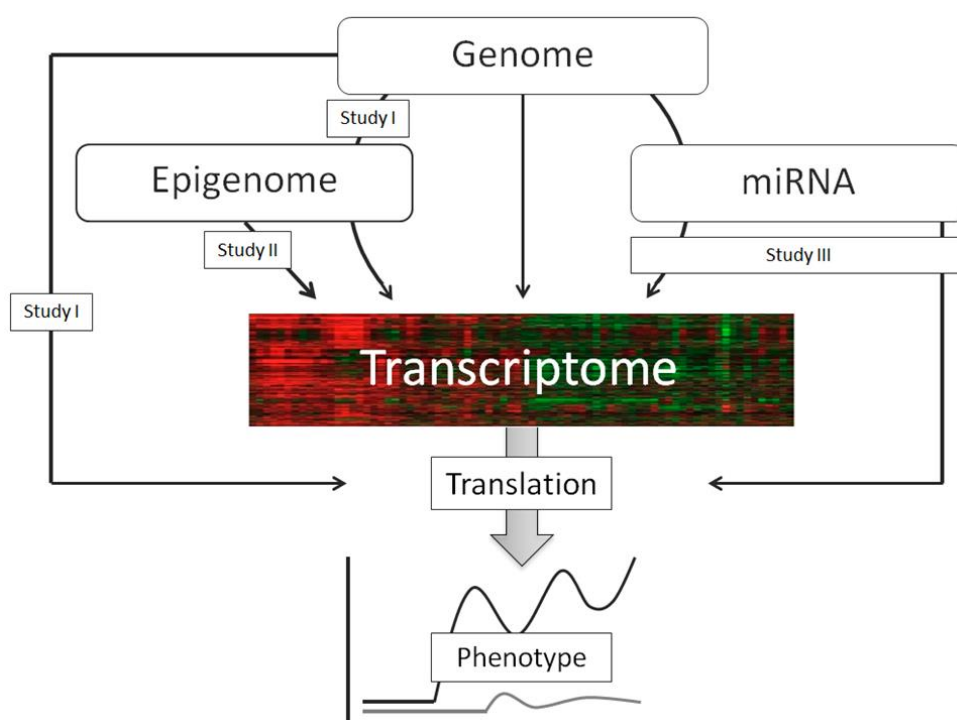
To facilitate the use of miR-150 as a biomarker in a clinical setting we investigated the potential of using a ‘miRNA pair’ to discriminate patients and controls, where the ratio of two miRNAs serves as a marker and thereby bypassed the need for spike-in controls. By comparing all possible miRNA pairs among the 15 tested miRNAs we concluded that the ratio of miR-150 and miR-204 best served this purpose. The miR-150/miR-204 ratio was found to have better potential to serve as biomarker (AUC 0.80), compared to miR-150 alone.

## 5 DISCUSSION AND CONCLUSIONS

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### 5.1 Epigenetics and miRNAs as modulators and mediators of neuroinflammation

The genetic diversity of humans and model animals has long been used to investigate the contribution of genetic regulation on disease susceptibility and severity. However, there is an ever-growing body of evidence for epigenetics and miRNAs being involved in shaping the transcriptome in pre- and post-transcriptional manners, during both physiological and pathological conditions. Epigenetics and miRNAs are likely to both modulate and mediate genetic information based on extrinsic and intrinsic signals. My studies have investigated the contribution of epigenetics to the heritability of experimental neuroinflammation both as mediators (Study I) and modulators (Study II). Furthermore, I have investigated miRNAs both as potential regulators of EAE susceptibility (Study III) and as biomarkers of MS (Study IV) (Figure 15).



**Figure 15:** Studies of this thesis demonstrate that both epigenetic factors and miRNAs can act as modulators of the information transmitted from the genetic code to the functional transcriptome (Study II and III). In addition, variations in the genetic information could potentially influence translation in a direct manner (Study I). Furthermore, dysregulated miRNAs have a great potential to act as biomarkers of disease (Study IV).

