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MicroRNA and MessengerRNA Expression Changes during Interferon-beta and Glatiramer Acetate Treatment in Peripheral Blood Cells of Multiple Sclerosis Patients

Dissertation

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Abbreviations

APC = Antigen-presenting cell

APL = Altered peptide ligand

BBB = Blood brain barrier

BP = Biological process

CC = Cellular component

CDF = Chip definition file

CIS = Clinically isolated syndrome

CNS = Central nervous system

CSF = Cerebrospinal fluid

EAE = Experimental autoimmune encephalomyelitis

EDSS = Expanded disability status scale

EDTA = Ethylenediaminetetraacetic acid

FACS = Fluorescence-activated cell sorting

FDR = False discovery rate

GA = Glatiramer acetate

GEO = Gene expression omnibus

GO = Gene Ontology

HSP = Heat shock protein

IFN = Interferon

IL = Interleukin

i.m. = Intramuscular injection

MACS = Magnetic-activated cell sorting

MAID = MA plot-based signal intensity-dependent fold-change criterion

MBP = Myelin basic protein

MF = Molecular function

MHC = Major histocompatibility complex

mRNA = Messenger ribonucleic acid

miRNA = Micro ribonucleic acid

MRI = Magnetic resonance imaging

MS = Multiple sclerosis

ncRNA = Non-coding ribonucleic acid

NGS = Next generation sequencing

NK cell = Natural killer cell

PBMC = Peripheral blood mononuclear cells

PCR = Polymerase chain reaction

pre-miRNA = Precursor-miRNA

pri-miRNA = Primary-miRNA

PWM = Position weight matrix

RIN = RNA integrity number

RISC = RNA-induced silencing complex

RRMS = Relapsing-remitting multiple sclerosis

s.c. = Subcutaneous

SD = Standard deviation

SNP = Single nucleotide polymorphism

TCR = T-cell receptor

TF = Transcription factor

TFBS = Transcription factor binding site

Treg cell = T regulatory cell

1. Introduction

Multiple sclerosis (MS) is an immune-mediated demyelinating disease of the central nervous system (CNS). MS is thought to be a result of the body's immune system crossing the blood brain barrier (BBB) into the CNS where it attacks the myelin sheath, a protective insulation surrounding nerve cells. The demyelination of the axons leads to decreased conduction velocity of action potentials or in the worst case a complete stop of the signal. MS is characterized by inflammatory axonal demyelination of neurons which can be detected as MS lesions which occur predominantly in the white matter region of the brain and spinal cord [1, 2]. These lesions can be analyzed by magnetic resonance imaging (MRI). Formation of new lesions results in physical disabilities and decline in cognitive functions.

The onset of MS disease typically occurs at 20 to 50 years of age [3]. MS affects more than 2.5 million people worldwide. Geographically MS is highly prevalent in northern and southern hemispheres. Gender plays a role in the occurrence of MS, incidence is higher in women compared to men with a ratio of at least 3:1 [4].

There are several factors which could be involved in the pathogenesis of MS. Environmental factors such as infection by Epstein-Barr virus [5] and low levels of vitamin D [6] were proposed to trigger the pathogenesis of MS. Genetics is also considered as a key factor for MS pathogenesis. For example, mutations in the HLA-DRB1 gene are compromising the immune systems response to differentiate between self-proteins and foreign proteins [7]. The genotype HLA-DRB1*15:01 increases the risk of MS more than 3-fold [8]. In another study mutations in the IL7R gene resulted in changes in growth, proliferation and survival of immune cells [9]. In the immunological aspect Th2 cells, regulatory CD4+ T cells and NK cells among others are thought to be responsible for the relapsing-remitting and chronic progressive nature of the disease [10].

Diagnosis of MS is done clinically by neurological examinations and para-clinically by MRI, CSF analysis (spinal tap) and evoked potentials. MS can be diagnosed using McDonald

criteria [11] and the disability is usually scored with the expanded disability status scale (EDSS) [12].

There are four main types of MS [13] which are characterized by the progression of the disease: relapsing-remitting MS (RRMS) is characterized by relapses (attacks) followed by remission (recovery), secondary-progressive MS (SPMS) happens following an initial RRMS period and a phase in which the disability worsens constantly regardless of any new relapse episode taking place or not, primary-progressive MS (PPMS) is characterized by a steady worsening of neurological functions from the beginning of the disease onset and there are no relapses. Before the diagnosis of RRMS there is often a condition called the clinically isolated syndrome (CIS). CIS can be a precursor condition of clinically definite MS. It is caused by inflammation or demyelination of nerve tissue and it is typically the first neurological episode occurring in a person.

There are several disease modifying drugs in the market for the treatment of MS (table 1). First line medications for MS are IFN-beta-1a either in an intramuscular once a week dosing or in a subcutaneous three times a week dosing, IFN-beta-1b s.c. every other day subcutaneously or GA (daily subcutaneously). In SPMS mitoxantrone intravenous infusions are sometimes prescribed. Recently there were new drugs approved for the treatment of MS [14].

Transcriptome profiling is the study of total RNA molecules within a particular biological system. Genetic information is primarily stored in DNA which can be copied by replication during cell division. Genes are segments of DNA which code for a particular product which can be just a transcript (ncRNA) or a transcript that is further translated into a protein (mRNA). There are around 20000 genes in a human genome [15]. RNA is transcribed from a template strand of DNA by the RNA polymerase enzyme and the transcription process is the first step in gene expression. Messenger RNA can be decoded by the ribosome into long amino acid chains called proteins. A transcript may also encode for non-coding RNA such as microRNA (miRNA), ribosomal RNA (rRNA) or transfer RNA (tRNA). Figure 1 shows the central dogma of molecular biology where RNA is transcribed from the DNA and later

translated into a protein. microRNAs are small RNA which are involved in the regulation of genes by mRNA cleavage or by translational repression [16].

<table>
<tr><th>Indication</th><th>CIS</th><th colspan="2">RRMS</th><th colspan="2">SPMS</th></tr>
<tr><td></td><td></td><td colspan="2"></td><td>With relapse</td><td>Without relapse</td></tr>
<tr><td rowspan="2">Escalation therapy</td><td rowspan="2"></td><td>Option 2</td><td>Mitoxantrone
Cyclophosphamide</td><td rowspan="2"></td><td rowspan="2"></td></tr>
<tr><td>Option 1</td><td>Fingolimod
Natalizumab</td></tr>
<tr><td>First-line therapy</td><td>IFN-beta 1a i.m.
IFN-beta 1a s.c.
IFN-beta 1b s.c.
GA</td><td colspan="2">IFN-beta 1a i.m.
IFN-beta 1a s.c.
IFN-beta 1b s.c.
GA</td><td>IFN-beta 1a s.c.
IFN-beta 1b s.c.
Mitoxantrone
Cyclophosphamide</td><td>Mitoxantrone
Cyclophosphamide</td></tr>
<tr><td rowspan="2">Relapse therapy</td><td>Option 2</td><td colspan="4">Plasma separation</td></tr>
<tr><td>Option 1</td><td colspan="4">Methylprednisolone</td></tr>
</table>

Table 1: Recommended multiple sclerosis disease modifying treatments. *IFN-beta or glatiramer acetate (GA) are first-line treatments for relapsing-remitting multiple sclerosis (RRMS) and clinically isolated syndrome (CIS), and fingolimod, natalizumab and mitoxantrone along with cyclophosphamide are used as escalation therapies for RRMS. For secondary-progressive multiple sclerosis (SPMS) mitoxantrone along with cyclophosphamid are used as treatments. (Adapted from the guidelines and recommendations of the Deutsche Gesellschaft für Neurologie. Reference: URL: www.dmsg.de/dokumentearchiv/dgnkknms_msll_20120412_final.pdf)*

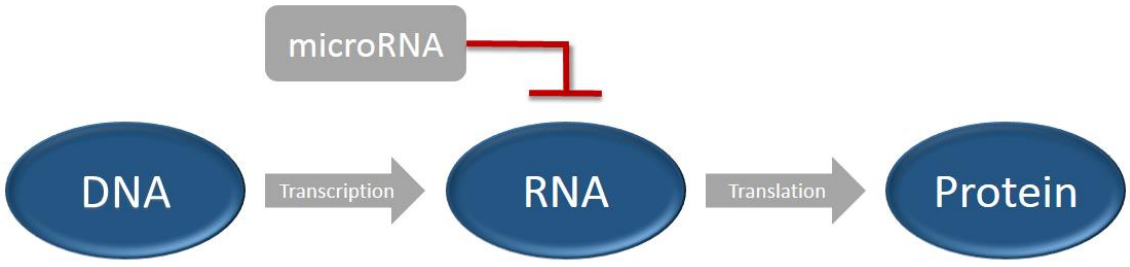

Figure 1: Adapted central dogma of molecular biology. *The sequence information flows from DNA to RNA to protein. DNA is copied as RNA in a process called transcription and RNA is translated into proteins. MicroRNAs are involved in the regulation of gene expression.*

In recent years there were several studies related to the gene expression profiling of various human diseases [17, 18, 19]. These studies were possible because of the development of various high-throughput platforms such as DNA microarrays [20] and TaqMan Array cards [21]. DNA microarrays allow to simultaneously measure the genome-wide changes of thousands of genes. In the same way TaqMan Array cards are used to quantify hundreds of miRNAs and mRNAs based on the more sensitive polymerase chain reaction (PCR) a method to analyze a short sequence of DNA or RNA quantitatively. In the studies reported in this dissertation both of these platforms were used to study the gene expression profile of MS patients.

IFN-beta and GA treatments bring down the number of relapses in MS but their mechanism of action and the effects of the treatments at the molecular level are yet to be fully understood. The previously known effects of IFN-beta and GA therapy on the immune system are explained in section 1.1 and 1.2. The studies reported in this thesis revealed various changes which happen at the gene expression level of monocytes and peripheral blood mononuclear cells (PBMC) from patient blood during GA or IFN-beta-1b s.c. treatment. MS patients who were about to start GA or IFN-beta-1b s.c. therapy were recruited and blood samples were collected at various time points in a longitudinal fashion. Monocytes or PBMC were isolated and transcripts (mRNA and miRNA) were isolated. Using DNA microarrays and real-time PCR the transcripts were analyzed quantitatively. The differentially expressed genes identified were investigated for their

functions and gene interaction networks were conceived. Figure 2 gives an overview of the studies reported in this thesis.