## 5.2 Kdm3a is not a master regulator of EAE

We hypothesized that *Kdm3a* acts as a ‘master regulator’ mediating diverse immunological phenotypes through epigenetic modifications (Study I). Given its role as a histone modifier, Kdm3a could epigenetically mediate regulation by altering the chromatin state. Kdm3a, a member of the Jumonji C domain-containing family proteins, is known to catalyze the reversal of H3K9 mono- and di- methylation. H3K9 methylation, which is associated with transcriptional repression<sup>14,251</sup>, has been shown to be essential for T and B cell development<sup>252</sup> as well as being important in the regulation of effector molecules such as IL-2<sup>253</sup>. Owing to this potential role of Kdm3a we were surprised to observe that complete removal of the gene did not result in any observed impact on the immune system or on EAE pathogenesis in mice. This could indicate that H3K9 methylation is of less importance in the context of EAE. Alternatively, this negative finding might just result from a difference between model species or that *Kdm3a* has important roles in the immune system other than those investigated. Another possibility is that the use of a full knockout results in the activation of compensatory mechanisms. Using a conditional knockout of *Kdm3a*, depleting *Kdm3a* in a specific cell type, for instance in macrophages, might have resulted in an effect on EAE pathogenesis. However, a conditional *Kdm3a* knockout mouse was not available at that time. Despite the disappointing results in the mouse model, *Kdm3a* could still be a candidate gene underlying the observed effects in rats. Historically, mouse have been the models of choice to study gene function, as tools for genetic manipulation *in vivo* were readily available in mice and gene targeting in rats was proven very difficult<sup>254</sup>. However, with recent advancements in genetic engineering, especially with the CRISPR/Cas9 system, it is now possible to modify the *Kdm3a* SNP in the DA strain. This would enable investigation of both nucleotide variations on the same genetic background. Genetic engineering could also be utilized to investigate the contribution of the other genes and regulatory regions within the disease promoting QTL.

Although *Kdm3a* is not regulating EAE through epigenetic mechanisms in mice, we found a synonymous coding SNP in the rat *Kdm3a* gene that may potentially directly regulate translation of the gene. The seemingly silent SNP had dramatic effects on PCR efficiency as a result of altered cDNA folding, demonstrating the importance of examining PCR parameters and sequence differences between sample subjects. The finding that a synonymous SNP can affect mRNA quantification and RNA folding has previously been indicated<sup>255,256</sup>, yet synonymous SNPs are rarely accounted for in studies of gene expression. In almost all of the



experiments of translation potential we observed a lower *Renilla Luciferase* activity with the DA *Kdm3a* SNP, indicating that the SNP may affect translation. Experiments *in vitro* all focused on the 181bp amplicon harboring the SNP, thus whether this also occurs for the full-length mRNA *in vivo* remains uncertain. However, there is some evidence for synonymous SNPs altering protein translation<sup>255</sup> and Shape Probability Shift analysis suggests that the SNP in *Kdm3a* has a significant impact on RNA folding.

This study sheds more light on the complexity of genomic variations. In the age of genomics and genome-wide association studies (GWAS) there is an ever-growing number of SNPs identified and associated with complex diseases. Initially, attention was given to SNPs that affect protein structure, and synonymous SNPs have largely been ignored. Here we demonstrate that by disregarding such variation, important biologically relevant aspects could be overlooked, such as in our case the implication of silent SNPs on the downstream nucleic acid folding and translation. Additionally, majority of the identified genetic variations that associate with diseases are located in intergenic regions. Apart from their obvious impact on regulatory elements their influence on other transcribed RNA products are yet to be discovered. Given that we now know that most of the genome is being transcribed, such mechanisms may be of more importance than originally believed.

### **5.3 Evidence for parent-of-origin effects in EAE**

Another mechanism of epigenetically mediated regulation of the transcriptome is parent-of-origin effects. In Study II we show that there is a large contribution of such effects in EAE etiology. Using a reciprocal backcross strategy we could demonstrate that 37-54% of the identified disease-predisposing loci depend on parental transmission and that accounting for parental transmission could further explain the disease variance by 2-4 fold. The identified preferential maternal pattern of disease inheritance has also been observed in MS<sup>134-136</sup>. Taken together we clearly demonstrate that accounting for parental origin can help to discover more risk loci and can further explain more of the previously unknown genetic regulation of underlying EAE. Furthermore, the fact that we observe EAE susceptibility alleles being transmitted from the PVG suggests that both strains contain both risk genes and protective genes and that it is the balance of these that ultimately determines susceptibility. The significant impact of parent-of-origin effects implicates epigenetic mechanisms (e.g.