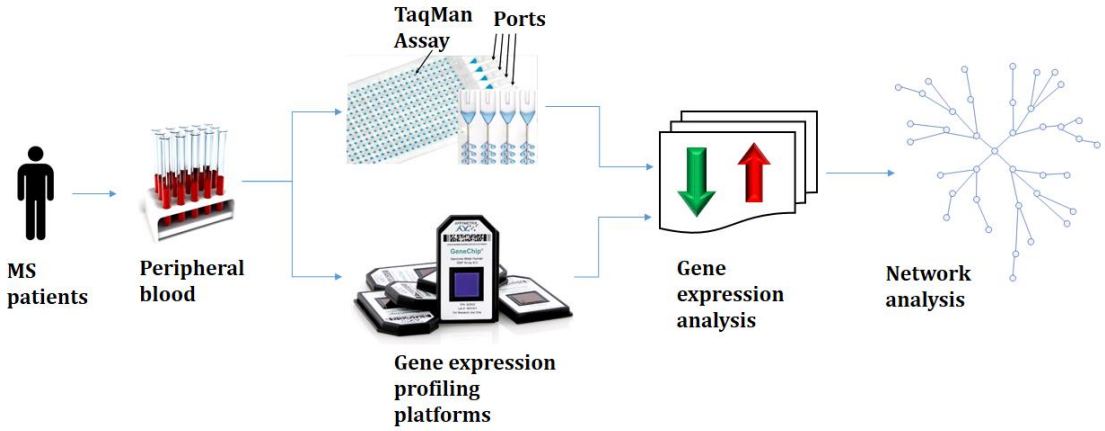

Figure 2: Study design. *Overview of the study design which is common to the GA and the IFN-beta-1b study. MS patients who were about to start a disease modifying therapy with GA or IFN-beta-1b were studied. Blood samples were collected and using different gene expression profiling platforms such as TaqMan real-time PCR and Affymetrix GeneChips the expression profiles of mRNAs and miRNAs were acquired. The data were analyzed comparing the expression before and during therapy. The differentially expressed genes were filtered and used in the construction of gene interaction networks.*

1.1 Glatiramer acetate

Glatiramer acetate (Copaxone® or Copolymer 1) is a peptide drug used in the first-line treatment of MS. There are four different amino acids which are the building blocks of GA. They are glutamic acid, lysine, alanine and tyrosine (G-L-A-T) in a molar ratio of 1.5:3.6:4.6:1.0. These amino acids are synthesized into random peptide sequences which are 40-100 residues long [22]. The peptides in GA were initially intended to trigger experimental autoimmune encephalomyelitis (EAE) by imitating myelin basic protein (MBP) but instead GA inhibited EAE [23]. EAE is an animal model for brain inflammation and it is also used to study T-cell mediated autoimmune diseases [24]. GA has become a regular therapy for MS patients due to its beneficial clinical effects and its safety profile

[25]. Despite many studies trying to understand the mechanisms of action of GA the molecular mechanism still remains unclear.

GA is believed to act as an altered peptide ligand (APL). An APL is a peptide variant which possesses properties similar to a particular antigen but when bound to T-cell receptors (TCR) it provokes a different response. During GA therapy, the partial activation of T-cells specific for MBP and other myelin antigens can distract the T-cell response, and this may contribute to the clinical effects of GA [26]. There are many studies which have shown that GA can bring a shift in T-helper cells by changing the cell population from Th1 to Th2 cells and this shift towards GA-reactive Th2 may be a main mechanism of action [27]. Th2 cells are anti-inflammatory in nature and they are capable of migrating across the blood brain barrier where they suppress inflammatory factors. This is called local bystander suppression. This process is thought to actively damper the activation of auto-reactive immune cells [28, 29]. Despite our understanding the precise mechanism of action of GA remains unclear.

The influence of GA on antigen presenting cells (APC) has been studied as well. APC, such as dendritic cells and macrophages, differentiate from monocytes [30]. These monocytes are believed to be a key population playing a role in the immune-modulatory effects of GA therapy [31]. One of the most rational mechanism of action of GA is its ability to bind to major histocompatibility complex (MHC) molecules and this means that it competes with myelin antigens in order to be presented on APC. GA can act as an antagonist of MBP/MHC at MBP-specific T cell receptors [32]. Weber *et al.* showed that monocytes when treated with GA switch from a pro-inflammatory to an anti-inflammatory type, and this change is characterized by the down-regulation of pro-inflammatory cytokines (e.g. IL-12) and the up-regulation of anti-inflammatory cytokines (e.g. IL-10) [26, 33]. Interestingly Monocytes produce 5- to 10-times more IL-12 and IL-18, in progressive multiple sclerosis [34]. In various monocyte studies the GA treatment resulted in an increase in anti-inflammatory type II monocytes. Type II monocytes are also characterized by increased secretion of transforming growth factor (TGF)-beta, and decreased

production of tumor necrosis factor (TNF). The suppressor functions of these monocytes were shown to contribute to Th2 deviation of naive T-cells in the CNS of MS patients [26, 33]. It was also shown that GA can change the expression of monocytes by increasing the expression of IL1RA while decreasing the production of IL-1β [35]. Changes in monocytes can be due to direct effects of GA or can be indirectly facilitated by cytokines produced by GA-specific Th2 cells as there may be active feedback loops connecting human T-cell and APC responses [26, 27].

There are more studies needed to understand the mechanism of action of GA. There are not many studies in the past which explored the effect of GA on the gene expression of monocytes. The *in vivo* gene regulatory effects of GA on monocytes have so far not been examined in a genome-wide and longitudinal manner.

1.2 Interferon-beta

Interferons belong to a family of cytokines produced in the human body during the innate immune response against viral infections (pathogens) [36]. Interferon-beta is effective in the suppression of the symptoms of MS [15, 37]. Injectable recombinant IFN-beta is used as first-line treatment for RRMS, CIS and SPMS with relapses. IFN-beta treatment has been shown to be effective in bringing down the number of relapses and to reduce the formation of new inflammatory lesions in the brain. There are different types of IFN-beta therapies in the market. They differ in dosage, mode and frequency of administration, but they are very similar in therapy effectiveness [38, 39].

The exact mechanism of action of interferon-beta proteins is still not fully understood. One proposed mechanism of action is that IFN-beta therapy decreases the expression of major histocompatibility complex (MHC) class II molecules on APC [40] as well as co-stimulatory molecules such as CD80 and CD28 on lymphocytes and APC which directly affects the activation of T-cells by decreasing their auto-reactive behavior thus inhibiting T-cell proliferation. Interferon-beta also changes the delicate balance of cytokines. IFN-beta treatment suppresses the pro-inflammatory Th1 and Th17 cells and their expression of IL-2, IL-12, IL-17 and interferon-γ while promoting the production of anti-inflammatory

Th2 cytokines (IL-10 and TGF-β). Interferon-beta can inhibit the expression of the adhesion molecule VLA-4 on T-cells and the release of matrix-metallo-proteinases (MMP) by T-cells which stops the migration of activated immune cells crossing the BBB from the blood stream [41].

IFN-beta has been shown to clearly affect the gene expression of various blood cells [41, 42, 43]. More than a hundred genes have been consistently found to be differentially expressed during treatment [44]. The transcript levels of most of these genes are up-regulated within a few hours after IFN-beta injection, and they return to pre-treatment or normal levels after a few days [45]. The IFN-beta-responsive genes are involved in the beneficial effects of the treatment and they express it by immunomodulatory, anti-proliferative and anti-pathogenic processes. However, there was so far no study that investigated the expression of miRNAs in the course of IFN-beta-1b s.c. therapy. As a part of this dissertation microarray technology was used to analyze the gene expression dynamics in the peripheral blood of MS patients in response to IFN-beta therapy. In this study the expression profiles of mRNAs and miRNAs were for the first time simultaneously measured. It is important to study the transcriptome profile before and during the treatment in order to better understand the delicate changes brought by the treatment. The study presented in manuscript 2 focused on differentially expressed miRNAs and their involvement in the mechanism of action of IFN-beta-1b s.c therapy.

1.3 MicroRNA in multiple sclerosis

MicroRNA are small non-coding RNA molecules about 22 nucleotides in length. Their main role is to control the expression of genes by translational repression or by target mRNA cleavage. A single miRNA can regulate hundreds of miRNA. The miRNA regulation of genes depends on sequence specificity [46]. The biogenesis of a miRNA starts with a RNA polymerase II enzyme that transcribes the primary RNA (pri-miRNA) from the miRNA genes in the genome. The pri-miRNA is then matured by the enzymatic activity of ribonuclease III (RNase III) which results in about 70 nucleotides long precursor miRNAs (pre-miRNA). Pre-miRNAs are then exported into the cytoplasm and by the action of the

enzyme Dicer and other protein complexes the pre-miRNA is further shortened to about 22 nucleotides which when loaded to an RNA-induced silencing complex (RISC) become active. The complex then binds to the 3' untranslated region of a target mRNA. In consequence, the target mRNA is either cleaved directly by the RISC or translationally repressed.

So far there are only relatively few miRNA-mRNA interactions which have been experimentally validated. It is thus necessary to computationally predict putative target genes for miRNAs [47, 48, 49]. miRNAs contain a seed region located at the 5' end of the strand. Mostly the 2nd to 8th nucleotides are called the seed region and this is the region which recognizes the target sequence. In most of the prediction algorithms the seed region forms the core idea along with the thermodynamics of the microRNA-mRNA interaction.

miRNAs are often found in clusters in the genome, as they are often found in close vicinity [50]. There are about 2500 unique mature microRNAs (miRBase 20 release) reported for humans. There are several miRNA target gene prediction databases available. The miRWalk database [51] allows to compare the predictions of 10 different algorithms. Experimentally verified interactions from the literature can be found in other databases such as miRTarBase [52] .The miR2Disease database helps in finding out miRNAs which are involved in MS and other diseases (http://www.mir2disease.org/). In the recent years there has been a tremendous development in miRNA databases and miRNA expression profiling platforms [53].

MicroRNA in MS and EAE is a relatively new field, a more complete picture is presented in manuscript 3 and manuscript 4. However it is interesting to note that there were only two small studies so far which reported about MS therapy effects on miRNA expression. A study by Waschbisch *et al.* [54] on 5 selected mature miRNAs found that GA treatment normalizes dysregulated miRNA expression in RRMS and in another study by Sievers *et al.* [55], differentially expressed miRNAs in B cells of patients treated with natalizumab were found. In the IFN-beta study reported in this thesis (manuscript 2) TaqMan microfluidic

cards as well as miRNA microarrays were used in the analysis. The microRNAs which were found to be differentially expressed upon IFN-beta treatment were further analyzed for target interaction networks.

2. Aims and objectives

The studies reported in this thesis are inter-related and they focus on a common goal to find out how first-line disease-modifying therapies prescribed for MS influence the blood transcriptome (*in vivo*). GA and IFN-beta were the two drug candidates analyzed. The expression profiles of mRNAs and miRNAs were measured. As gene expression profiling platforms, microarrays and TaqMan microfluidic cards were used. The data obtained from these experiments were analyzed for differentially expressed genes and miRNAs during therapy. The studies were longitudinal in design and had different time points depending on the therapy GA or IFN-beta. The mRNA and the miRNA expression data were acquired and the up- and down-regulation in relation to pre-treatment levels was analyzed. The up- and down-regulated mRNAs and miRNAs were then used in the construction of gene interaction networks. Three different interaction networks were constructed. The purpose of these studies was to better understand the mechanism of action of GA and IFN-beta-1b s.c. and to find biological markers of therapy response.

Study 1: Glatiramer acetate treatment effects on gene expression in monocytes of multiple sclerosis patients.

Background: This study is the first genome-wide RNA profiling of monocytes under GA therapy. Monocytes are part of the innate immune system. Changes in the gene expression of monocytes could directly affect the pathophysiology of MS. Monocytes are known to shift their cytokine profiles from pro-inflammatory to anti-inflammatory when treated with GA. Monocytes secrete less TNF-alpha and IL-12 but more IL-10 and TGF-β on GA treatment. This longitudinal study was designed to investigate the differentially expressed genes within the first 2 months into GA treatment in monocytes from the blood of 8 RRMS patients from the Department of Neurology, University of Rostock.

Aim: To find out the effects of GA treatment on monocytes at the level of transcriptome. The aim was to find out the role of monocytes in the therapeutic outcome of GA and to

better understand its mechanism of action and to validate previous findings of other research groups on specific genes.

Study 2: MicroRNA expression changes during interferon-beta treatment in the peripheral blood of multiple sclerosis patients.

Background: This is the first study to report on miRNA expression during IFN-beta-1b s.c. therapy. Injections of recombinant IFN-beta-1b s.c. are a first-line option in the treatment of RRMS. IFN-beta-1b s.c. has been shown to reduce the number of relapses and to suppress the accumulation of new inflammatory lesions in the brain. There were several studies in the past which showed that there is a clear modulation of IFN-regulated genes during treatment, but there were no studies which analyzed miRNA-mRNA interactions in the presence of IFN-beta-1b s.c. treatment in MS patients.

Aim: To identify microRNAs and mRNAs which are differentially expressed in blood when MS patients are treated with IFN-beta-1b s.c. and to find out the most relevant miRNA-mRNA interactions and their involvement in the pathophysiology of MS.

Study 3: Integration of microRNA databases to study microRNAs associated with multiple sclerosis.

Background: MicroRNAs are small molecules which are known to have a broad influence in the regulation of gene expression. In many diseases they have been discussed as potential biomarker candidates. For the majority of microRNAs the target genes are yet to be validated but there are databases which predict target sequences. This study integrates different databases to find out the MS-relevant miRNA targets.

Aim: The aim was to establish a work-flow that integrates the information of different databases and to find out miRNA-mRNA target interactions using online target prediction databases. MS-associated miRNAs were used in the analysis to find out their molecular interaction network.

Study 4: MicroRNAs in multiple sclerosis and experimental autoimmune encephalomyelitis.

Background: MicroRNA's role in the MS disease is an emerging field. MicroRNAs are known to play a central role in the pathogenesis of the disease. This is one of the earliest articles which reviewed the interesting connection between miRNAs and MS.

Aim: To find out MS-relevant miRNAs which were reported in the literature and review their involvement in MS and EAE. To reflect the current state of the art in the field of miRNA in MS. To review the findings of studies using blood cells or brain tissues to find differentially expressed miRNAs in MS.

3. Materials and methods

3.1 Study population and blood sample collection

Two studies dealing with MS patients were performed: a study on IFN-beta and a study on GA. Eight patients diagnosed with RRMS were recruited for the GA study (manuscript 1) and six patients (CIS *n*=2 and RRMS *n*=4) were recruited for the IFN-beta study reported in manuscript 2. These patients were diagnosed according to the 2010 revised McDonald criteria [11].

For the GA study, patients started treatment with GA in 20 mg doses given daily as subcutaneous injection. For the IFN-beta-1b s.c. study, the patients started with the Betaferon® titration pack, and gradually reached a full dose of 250 µg. Blood samples were used in these studies as these drugs are known to mainly target immune related cells. Blood samples for GA study were collected for each patient at 5 different time points: before the first injection of GA (baseline) and after 1 day, 1 week, 1 month and 2 months. Blood was collected by venipuncture with ethylenediaminetetraacetic acid (EDTA). In the IFN-beta-1b s.c. study, 4 different time points were investigated: baseline, after two days, after four days and after 1 month. The rationale behind these time points was that they give a snapshot on the initial changes which occur at the start (early phase) of the treatment. Clinical follow-up data containing EDSS scores and the number of relapses were collected and recorded. The studies were approved by the ethics committee of the University of Rostock and performed according to the Declaration of Helsinki. All patients gave written informed consent to be included in the study.

3.2 Cell isolation, RNA and miRNA preparation and gene expression profiling

For the GA study, monocytes were isolated from the blood samples using erythrocyte lysis buffer (Qiagen), followed by magnetic-activated cell sorting (MACS). For the IFN-beta-1b s.c. study, PBMC were isolated using the Ficoll gradient method. In the IFN-beta-1b s.c. study, total RNA enriched with small RNA was isolated using the mirVana miRNA isolation

kit (life technologies, USA). RNA from monocytes was isolated using RNeasy columns and the RNA quality and quantity was measured. In both studies, the microarray experiments for mRNA quantification were performed with an Affymetrix platform. HG-U133 Plus 2.0 GeneChips were used to quantify the expression of about 19,000 genes. The microRNA expression was quantified using TaqMan Array Human MicroRNA cards. These cards are based on real time PCR. They can quantify the expression of 651 mature microRNAs. For validation of the miRNA data, samples of *n*=3 MS patients at two time points (baseline and after one month) which resulted in six samples were used. The RNA of these 6 samples was labeled and hybridized onto Affymetrix GeneChip miRNA 2.0 arrays. To verify the results in an independent cohort of 12 MS patients, microRNAs were also measured using TaqMan single-tube assays. The *hsa-miR-191-5p* housekeeping miRNA was used for normalization.

3.3 Data analysis and databases

The microarray data pre-processing and quality control was done with the Affymetrix GeneChip operating software and MAS5.0 statistical algorithms (Microarray Analysis Suite 5.0). Since the design of the microarrays the annotation of genes has changed. There are probes on the microarrays which detect no transcript and probes which correspond to multiple transcripts. To remove such probes from the analyses a custom chip definition file (CDF) was used, which was based on the GeneAnnot database [56]. Every probe set in the custom CDF matches a single gene. Normalization of the data was performed by a loess fit to the data using the R package “affy”.

Two analyses were performed to filter genes which are differentially expressed in the data: I) paired *t*-tests comparing the expression at baseline with the expression at the time points during therapy were calculated. II) In the second analysis the data was analyzed with the MAID filtering method, which calculates MA plot-based signal intensity-dependent fold-changes (MAID-scores) for each time point comparison [57]. To find out significantly up-regulated or down-regulated genes compared to baseline, both MAID-score and paired *t*-test outputs were combined.

In the miRNA interaction study described in manuscript 3 (chapter 8.3), MS-relevant miRNAs were collected from the miR2Disease database [58]. miRBase [59], the central database for pre-miRNAs and mature microRNAs, was used to collect sequence information about the miRNAs and also to find out the genomic location of the miRNAs. The target mRNAs were predicted using the miRWalk database [51]. miRWalk database was used for the study because the database has its own target prediction algorithm along with nine other algorithms from other target prediction databases. miRTarBase was used to acquire experimentally validated miRNA target interactions [52]. miRNA regulating transcription factors were acquired from the miRGen database [60]. smiRNAdb is a database containing expression profile information of miRNAs in various cell types and tissue samples [61].

3.4 Gene Ontology analysis

A Gene Ontology (GO) term enrichment analysis was performed to analyze the function of the genes filtered in the GA study. The analysis was based on the association of functional annotations for each gene in the GO database [62]. The R package GOstats was used to test GO terms for overrepresentation. All the genes which were measured with the HG-U133 Plus 2.0 microarray were used in the analysis as ‘gene universe’.

3.5 Molecular network analysis

The Pathway Studio software version 7.1 from Ariadne Genomics was used in the network construction presented in manuscript 1. The gene interactions were automatically obtained from the literature by text mining. The interactions were then exported and fed into Cytoscape, which is an open source software for visualization [63]. The resulting interactions were represented in various shapes to indicate the regulatory effects such as positive, inhibitory and binding interactions.

To construct regulatory networks between miRNAs and genes (manuscript 2 and manuscript 3), the miRNAs were subjected to interaction analysis using various miRNA databases. Computational target predictions were found using miRWalk database [51]

and for experimentally validated miRNA targets miRTarBase was used. miRWalk database integrates 10 different prediction algorithms. Only interactions which were predicted consistently by several of the algorithms were further used. This helped to reduce the high number of putative and maybe false positive targets. These interactions were visualized again using the Cytoscape software.

4. Results

This thesis consist of two main studies and two reviews which focused on expression profiles of genes and miRNAs, and their interactions in MS and during therapy. One major objective was to find out the effects of two disease modifying drugs: I) GA therapy and how does it affect monocytes at the gene expression level, and II) IFN-beta-1b therapy and how it affects PBMC at the miRNA and mRNA level. The gene expression data of monocytes and PBMC were acquired before and during the treatment of GA or IFN-beta-1b. Analyses of the expression data helped to better understand the mechanisms of action of GA and IFN-beta-1b. The studies were longitudinal and recorded the individual gene expression changes across time. The results reported in this synopsis can be summed up into three categories: filtering of genes, visualization of mRNA/miRNA dynamics and interaction network analysis.

4.1 Differential expressed genes and microRNAs

In the GA study (n=8 RRMS patients) (manuscript 1), there were five different blood sampling time points. The monocyte transcript levels were always compared with the pre-treatment levels (baseline). The Affymetrix microarrays measured 18.862 different genes. When the baseline levels were compared with those at day one, 438 genes were filtered with the MAID analysis criterion (|MAID-score|>2) and 1124 genes were filtered with the paired *t*-test criterion (*p*-value<0.05). In the combined analysis 171 genes were filtered of which 101 genes were up-regulated and 70 genes were down-regulated. Altogether four time points were compared with the pre-treatment levels using *t*-test and MAID-scores resulting in different gene lists and when aggregated there were a total of 463 different genes identified to be differentially expressed during the first 2 months of GA treatment (figure 3).