DNA methylation and non-coding RNAs, that commonly control imprinting) modulating the genetic information in autoimmune inflammation.

Several of the parent-of-origin QTLs overlap with known imprinted genes, some of which with known importance for the immune system. One of these, underlying the maternally transmitted QTL on chromosome 2, is the maternally expressed *Igf2r*<sup>257</sup> gene. *Igf2r* is known to be important for T cell activation<sup>258</sup> and for regulatory T<sup>259</sup> and B cell<sup>260</sup> function. The paternally transmitted locus on chromosome 6 overlaps with the imprinted *Dlk1-Dio3* cluster containing paternally expressed genes. Using a transgenic model we could identify *Dlk1* as a novel candidate gene for EAE, predisposing for more severe disease when paternally transmitted in rats. *Dlk1* is an atypical Notch ligand, which is suggested to inhibit signaling through the Notch pathway<sup>261,262</sup>. Notch signaling has previously been implicated in the pathogenesis of MS and EAE<sup>263,264</sup>, where the signaling pathway is known to regulate key players in the immune response<sup>265-267</sup>, as well as oligodendrocytes<sup>268</sup>, thereby potentially affecting both demyelination and remyelination processes. In addition to coding genes, this imprinted region also contains a large cluster of miRNAs, all of which are maternally expressed<sup>241</sup>. Two of these, miR-127 and miR-136, are important during development where they target the *Rlt1* gene on the paternally inherited allele, thereby silencing it<sup>269</sup>. Furthermore, several miRNAs in this cluster have been associated with human malignancies<sup>270</sup>, and identified as differentially expressed in EAE (Study III). The latter indicates a role of imprinted miRNAs in immune cell regulation and in experimental neuroinflammation.

This is the first study to demonstrate the contribution of parent-of-origin effects in regulating susceptibility to EAE in rats. By controlling for the environment we exclude external contribution to the observed effects, implicating that risk factors of EAE development can be modulated by epigenetic mechanisms. Our findings also suggest that accounting for parent-of-origin effects in human genetic studies would likely result in identifying additional risk genes and explaining more of the heritability of complex diseases.

## 5.4 MiRNAs as regulators of pathogenic immune response

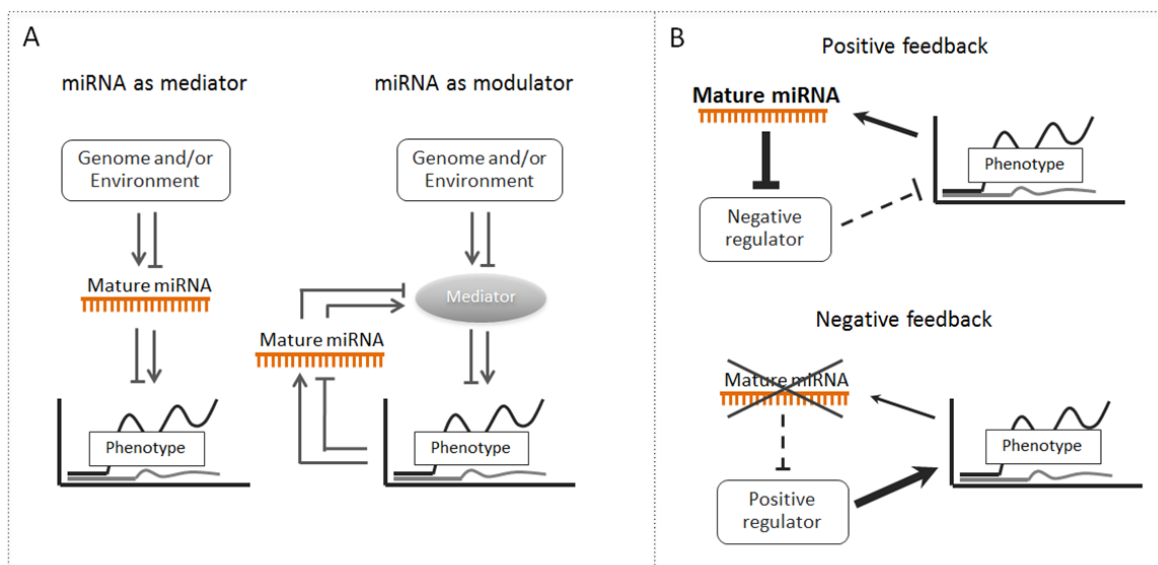
It is now well established that miRNAs play an important role in regulating biological processes, including the immune system. Studies of miRNA regulation during EAE have

primarily been focusing on the role of miR-155<sup>58,203,204,271</sup> and miRNA as regulators of Th1 and Th17 cells<sup>197-200</sup>. In Study III we established a miRNA profile regulating susceptibility to develop EAE, determining both miRNAs that mediate a pathogenic immune phenotype and the potential target genes modulated by the differentially expressed miRNAs (Figure 16A). The miRNA profile demonstrated that the majority of the miRNAs had higher expression in the susceptible strain. Together with the different expression kinetics and cellular source of miRNA expression, we conclude that miRNA regulation of EAE susceptibility is likely a result of both miRNAs promoting disease and miRNAs expressed to regulate ongoing inflammation.

Even though we observed quite a large number of differentially expressed miRNAs, the fold-change between the strains was modest. This is not surprising as miRNA dysregulation in diseases, including MS, is rarely that extensive<sup>247,272</sup>. This might be due to the feedback mechanisms that regulate miRNA levels. Given that each miRNA can regulate a vast number of genes, it is not surprising that their expression is under tight regulation. The feedback loops regulating miRNA levels further demonstrate the role of miRNAs in fine-tuning biological processes<sup>273</sup>. One of the most studied miRNA in inflammation, miR-146a, which was higher expressed in DA rats, is an example of this. A negative feedback loop is generated as miR-146a, which is upregulated by immune activation, downregulates factors of the immune signaling pathway and thereby controls the immune activation<sup>59</sup>. In view of the central role that miRNAs have in fine-tuning the immune response it is not unexpected that miRNA dysregulation can disrupt the intricate feedback system and ultimately cause disease (Figure 16 B). Given the high conservation of miRNAs between species and possibly shared functions in MS and EAE pathogenesis<sup>197</sup>, miRNAs identified in EAE susceptibility might also be important in the early stages of MS. In animal models they play a crucial role during this early time-point, prior to diagnosis, which is not possible to study in human disease.

To understand how the alterations in miRNA expression can lead to disease we investigated the functions and pathways regulated by targets of the differentially expressed miRNAs. By integrating expression analysis with miRNA target predictions we identified miRNA-regulated genes important for EAE susceptibility. It is now shown that the binding of miRNA to the 3'UTR of mRNA primarily results in mRNA deamination and decay<sup>274</sup>, justifying the pursuit for miRNA targets with inverse correlated expression. However, translational repression occurs without change in mRNA levels<sup>275</sup> and these targets will therefore be overlooked. By demonstrating a direct interaction of one miRNA with three of its predicted

target genes we further demonstrate that the targets we identified are likely to be true. Several pathways and functions were enriched in the list of miRNA targets. Targets of miRNAs with higher expression in the susceptible DA strain were involved in functions related to the migration and homing of immune cells. The potential for the autoreactive immune cells to migrate and infiltrate the CNS is one of the hallmarks of MS and EAE. The miRNA were shown to target several important chemokines and their receptors, such as CXCL13, CCL3L1, CXCR3, all of which are have previously been associated with MS and EAE<sup>172,276,277</sup>.



**Figure 16:** A) Illustration of how miRNA can mediate and modulate the transmission of information. B) MiRNAs work in feedback systems to fine-tune biological processes. Dysregulation of miRNA levels can both reduce negative control regulation and increase positive control regulation, thereby further enhancing the phenotype.