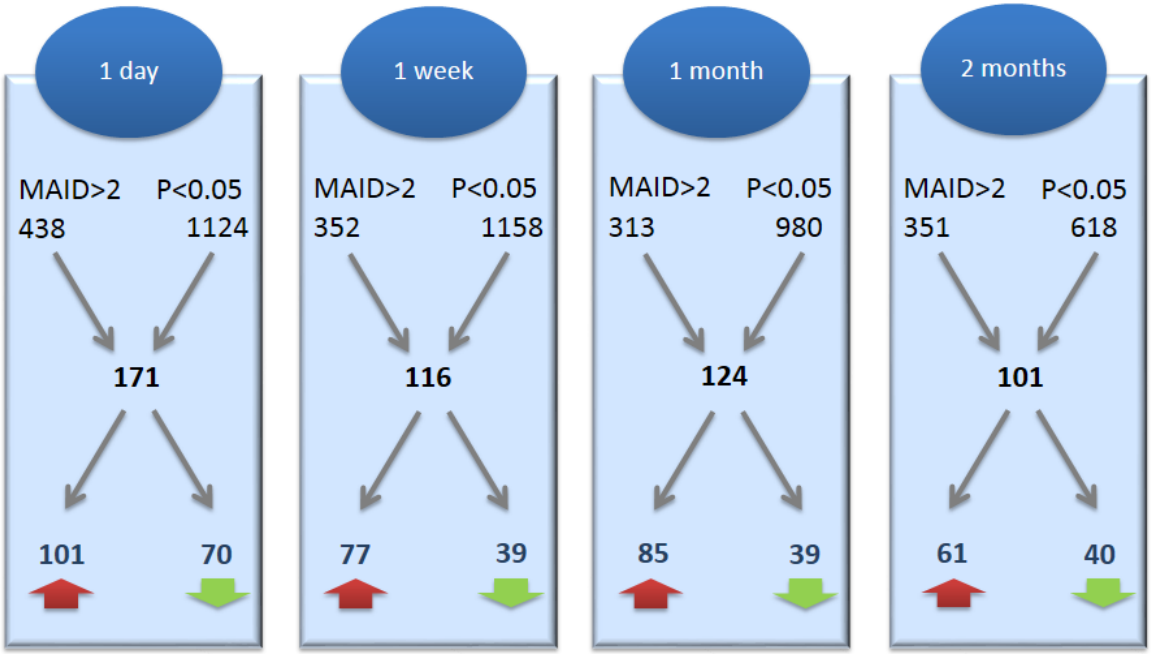

Figure 3: GA therapy - Filtering of genes. *Using Affymetrix microarrays the transcript levels of 18.862 genes were analyzed in monocytes. GA was prescribed 20 mg once daily at study onset. Five different time points were analyzed, before the start of GA treatment (baseline) and after one day, one week, one month and two months of GA treatment. t-test p-values and MAID-scores were used to determine differentially expressed genes. The expression levels at baseline were compared with the expression levels during GA therapy. The number of up-regulated genes is indicated by the red arrow and the number of down-regulated genes is indicated by the green arrow. Adapted from manuscript 1 [64].*

In figure 4 represents the mean mRNA expression dynamics of 23 genes from the GA study. From the 463 genes filtered in total by the previous analysis, these 23 genes were found to be differentially expressed in at least two consecutive time point comparisons. Out of the 23 genes there were 18 genes which were up-regulated and 5 genes which were down-regulated.

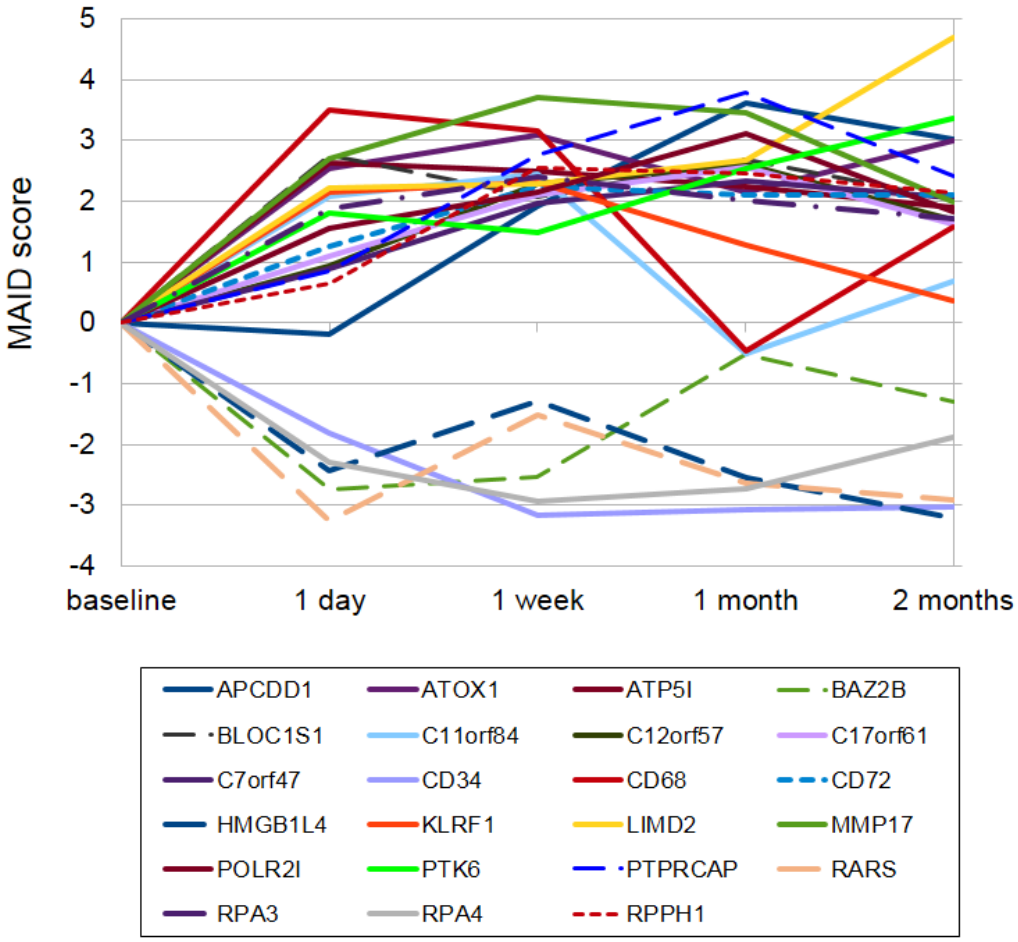

Figure 4: mRNA dynamics of selected differentially expressed genes during GA therapy. *Out of the 463 filtered genes these 23 genes were differentially expressed at least in two consecutive time point comparisons. There are 18 up-regulated and 5 down-regulated genes. The gene expression dynamics are shown in relation to the time points. The MAID-score, a fold change variant that is calculated from the data of all patients in the GA study, is represented on the y-axis and the time points are represented on the x-axis. Adapted from manuscript 1 [64].*

In the IFN-beta-1b s.c. study the PBMC gene expression levels during IFN-beta-1b s.c. treatment were compared with baseline. In figure 4A the numbers of differentially expressed mRNAs are presented in the table along with a bar plot. In figure 4B the numbers of differentially expressed miRNAs are presented along with a bar plot. The row "Total" gives the union set over all the three time point comparisons. In total, there were 95 genes found to be differentially expressed in response to IFN-beta-1b s.c. treatment.

Out of them *n*=75 genes were found to be up-regulated. The strongest changes in the expression of genes were observed at one month versus baseline. The filtering further identified more down-regulated miRNAs (*n*=13) than up-regulated miRNAs (*n*=7) during the IFN-beta- treatment. The maximum changes in miRNA expression occurred after one month of therapy (figure 5).

A mRNA filtering results

	Up	Down	Total
2 days	10	4	14
4 days	22	12	34
1 month	60	6	66
Total	75	20	95

B miRNA filtering results

	Up	Down	Total
2 days	1	1	2
4 days	0	6	6
1 month	6	8	14
Total	7	13	20

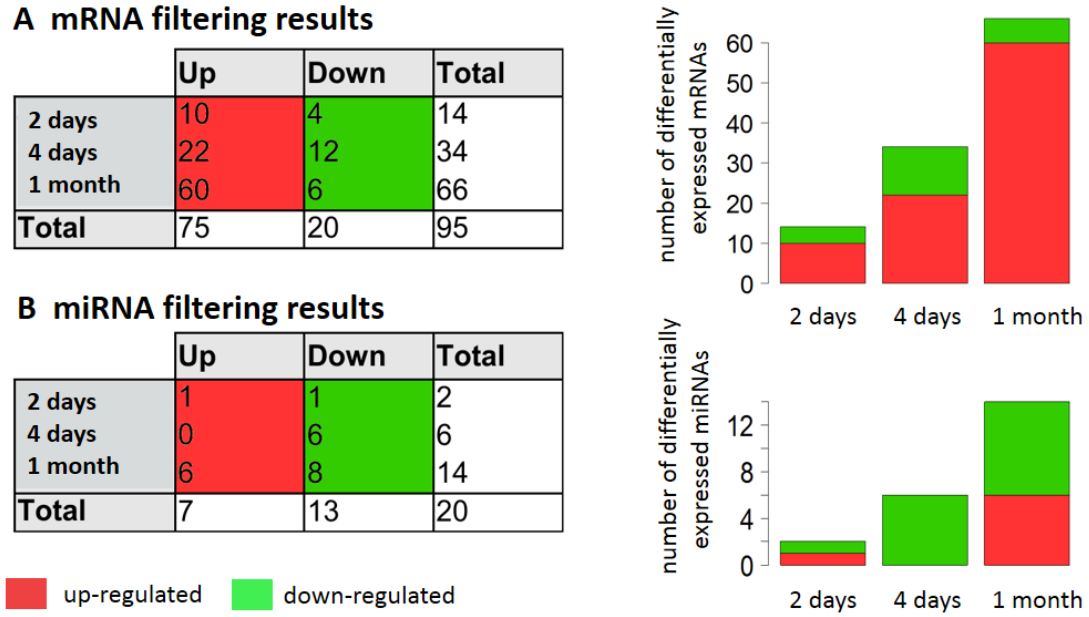

Figure 5: IFN-beta-1b s.c. therapy - miRNA and mRNA filtering. *PBMC samples were used for the analysis. The IFN-beta-1b treatment effects on PBMC were analyzed. (A) & (B) show the number of IFN-beta-1b responsive genes and miRNAs, respectively. (A) Shows the number of mRNAs which were found to be differentially expressed across the time points. In (B) the number of miRNAs which were differentially expressed in relation to the time points are given. There were more mRNAs which were up-regulated in response to IFN-beta-1b treatment in contrast to a relatively higher number of miRNAs which were found to be down-regulated. Adapted from manuscript 2 [65].*

In the IFN-beta-1b s.c. study the miRNAs expression profiles were analyzed and the most significant finding is presented in figure 6. The expression dynamics of *hsa-miR-29c-3p* in the first month of the IFN-beta treatment are visualized. The down-regulation of *hsa-miR-*

29c-3p is consistent across three different analysis. Initially six patients were analyzed with TaqMan miRNA cards, and this revealed the down-regulation of this miRNA in PBMC. Samples of three patients out of the 6 patients were re-analyzed using Affymetrix miRNAs arrays and the results were consistent with the findings from the TaqMan miRNA cards. In the last analysis the down-regulation of *hsa-miR-29c-3p* was confirmed in an independent validation cohort of 12 patients and their PBMC samples were analyzed using TaqMan single-tube assays.