Although the list of miRNA downregulated genes in the PVG was shorter, pathway analysis was still informative and suggested miRNA associated regulation of B lymphocyte differentiation and apoptosis. The first is mainly regulated by miR-150, which is known to regulate T and B cell differentiation<sup>52,64</sup>. The levels of miR-150 are reduced as the lymphocytes differentiate, possibly indicating that PVG produces less fully differentiated effector cells. Furthermore, the amount of autoreactive cells in the PVG could be controlled by miRNA-mediated regulation of apoptosis. Taken together, the miRNAs and their targets give new insight into the complicated regulation that occurs during immune activation and that altered expression of miRNAs can contribute to EAE susceptibility. The direct

mechanism through which the individual miRNAs influence the immune systems deserves further investigation.

## 5.5 Circulating miRNAs as biomarkers of neuroinflammation

For complex diseases, where the cause is unknown, there is a high demand for good biomarkers. Biomarkers of disease are of extra relevance in MS given the inaccessibility of the target organ. A large number of molecules identified in blood and CSF of MS patients has been proposed as biomarkers<sup>278</sup>, although few have been extensively evaluated in a larger sample cohort<sup>278</sup>. Circulating miRNAs as biomarkers in the CSF have primarily been identified in neurodegenerative disorders such as Alzheimer's and Parkinson's diseases<sup>72,279</sup>. To date, only one study has investigated circulating miRNAs in the CSF of MS patients and controls<sup>155</sup>. In pooled CSF samples from MS patients and controls we could reliably detect 88 miRNAs. Detected miRNAs displayed poor overlap with those miRNAs detected in the study by Haghikia *et al*, but we observed a substantial overlap (63%) with miRNAs detected in active and inactive lesions<sup>148</sup>, indicating that our miRNA profile mirrors events happening in the CNS during MS.

In a uniquely large sample cohort we demonstrated miR-150 as a putative new biomarker for MS. MiR-150 has previously been found downregulated in PBCMs and T cells in MS compared to healthy controls<sup>248,249</sup>. Levels of miR-150 in the CSF correlated with immunological parameters, indicating that miR-150 is either released from infiltrating immune cells or by CNS resident cells in an attempt to downregulate the inflammation. Both monocytes and lymphocytes release miR-150. MiR-150 released by monocytes promote endothelial cells migration and angiogenesis<sup>280,281</sup> and miR-150 secreted by B cells<sup>282</sup> could potentially be taken up by CD8<sup>+</sup> T cells<sup>283</sup>. More and more evidence suggests an important role of B cells in the pathophysiology of MS, an observation further supported by the therapeutic potential of B cell-depleting anti-CD20 monoclonal antibodies (Rituximab) in RRMS<sup>102</sup>. We determined positive association of miR-150 to OCBs, IgG index and CXCL13, all of which are markers of B cell immunopathology<sup>96,101,284</sup>. This indicates that miR-150 may be released from infiltrating B cells or from other cells that impact on B cell function. These are as yet speculations and further investigation of the role of miR-150 in MS is warranted.

Extracellular miRNAs in biofluids have a great potential as biomarkers due to their stability and accessibility. As opposed to protein biomarkers, several miRNAs can be detected in a single isolation procedure from a fairly small sample volume. To aid clinical implementation we reveal how challenges with miRNA normalization in circulation can be circumvented with the use of 'miRNA pairs' as diagnostic markers. The miR-150/miR-204 ratio reflects variations in both miRNAs and actually provided a better marker than miR-150 alone.

## 6 CONCLUDING REMARKS AND POINTS OF PERSPECTIVE

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Diseases such as MS are challenging to study as they arise from a complex interaction of numerous genes and environmental factors. The fact that most of the mechanisms underlying this interaction remain undiscovered, and that the pathogenic triggers are likely to vary among patients, further adds to the complexity. The epigenome integrates signals from the genome and the environment, thereby shaping the transcriptome. Through continuous exploration of epigenetic mechanisms and miRNAs involved in MS etiology, we are likely to unravel more of these underlying mechanisms.

In the first study we were unable to demonstrate the potential of the histone demethylase Kdm3a in regulating autoimmunity in mice. Histone modifications have been implicated in both MS and EAE, but the histone code is comprised of a multitude of modifications regulated by a large number of enzymes, and not all of them are likely to be important in immune system regulation. However, whether Kdm3a is equally dispensable in rat EAE has yet to be investigated. With novel techniques of gene targeting, similar investigations in rats are now possible.