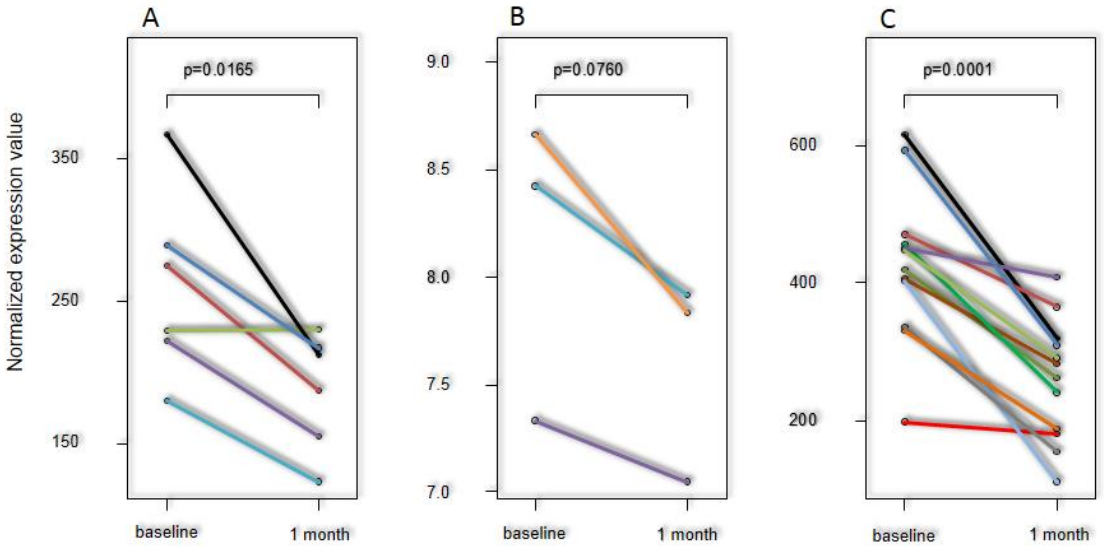

Figure 6: hsa-miR-29c-3p expression dynamics during IFN-beta-1b s.c. therapy. *Down-regulation of hsa-miR-29c-3p in PBMC in response to IFN-beta-1b s.c. treatment is shown across three different techniques (A - TaqMan miRNA cards, B - Affymetrix miRNA arrays and C - TaqMan single tube assays). The Affymetrix data are in log2 scale and the TaqMan data are in linear scale due to a different data pre-processing. P=paired t-test p-values are given in each graph. Each colored line represents a patient. Adapted from manuscript 2 [65].*

4.2 Gene interaction network analysis

The shortlisted genes from the GA study were used as input for the network analysis. The network was constructed using Pathway Studio software [66]. The software constructs the network based on text-mining information. The output resulted in 41 genes with 59 interactions. The interactions between the genes were visualized as a network. The interactions consisted of 43 positive regulatory interactions, 14 inhibitory interactions and 2 binding interactions (figure 7).

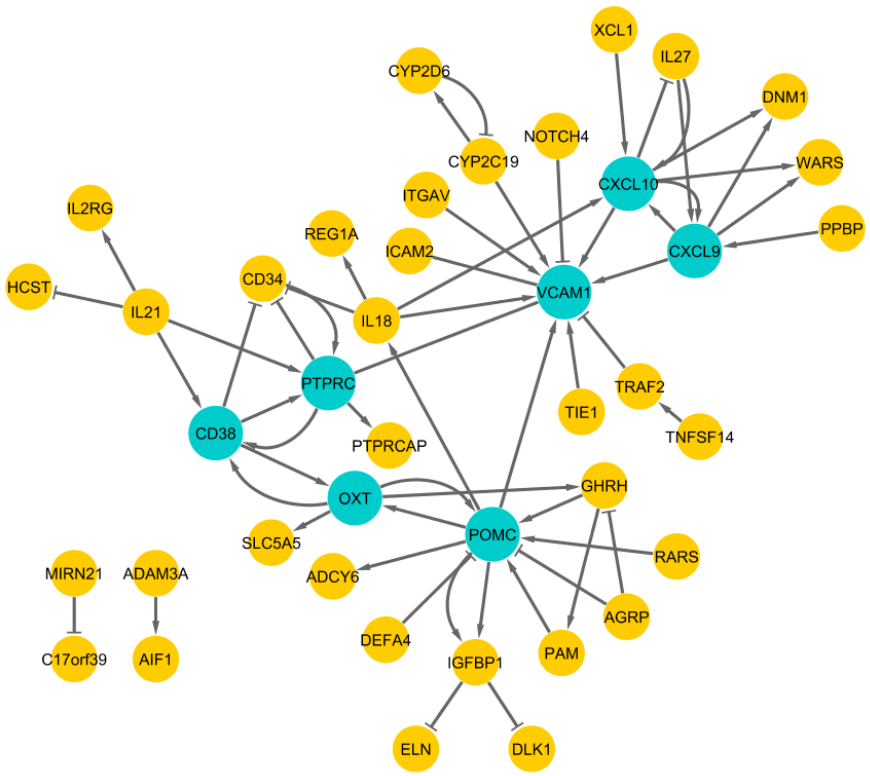

Figure 7: Interaction network of differentially expressed genes during GA therapy. *The differentially expressed genes (n=463) which were filtered in 4 time point comparisons were used as input for the Pathway Studio software. The software found 41 genes (=nodes) which had 59 interactions (=edges). The other genes had no interaction with the other differentially expressed genes. Different types of interactions (=edges) are shown differently. There are seven nodes, indicated blue in color, which are involved in the regulation of many other genes in the network. → = positive effect (n = 43), ⊣ = negative effect (n = 14), – = binding (n = 2). Adapted from manuscript 1 [64].*

The manuscript 3 focused on miRNAs that were found to be dysregulated in the blood of MS patients in four studies [67, 68, 69, 70]. The molecular interaction network was constructed using MS-associated miRNA, target genes and transcription factors (TF) acquired from various databases. TF-miRNA interactions were obtained from the miRGen 2.0 database [60]. Validated and predicted miRNA-mRNA interactions were obtained from miRTarBase [52] and miRWalk [51] databases. In the network *hsa-miR-20a-5p* and *hsa-miR-20b-5p* are very closely related to each other and were predicted to regulate many common target genes. They appear close to *hsa-miR-17-5p*, whose targets were obtained from miRTarBase only and which belongs to the same miRNA cluster as *hsa-miR-20a-5p*. The network revealed several feedback loops in the network, e.g. SOX9 was seen both as transcriptional regulator and as a target gene of *hsa-miR-145-5p* (figure 8).

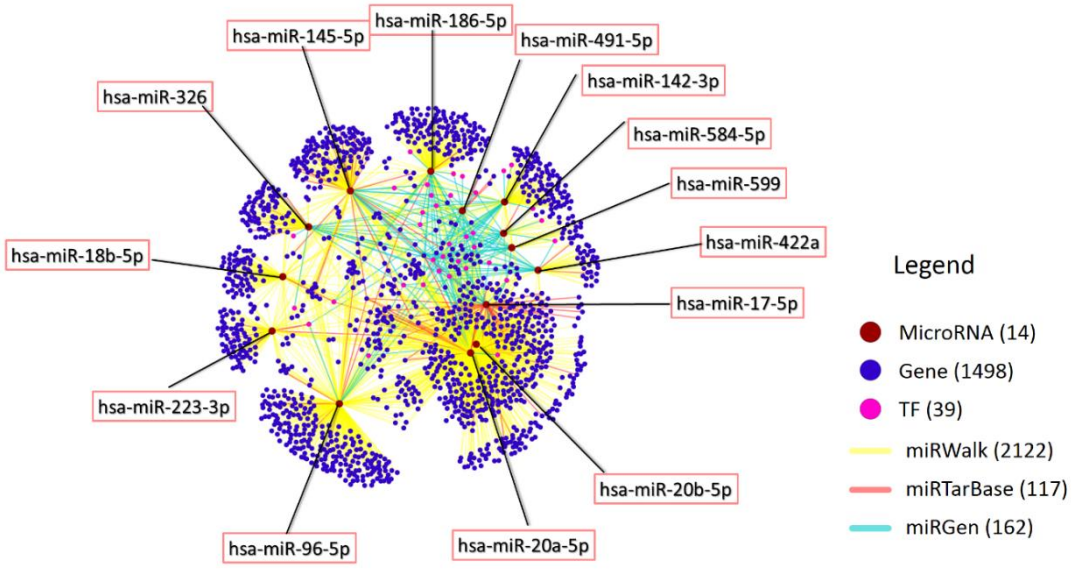

Figure 8: MS associated miRNA, target gene and transcription factor interaction network. *Cytoscape interaction network of MS-associated miRNAs (n=14) and their target genes (n=1498) and regulating TFs (n=39). TF – miRNA interactions were retrieved from miRGen 2.0 database. Validated and predicted mRNA – miRNA interactions were found using miRTarBase and miRWalk databases. The network visualizes the complexity of molecular interactions associated with miRNAs that were described in the literature to be higher or lower expressed in the blood of patients with MS. Adapted from manuscript 3 [71].*

5. Discussion

MS is a chronic inflammatory autoimmune disease of the CNS. The studies reported in this thesis focused on the transcriptome profiling of MS patients' blood and the gene expression changes during therapy. High-throughput profiling platforms such as Affymetrix microarrays and very sensitive methods such as TaqMan real-time PCR were used in these studies. The transcriptome analysis comprised mRNA expression and miRNA expression. The GA study and the IFN-beta-1b study were longitudinal consisting of different time points at which the blood samples were collected. In the GA study monocytes isolated from the blood samples were analyzed and in the IFN-beta-1b study PBMC samples were used. Differentially expressed mRNAs and miRNAs were filtered as up- or down-regulated. They were then used in the construction of molecular interaction networks.

MS is a complex disease and still there is no cure for MS [72]. There are several disease modifying drugs (table 1) used in the treatment of MS. Diagnosis of MS is not always straightforward as it is generally an elimination process. Diagnosis of MS is time consuming and other CNS disorders must be ruled out. The studies reported in this thesis focused on understanding more about the underlying changes within the immune cells as a result of the treatment with GA or IFN-beta-1b therapy. The mechanism of action of both of the drugs is not fully understood. In the studies monocytes and PBMC populations were analyzed longitudinally before and during GA or IFN-beta-1b treatment.

In the GA study, 463 genes were filtered as differentially expressed across the five different time points (before GA therapy as well as one day, one week, one month and two months under GA treatment). Gene Ontology (GO) analysis showed the overrepresented GO terms and they included 'lymphocyte proliferation' and 'regulation of T-cell proliferation', explained in detail in manuscript 1. The GO findings are consistent with earlier studies that showed that GA lowers the lymphocyte proliferation by modulation of monocytes and monocyte-derived dendritic cells, hence reducing the number of auto-reactive T-cells [73, 74, 75].

The GA study reported here was compared with the study by Achiron *et al.* which was another microarray study analyzing the gene expression in PBMC during GA treatment [76]. In the Achiron *et al.* study only two time points were compared, before and after 3 months of GA treatment [76]. In this thesis the GA treatment effects were studied at five time points and this helps to understand the variation in mRNA dynamics in a higher resolution which was not previously possible. Using Affymetrix microarrays, Achiron *et al.* found 480 genes to be differentially expressed in response to GA treatment in PBMC. When comparing the gene lists of both of the studies there were only five genes (BAT1, ELOVL5, ETV7, MT1E and PCBD1) in common. Therefore the GA study could not validate the results by Achiron *et al.* as there are very few gene overlapping between these two independent studies. An explanation can be that GA possibly acts selectively on other subsets of circulating cells. A possible hypothesis could be that GA might mainly alter the functional properties of (auto-reactive) T-cells, and this could be the reason behind a slightly stronger influence on the gene expression of PBMC than of monocytes from GA-treated patients.

In another study by Weber *et al.,* it was shown that monocytes when treated with GA shift to anti-inflammatory type II monocytes [33]. They have shown in a mouse model that there is an increased secretion of IL-10 and TGF-beta and decreased levels of IL-12 and TNF-alpha. In the study described in manuscript 1, there were few cytokines like CXCL9 and CXCL10, which showed increased expression the day after the start of GA therapy. CXCL10 was also previously shown to be induced in PBMC when treated with GA [77]. However, the cytokines IL-10 and IL-12 were stable in expression and did not change upon treatment *in vivo*, thus not supporting a significant shift in the cytokine profile of monocytes. The GA study could not find a stable pattern of differentially expressed genes which could support the notion of an increase of type II monocytes in the 2 months long study.

It is quite clear that GA treatment could influence the gene expression profile in monocytes but the transcriptome changes were not consistent across the time points.

Out of the 463 genes there were only 23 genes which were differentially expressed in at least two consecutive time point comparisons, but nevertheless these 23 genes can be regarded as good candidates for molecular markers of GA activity. These candidate genes need further studies to understand their role in the mechanism of GA. When looking at the overall effect of GA on the gene expression profile it is clear that the drug has only moderate influences on monocytes.

GA's influence on the gene expression level is not yet clearly understood. There were very few studies in the past which have reported on differentially expressed genes during GA therapy. In the GA study a clear snapshot of the gene expression dynamics across five different time points before and after the start of GA therapy was generated. The study was able to report moderate changes in expression when compared with the pre-treatment levels. The gene expression was altered but there was no gene consistently modulated across all the time points. However the 23 genes which were differentially expressed in at least two time point comparisons are potential molecular marker candidates which need to be studied in more detail.

In the IFN-beta-1b s.c. study, which was a longitudinal genome-wide study examining the *in vivo* effects of IFN-beta-1b on PBMC, mRNA and miRNA expression profiles were analyzed simultaneously. Information from various databases was integrated to examine how miRNAs affect the expression levels of their target mRNAs. The study was able to show that the mRNA results were quite consistent with results published before. Out of the 95 differentially expressed genes filtered in this study (figure 5), in the Goertsches *et al.* study 63 differentially expressed genes were also reported [78] and in the Hecker *et al.* study 49 of the 95 genes were already identified as transcriptionally modulated in response to IFN-beta-1a s.c. [42]. Out of the 95 genes reported in the IFN-beta-1b s.c. study 75 were found up-regulated. In contrast, in the miRNA data, the filtering method could find more down-regulated than up-regulated miRNAs during therapy. There were 20 miRNAs filtered as differentially expressed miRNAs and 7 were up-regulated. In the

analysis four miRNA (*hsa-miR-29a-3p, hsa-miR-29c-3p, hsa-miR-193a-3p and hsa-miR-532-5p)* were confirmed to be down-regulated.

The expression of the mir-193 family members *hsa-miR-193a-3p* and *hsa-miR-193a-5p* was repressed during the therapy. A study by Lindberg *et al.* showed increased expression of *hsa-miR-193a-5p* in CD4+ T cells of RRMS patients when compared to healthy controls [79]. Otaegui *et al.* described that *hsa-miR-193a-3p* is related to the remission stage of MS [80]. *hsa-mir-193a* controls the apoptotic processes by helping CASP3 activation stimulated by TNFSF10 signaling [81]. TNFSF10 (=TRAIL) is a known IFN-beta-induced gene and it was transcriptionally up-regulated in the patients' PBMC. The regulation of mir-193 miRNAs thus could play an important role in the molecular mechanisms of action of IFN-beta therapy.

let-7 family members (*hsa-let-7a-5p* and *hsa-let-7b-5p)* were found to be expressed at higher levels during treatment in the IFN-beta-1b s.c. study. In another study it was shown in primary macrophages that *hsa-let-7b-5p* is up-regulated in response to IFN-beta *in vitro* [82]. In another study miRNAs of the let-7 family were shown to activate TLR7 signaling in microglia and macrophages which induces neurodegeneration [83]. Gandhi *et al.* [84] in their study reported variations in expression levels of *hsa-let-7a-5p* in the blood plasma of patients with secondary progressive MS when compared with patients with RRMS. In CD4+ T cells, let-7 miRNA reduce the expression of IL-10, a cytokine with anti-inflammatory properties [82].

The IFN-beta-1b s.c. study was an interesting integration of different molecular layers that are orchestrated to a tapestry of genes. The study integrated mRNA and miRNA expression profiles before and during IFN-beta-1b s.c. treatment across different time points. This was the first time such a study has been performed. The study definitely improved the understanding of the molecular effects of IFN-beta-1b s.c. treatment in MS patients.

In the third study, MS-related miRNA-mRNA interactions were studied. The regulatory network between mRNAs and MS-associated miRNAs was constructed using information from different databases. In this study 16 miRNAs were found in the miR2Disease database as higher or lower expressed in blood cells of MS patients. CDKN1A (p21) was the gene with the maximum number of interactions in the network. CDKN1A interacts with five miRNAs which are associated with MS. For instance the miRNAs *hsa-miR-20a-5p* and *hsa-miR-20b-5p* are known to interact with CDKN1A according to miRTarBase and miRWalk databases. The function of CDKN1A is to block the induction of the S-phase in T-cells. Thus any faulty regulation of this gene can result in autoimmune processes [85]. Another study by de Santis *et al.* [86] showed that CDKN1A expression can also be regulated by miRNA *hsa-miR-25-3p* and *hsa-miR-106b-5p*, thereby controlling the TGF-beta pathway and influencing the development of regulatory T-cells in MS patients. In the network the RUNX1 gene is interacting with four miRNAs. It is involved in various aspects of T-cells including development and function. It is a central factor in the differentiation of Th17 cells with a dual effect on IL17 transcription [87].

In the network four miRNAs target TF E2F1. E2F1 helps to regulate the start for antigen-stimulated activation of T-cells and also plays a role in the negative selection in the thymus [88]. FOXO1 has five interactions and FOXO3 has three interactions in the network. FOXO1 and FOXO3 belong to the fork head family of TFs. The function of FOXO1 is to suppress the activation, proliferation and development of T-cells. Down-regulation of FOXO1 by *hsa-miR-182-5p* was described to be necessary for T-cell clonal expansion [89]. Deletion of FOXO1 along with FOXO3 in mice was shown to result in inflammatory diseases and decreased Treg cell differentiation [90].

The mechanisms of GA and IFN-beta are not fully understood. The GA and IFN-beta-1b studies reported here gave new insights to better understand the mechanisms of action of these drugs. The studies reported in this thesis have several interesting findings, for instance the down-regulation of *hsa-miR-29c-3p* in response to IFN-beta therapy. Secondly, *hsa-let-7a-5p* and *hsa-miR-16-5p* were found highly expressed in peripheral

blood and brain and their precise relevance to MS needs to be studied further. Thirdly, the 16 MS-relevant miRNAs and their 1498 target genes along with their 39 TFs definitely delivered a wider understanding of the possible interactions taking place at the molecular level. Fourthly, in the GA study the 23 genes which were differentially expressed across two time points may represent useful biomarkers of the biological response to GA. Most of the findings reported here are novel and will help us to better understand MS pathophysiology and MS treatment.

6. Summary

MS is an inflammatory disease of the central nervous system. There are many types of disease modifying drugs available for MS but none of them can cure MS. The medications are taken for the rest of the life. Despite that our understanding about MS is still unclear. Genome-wide transcriptome studies are vital to understand the changes happening at the molecular level during treatment. Due to the advancement of technology it is now possible to precisely measure the expression levels of thousands of genes simultaneously. The studies reported in this thesis analyzed the changes which happen at the transcriptome level during the course of treatment. Glatiramer acetate and interferon-beta-1b s.c. were the drugs analyzed in two independent studies. The studies reported revealed several differentially expressed transcipts which included both mRNAs and miRNAs. The differentially expressed genes and miRNAs can be considered as biomarker candidates for therapy monitoring which can also help to better understand the mechanism of action of GA and IFN-beta-1b s.c.

GA is a peptide drug used in the treatment of RRMS. Its mechanism of action is still not clearly understood. The study in this thesis gave a snapshot of changes which happened during the treatment in monocytes at the gene expression level. Affymetrix GeneChips were used to analyze the gene expression of 18.862 genes in the blood monocyte sub-population. Monocytes from eight MS patients were isolated. Total RNA was then isolated from those samples. GA was prescribed 20mg once daily. The study was longitudinal in design with five different blood sampling time points, baseline – before the start of the treatment, after one day, after one week, after one month and after two months. There were more than 400 genes which were filtered as differentially expressed genes when compared with their pre-treatment levels. These genes were not reported before in any genome-wide study on GA therapy Out of them only 23 genes appeared in at least two consecutive time point comparisons. The differentially expressed genes were analyzed further for interactions and a gene interaction network was constructed using Pathway Studio software. The network revealed interactions between the GA-responsive

differentially expressed genes VCAM1, CXCL9, CXCL10, POMC, OXT, PTPRC, CD38 and 34 other genes. This interaction network can help to better understand the gene regulatory changes during GA treatment. The network interactions need to be further studied with wet lab experiments to understand the cascade of events taking place within the cell during GA therapy.

The IFN-beta-1b study design was longitudinal with four different time points (baseline, after 2 days, after 4 days and after 1 month). PBMC were isolated from the patient blood and total RNA was isolated from them. Using Affymetrix microarrays and TaqMan microfluidic cards miRNA and mRNA gene expression were measured. miRWalk and miRTarBase databases were used to find out the interactions between the differentially expressed mRNAs and miRNAs. The interaction predicting database used different types of algorithms to find the most likely miRNA target genes. In the filtering of IFN-beta-1b-responsive miRNAs and mRNAs there were 95 mRNAs of which 75 were up-regulated and 20 were down-regulated. Additionally, there were 20 miRNAs which were filtered. Out of them 7 were up-regulated and 13 were down-regulated. The study found that there were more mRNA which were up-regulated and contrastingly there were more miRNAs which were down-regulated. The miRNA *hsa-miR-29c-3p* was confirmed to be significantly down-regulated in response to IFN-beta-1b treatment. Further investigation of the miRNA-mRNA interactions with *in vivo* miRNA target assays such as the luciferase assay are needed.

The manuscript 3 presented different databases in the context of miRNA, their contents and utility. The target predicting databases use different types of algorithms to predict miRNA targets. miRWalk database was extensively used in the study for target prediction and the main reason was that it combines information from 10 target prediction databases. The miRNA targets reported in the study were at least predicted by 7 different databases consistently. The review explains the information which can be collected from different miRNA databases and how to integrate the heterogeneous information to

investigate functions and interactions of miRNAs. This may serve as a guideline for similar studies on different issues or diseases.

The studies reported in this thesis brought a wealth of new information to the table. There are more studies needed to improve our understanding on the mechanisms of action of these drugs and to improve them. The findings reported in this thesis will hopefully help in the quest to find a better treatment for MS.

7. References

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8. Publications

8.1 Manuscript - 1

Glatiramer acetate treatment effects on gene expression in monocytes of multiple sclerosis patients

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Glatiramer acetate treatment effects on gene expression in monocytes of multiple sclerosis patients

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Abstract

Background: Glatiramer acetate (GA) is a mixture of synthetic peptides used in the treatment of patients with relapsing-remitting multiple sclerosis (RRMS). The aim of this study was to investigate the effects of GA therapy on the gene expression of monocytes.