During the past decades a large focus has been on identifying risk factors for MS such as genetic variations and environmental influences. Genetic studies have largely focused on factors that follow Mendelian inheritance and which affect either regulatory regions, and hence levels of gene transcription, or protein structure. My work demonstrates the importance of extending these investigations to account for ‘silent’ variations and miRNAs that eventually impact the proteome. Additionally, my work highlights a necessity to consider non-Mendelian inheritance and the fact that, among others, risk genes will also be modulated by epigenetic mechanisms.

Study I demonstrated how a seemingly silent SNP can affect the nucleotide secondary structure and potentially protein translation. This finding implicates a new impact of the previously largely overlooked synonymous SNPs. Although they do not change the protein product, they may change the rate at which they are translated. Assumptions of candidate genes underlying QTLs generally include identification of non-synonymous SNPs that may

result in altered proteins. However, in the light of our findings synonymous SNPs may also be informative, and should be investigated accordingly. Furthermore, although it was previously known that SNPs can alter the secondary structure, they are not accounted for when designing experiments to measure RNA expression. Tools should be developed so that SNP effects on RNA secondary structure can be modulated and interpreted.

In Study II we demonstrate significant parent-of-origin effects in inheritance of EAE, corroborating similar suggested effects in MS, which were questioned due to potential confounding factors in epidemiological studies. By accounting for parent-of-origin effects, novel risk genes/regions are likely to be discovered. The identification of parent-of-origin regulated loci may reflect genomic areas that are open to regulation by the environment, and so present an opportunity to further explore gene-environment interactions. Although identification of candidate imprinted genes underlying the parent-of-origin dependent EAE QTLs proved to be challenging, ongoing efforts to identify genome-wide imprinted genes in relevant immune cells using RNA sequencing in reciprocal F1 hybrids is ongoing. These experiments will not only identify the genes underlying the parent-of-origin EAE QTLs, but also address the impact and functions of imprinting in immune cells.

MiRNAs are known to be dysregulated in most diseases and the role of a few miRNAs has been demonstrated in EAE. The findings of Study III demonstrate that in the context of complex diseases, several miRNAs are more likely to act together in a fine-tuning manner rather than a single or a few miRNAs exerting dramatic effects. The miRNA profile identified is different from that reported in other studies, as it demonstrates miRNAs that are differentially expressed between pathogenic and non-pathogenic immune activation states, as opposed to differences between disease and healthy states. We identified several miRNAs with known immune functions, and many with less well-documented functions. Further studies are required to investigate their roles in regulating autoimmunity. Functional miRNA studies generally investigate the role of a single miRNA. However, our study demonstrates that miRNAs are likely both disease-promoting (pathogenic miRNAs) and disease-protective (regulatory miRNAs). Future functional studies could therefore be more informative if the net effect of several miRNAs is investigated. Targeting or over-expressing several miRNAs could also provide new therapeutic options in the future, especially considering development in small RNA interventions.



There is an ever-pressing need for surrogate markers of complex disease that can facilitate early diagnosis, better stratify patients in subgroups, and that can predict prognosis and treatment outcome. Study IV demonstrates the potential of circulating miRNAs in the CSF to serve this purpose. However, we clearly show that larger cohorts than those commonly used are required to fully evaluate the potential for each identified marker. Furthermore, given the heterogeneity of complex diseases it is likely that a panel of biomarkers is necessary to reflect different disease variations. As several miRNAs can be detected in a single isolation protocol, they could present a great feasibility in a clinical setting. In the case of miR-150, future investigation should test its potential as a biomarker of disease in other body compartments, which are more easily accessible, and its potential to serve as a marker for treatment response.

Ultimately, the goal in MS research is to understand the cause and the underlying disease mechanisms. Through understanding of the genetic factors, epigenetic mediators and modulators and how the environment interacts with these factors we can improve prediction and hopefully prevent and cure disease. The reversibility of epigenetic mechanisms makes them great targets for future development of ‘epigenetic medicine’.

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