Methods: Monocytes were isolated from the peripheral blood of eight RRMS patients. The blood was obtained longitudinally before the start of GA therapy as well as after one day, one week, one month and two months. Gene expression was measured at the mRNA level by microarrays.

Results: More than 400 genes were identified as up-regulated or down-regulated in the course of therapy, and we analyzed their biological functions and regulatory interactions. Many of those genes are known to regulate lymphocyte activation and proliferation, but only a subset of genes was repeatedly differentially expressed at different time points during treatment.

Conclusions: Overall, the observed gene regulatory effects of GA on monocytes were modest and not stable over time. However, our study revealed several genes that are worthy of investigation in future studies on the molecular mechanisms of GA therapy.

Keywords: Glatiramer acetate, Relapsing-remitting multiple sclerosis, Monocytes, Gene expression profiling, Microarray analysis

Background

Multiple sclerosis (MS) is a chronic immune-mediated disease of the central nervous system (CNS). The autoreactive behavior of the immune system in MS patients is associated with inflammatory lesions in the CNS and axonal demyelination. There is currently no cure for MS, but there are several therapies available as disease-modifying agents. The relapsing-remitting type of MS (RRMS) is mainly treated with immunomodulating drugs like interferon-beta (IFN-β) and glatiramer acetate (GA) [1,2].

GA (copolymer 1) is a first-line treatment option for RRMS. Different clinical trials have shown that GA treatment decreases the incidence of relapses and significantly reduces the number of gadolinium-enhancing lesions in magnetic resonance imaging (MRI) [3-6]. GA is not a defined chemical substance, but a standardized mixture of synthetic peptides. These peptides are made up of four different amino acids, glutamic acid, lysine, alanine and tyrosine (G-L-A-T), in a molar ratio of 1.5:3.6:4.6:1.0, assembled in a random order into polypeptide chains with a length of 40 to 100 residues [7]. This mixture of peptides was initially intended to mimic myelin basic protein (MBP) and to induce experimental autoimmune encephalomyelitis (EAE), the animal model of MS [8]. However, surprisingly, GA inhibited EAE in rodents and monkeys [9]. Today, GA has been well-

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established for MS therapy for more than a decade due to its beneficial clinical effects and its favorable safety profile.

According to pharmacokinetic studies, GA is quickly absorbed after subcutaneous administration, and it undergoes rapid degradation to amino acids and shorter peptides. Only 10% of the peptides remain at the site of injection after one hour [10]. A fraction of GA presumably enters the lymphatic circulation and reaches the regional lymph nodes where it modulates immune responses. Of note, patients treated with GA have the tendency to develop antibodies against it. However, the biological meaning of anti-GA antibodies remains controversial and it is unclear whether they may have a neutralizing or a beneficial effect in MS patients [11-14].

Different molecular mechanisms of action of GA have been proposed [7,15-18]. One postulated mechanism is that GA peptides act as altered peptide ligands (APL). An APL is a peptide, usually closely related to an agonist peptide in amino acid sequence, that induces a different function or partial response of T-cells specific for the agonist peptide as a result of the modified interaction with the T-cell receptor (TCR). During GA therapy, the partial activation of T-cells specific for MBP and other myelin antigens can induce peripheral tolerance, and this may contribute to the clinical effects of GA by preventing the attack of the myelin sheath around the nerves. Indeed, cross-reactivity of GA-specific T-cells with myelin antigens has been demonstrated, and there is evidence that prolonged exposure to GA results in anergy or depletion of GA-reactive cells that are possibly relevant in the pathogenesis of MS [19-23]. On the other hand, several studies have shown that GA induces a T helper cell type 1 (Th1) to Th2 shift in T-cells. Th1 and Th2 each produce a different combination of pro- and anti-inflammatory cytokines, respectively. The increase of GA-reactive Th2 cells during treatment is regarded as a central mechanism of action of GA [24-27]. These anti-inflammatory Th2-like cells were found to mediate regulatory functions as they can migrate into the brain and act suppressively at the sites of inflammation (local bystander suppression). This leads to a reduced activation and proliferation of auto-reactive immune cells, even if they recognize unrelated antigens and do not cross-react with GA [11].

Antigen-presenting cells (APC) are also believed to play a role in the immunomodulatory effects of GA therapy. The professional APC are dendritic cells and macrophages, which differentiate from circulating monocytes [28]. The interplay between APC and T-cells is fundamental in adaptive immune responses as well as in the pathophysiology of MS [29]. One suggested mechanism of GA is that it binds to major histocompatibility complex (MHC) molecules and thus competes with myelin antigens for their presentation on APC to T-cells. Specifically, GA can act as an antagonist of MBP/MHC at MBP-specific TCR and it is able to displace MBP from the binding site on MHC class II molecules [30,31]. On the other hand, GA was shown to change the properties of APC in such a way that they stimulate Th2-like responses. These APC are called type II APC. The effect of the drug on APC seems to depend on the cell type [11,32]. Weber *et al.* showed that GA inhibits monocyte reactivity and induces type II monocytes, which promote both Th2 differentiation and expansion of T regulatory cells (Treg). They observed that after GA administration in EAE, the pattern of cytokine production by monocytes switched towards an anti-inflammatory profile, characterized by down-regulation of pro-inflammatory cytokines (for example, IL12) and up-regulation of anti-inflammatory cytokines (for example, IL10) [33,34]. An increased IL10 production of monocytes has already been observed 72 hours post GA therapy initiation in a recent study by Ayers *et al.* [35]. Other studies confirmed that there is an increase in anti-inflammatory type II monocytes during GA therapy, and that the suppressor functions of these monocytes contribute to Th2 deviation of naive T-cells of MS patients [36-38]. Additionally, GA was shown to affect monocytes by increasing the expression of IL1RA while diminishing the production of IL1-β [39,40]. Recently, Caragnano *et al.* also observed a trend for IL1-β down-regulation in stimulated monocytes from GA-treated MS patients, and this was paralleled by lower levels of P2RX7, a receptor regulating cytokine production and apoptosis [41]. However, whether GA acts directly on monocytes *in vivo*, or whether the effects on monocytes are mediated by cytokines produced by GA-specific Th2 cells, is unclear as there is a dynamic feedback loop between human T-cell and APC responses [11,36].

However, GA not only modulates CD4+ T helper cell responses and binds to MHC class II molecules on APC. It has also been shown that GA incites an HLA class I-restricted, cytotoxic suppressor CD8+ T-cell response [42]. This may be mediated by heat shock proteins (HSPs) that bind extracellular antigens and mediate their cellular uptake. HSP-antigen complexes are then directed toward either the conventional class II pathway or the MHC class I pathway through cross-presentation [43,44]. In the similar way, GA peptides may bind to HSPs, and thus may be presented on MHC class I molecules resulting in an altered activation of T-cell subsets. This potentially leads to cytotoxic T-cells, which can kill CD4+ T-cells in a GA-specific manner [42]. In addition to its immunomodulatory effects, direct neuroprotective and even remyelinating properties have been ascribed to GA as well [45-49]. For instance, GA may foster repair after neurologic damage by stimulating the expression of neurotrophic factors like BDNF by various immune and CNS resident cells [50].

Over the last years, several research groups performed longitudinal gene expression profiling studies with microarrays to better understand the mechanisms of action of MS therapies. However, while the broad and rapid gene regulatory effects of IFN-β treatment in blood cells have been investigated extensively [51], there is only one such study for GA treatment: Achiron *et al.* measured the gene expression in peripheral blood mononuclear cells (PBMC) from 14 RRMS patients before and three months after initiation of GA therapy [52]. In their analysis, they identified 480 genes to be differentially expressed at the transcript level. They concluded that changes in the expression of immunomodulatory genes during GA therapy are important to reduce the activity of the disease [52].

The present study focuses on the effects of daily subcutaneous GA injections on the mRNA expression profile of monocytes in the peripheral blood. We were interested in monocytes because GA has been described as modulating these cells to promote Th2-like responses [11,31], but the *in vivo* effects have so far not been examined in a genome-wide and longitudinal manner. We obtained monocytes from RRMS patients immediately before as well as at four different time points after the start of GA therapy. The gene expression analysis was performed using microarrays. Genes that were found to be differentially expressed in response to GA therapy were then analyzed for biological functions and molecular interactions to derive new hypotheses on the molecular mechanisms of action of GA. This is the first study that investigates the transcriptome dynamics over the course of the therapy in a cell type-specific manner.

Methods

Blood sample collection

Eight Caucasian patients with diagnosed RRMS according to the revised McDonald criteria [53] were recruited for this study. The patients started a treatment with GA (Copaxone, Teva Pharmaceutical Industries Ltd., Petah Tikva, Israel) in 20 mg doses given daily as a subcutaneous injection. Five of the patients were not treated with any immunomodulatory or immunosuppressive drug prior to the onset of this study. Two patients (MS3 and MS5) received subcutaneous IFN-β, and one patient (MS8) received mitoxantrone (the last injection was four months ago) previously (Table 1). All patients were given routine care following the consensus treatment guidelines and recommendations of the German Society of Neurology (DGN). Blood samples were obtained from each patient at five different time points: before the first injection of GA (baseline) and after one day (that is, before the second injection) as well as after one week, one month and two months. Collection of blood was done by venipuncture with ethylenediaminetetraacetic acid (EDTA) as anticoagulant. An approximate volume of 15 ml whole blood was collected for each patient and each time point. In the clinical follow-up, the patients were assessed neurologically, monitored for relapses, and rated using the expanded disability status scale (EDSS) and cranial MRI. The study was approved by the ethics committee of the University of Rostock and carried out according to the Declaration of Helsinki. All patients gave written informed consent to be included in the study.

Monocyte isolation, RNA preparation and gene expression profiling

Monocytes were isolated from the blood samples using erythrocyte lysis buffer (Qiagen, Hilden, Germany), followed by magnetic-activated cell sorting (MACS). The CD14+ cells were magnetically labeled using CD14 MicroBeads and collected as positively selected cell fraction using the autoMACS Separator (Miltenyi Biotec, Teterow, Germany). Total RNA was then isolated from the monocytes using RNeasy columns (Qiagen, Hilden, Germany). RNA concentrations were measured using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scien-

Table 1 Demographic data and clinical data of the eight patients

Patient	Gender	Age at study onset	Disease duration	Previous treatment	EDSS at baseline	EDSS after 12 months	Relapses during first 12 months	cMRI at follow-up
MS1	Female	41	0	None	1.5	1.5	0	-
MS2	Female	50	10	None	1.5	1.5	1	New lesion
MS3	Female	37	33	IFN-beta sc.	1.0	1.5	0	stable
MS4	Female	38	9	None	1.0	1.5	0	New lesion
MS5	Female	38	89	IFN-beta sc.	3.5	2.0	1	stable
MS6	Female	47	1	None	1.5	1.5	0	New lesion
MS7	Male	35	16	None	1.0	1.0	0	New lesion
MS8	Male	25	55	Mitoxantrone	2.0	2.5	1	New lesion

The table shows gender, age, the previous treatment, and the EDSS scores at therapy initiation (baseline) and after 12 months. The duration from the diagnosis of definite MS to the start of GA therapy in months (disease duration) is also given. The gender ratio was 6:2 (female: male) and the average age at study onset was 38.9 ± 7.6 years (mean ± SD). Three patients had a relapse during the first 12 months after GA treatment initiation. Five patients showed a new brain lesion in the follow-up compared to pre-treatment. cMRI: cranial magnetic resonance imaging, EDSS: expanded disability status scale, sc.: subcutaneous, SD: standard deviation.

tific, Wilmington, DE, USA) and their integrity was assessed with an Agilent 2100 Bioanalyzer using RNA 6000 Pico LabChips. The microarray experiments were performed with an Affymetrix platform. HG-U133 Plus 2.0 GeneChips were used for this analysis. From each sample preparation, total RNA amounts ranging from 100 ng to 200 ng were used as starting material. RNA was converted to cDNA and later into biotinylated cRNA using the MessageAmp II-Biotin Enhanced Kit (Ambion, Foster City, CA, USA). The cRNA molecules were fragmented and 15 μg of cRNA were hybridized onto the GeneChips for 16 hours at 45°C. The GeneChips were later washed and stained in the Affymetrix Fluidics Station 450 and scanned with a GeneChip Scanner 3000 7G system. All these procedures were performed according to the manufacturer protocols (Affymetrix, Santa Clara, CA, USA).

Microarray data pre-processing

Initial data pre-processing and quality control was done using the Affymetrix GeneChip operating software (GCOS 1.4) and MAS5.0 statistical algorithms (Microarray Analysis Suite 5.0) (Affymetrix, Santa Clara, CA, USA). Since the annotation of genes has changed since the development of the microarrays, there are oligonucleotide probes on the chips, which match to no transcript, and probes, which match to multiple transcripts. Therefore, to exclude such probes from the analysis, we used a custom chip definition file (CDF), which was based on the GeneAnnot database version 1.9 (http://www.xlab.unimo.it/GA_CDF/, CDF version 2.1.0) [54]. Each probe set in the custom CDF matches a single gene. Data normalization was done by a loess fit to the data with span = 0.05 using the R package 'affy'.

Filtering of differentially expressed genes

To filter differentially expressed genes from the data, we applied two criteria. First, we computed paired *t*-tests comparing for each gene the expression at baseline with the expression at one day, one week, one month and two months. In the second analysis, we evaluated the data with the MAID filtering method, which calculates MA plot-based signal intensity-dependent fold-changes (MAID-scores) for each time point comparison [55]. To filter genes that are significantly up-regulated or down-regulated relative to baseline, we combined the MAID-score outcomes with the paired *t*-test outcomes. Genes with $|\text{MAID-score}| > 2$ and *t*-test *P*-value < 0.05 were filtered as differentially expressed.

Functional analysis of differentially expressed genes

To investigate the functions of the filtered genes, we performed a Gene Ontology (GO) term enrichment analysis, which was based on the association of functional annotations for each gene in the GO database. GOstats [56], a Bioconductor software package, was the application used to test GO terms for overrepresentation. GOstats computes a probability based on a hypergeometric distribution, which assesses whether the number of filtered genes associated with the term is larger than expected by chance. As a reference, that is, the gene universe, we used all genes that were measured with the HG-U133 Plus 2.0 microarrays (n = 18,862).

Text mining-based gene interaction network analysis

A gene interaction network was constructed for the filtered genes using the Pathway Studio software version 7.1 from Ariadne Genomics (Rockville, MD, USA) [57]. This software allowed the extraction of gene interactions that were automatically obtained from the literature by text mining. The information about interactions between the genes was exported from the Pathway Studio software as a table, which contains nodes (genes) and edges (interactions) as the building blocks of the gene network. Cytoscape, which is an open source software [58], was used to visualize the network. The edges were represented in various shapes to display the type of the interaction (positive, inhibitory and binding).

Results

Patient and sample information

Eight patients were included in this study (six females and two males). The patients were 38.9 ± 7.6 years of age (mean ± standard deviation) and had a mean EDSS of 1.6 (1.0 to 3.5) after a mean disease duration of 26.6 (0 to 89) months (Table 1). All patients started GA treatment at standard dose. During the follow-up period of 12 months, three patients had one relapse each and the other five patients had no relapse. There was only a moderate increase in disability when comparing the EDSS at study onset (baseline) with the EDSS after a follow-up of one year (Table 1). One patient discontinued the therapy in this period of time: MS5 had a severe relapse soon after study onset and, therefore, switched to natalizumab therapy (Tysabri, Biogen Idec, Weston, MA, USA) after three months. Cranial MRI scans were done for seven of the eight patients before the start of GA therapy as well as after a mean follow-up of 17.6 ± 9.5 months. Despite the therapy, five patients each had one new lesion.

From each blood sample, monocytes were isolated by MACS separation. For quality control, we analyzed the mRNA levels of genes, which are known to be specifically expressed by different blood cell types [59]. This revealed high CD14+ monocyte purities (Additional file 1: Figure S1). The RNA that was isolated from the monocytes was in general of high quality with an average RNA integrity number (RIN) of 9.6. The quality of RNA was poor for three samples (MS3/1 month, MS6/1 week,

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MS7/1 week) and so they were excluded from further analyses.

Differentially expressed genes

The pre-processing of the Affymetrix microarray data resulted in transcript levels for 18,862 different genes and 37 different samples. The data are available in the Gene Expression Omnibus (GEO) database with the accession number 'GSE42763'. The filtering of genes differentially expressed in response to GA therapy resulted in a gene list for each time point comparison. In total, 171 genes met the filtering criteria for one day, 116 genes for one week, 124 genes for one month and 101 genes for two months versus baseline (Figure 1, Additional file 2: Table S1). These four gene lists, when aggregated, resulted in 463 different genes (293 up-regulated and 170 down-regulated genes). We observed no accumulation of gene regulatory effects in the course of the therapy since similar numbers of genes were filtered at early (within the first week) and later time points (after one and two months). Moreover, unexpectedly, we found no stable signature of GA-responsive genes since only 45 of the 463 genes were repeatedly identified as differentially expressed, and there was no gene modulated in expression at all time points during therapy.

To further narrow down the list of genes, we selected only those genes that were expressed at significantly higher or lower levels compared to baseline at two or more consecutive time points. This resulted in a subset of 23 out of the 463 genes. Of these 23 genes, 5 genes were down-regulated (CD34, RPA4, HMGB1L4, BAZ2B and RARS) and 18 genes were up-regulated (for example, ATOX1, BLOC1S1, LIMD2, POLR2I and RPA3). The average mRNA expression dynamics of these genes during GA therapy are shown in Figure 2.

Functional annotation of the genes

A Gene Ontology (GO) term enrichment analysis was performed to determine the functions, which are characteristic for the genes filtered as differentially expressed in response to GA therapy (n = 463). The GO terms are classified into three major categories: biological process (BP), cellular component (CC) and molecular function (MF). In our analysis, we shortlisted the top 20 overrepresented GO terms according to the *P*-value (Table 2). Several genes appeared under multiple GO terms. The GO terms, which had the most gene members in the list of filtered genes, were 'extracellular region' (GO:0005576, *P*-value = 2.27E-07) and 'immune system process' (GO:0002376, *P*-value = 1.19E-04). The GO term 'extracellular region' contains genes whose protein products are secreted from cells, for example, cell communication molecules, and it was associated to 76 of the 463 genes. Out of them, 45 genes were up-regulated, for example, POMC, MMP17, LTB, XCL1 and APOL3, and 31 genes were down-regulated, for example, CD163, ADAMTS5, TNFSF14, CTSZ and PAM. The GO term 'immune system process' contained 48 of the genes. Out of them, 35 genes were up-regulated, for example, CD38, CXCL9, CXCL10, IL18 and ICAM2, and 13 genes were down regulated, for example, PTPRC, NCK2, C4BPA,

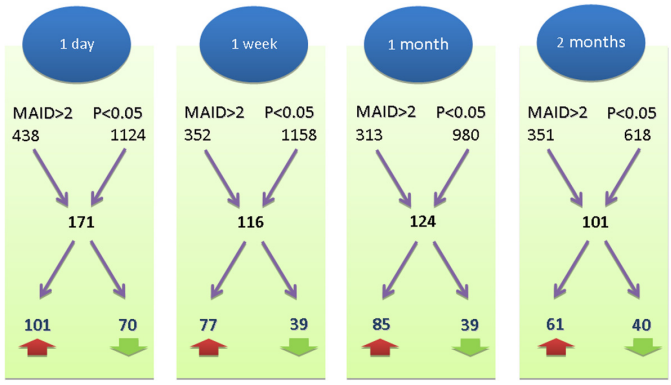

Figure 1 Longitudinal study design and gene filtering results. Blood was sampled from eight patients at five different time points: before the start of GA treatment as well as after one day, one week, one month and two months. The expression of 18,862 genes was measured in monocytes with Affymetrix microarrays, and transcript levels during therapy were compared to the pre-treatment levels. *t*-test *P*-values and MAID-scores were used to determine differentially expressed genes. For instance, when comparing the baseline levels with the expression levels after one day, 438 genes survived the MAID analysis criterion (|MAID-score| > 2) and 1,124 genes survived the paired *t*-test criterion (*P*-value < 0.05). In combination, this resulted in 171 filtered genes: 101 were up-regulated (red arrow) and 70 were down-regulated (green arrow). For each time point comparison, a similar number of genes were filtered. However, the overlap of these four gene lists was relatively small (n = 45). When taken together, 463 different genes were identified to be differentially expressed within the first two months of GA therapy.

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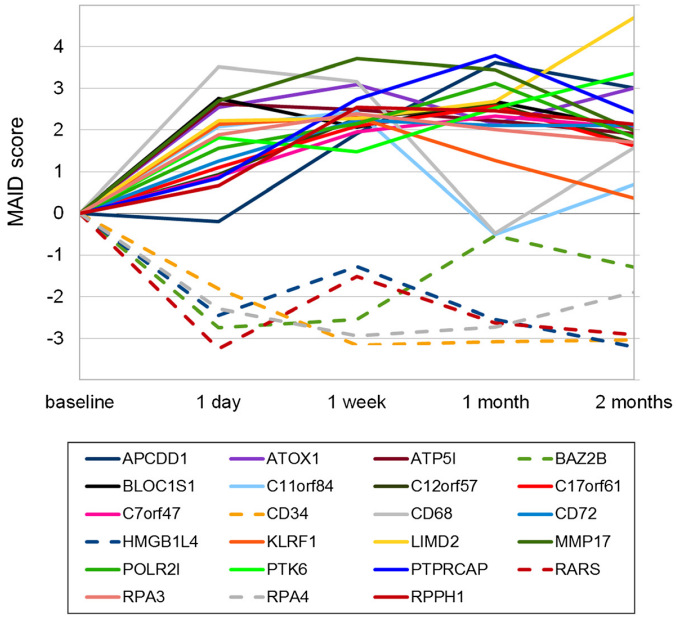

Figure 2 Dynamics of 23 genes consistently modulated in expression during glatiramer acetate (GA) therapy. A subset of the 463 filtered genes appeared repeatedly as differentially expressed in consecutive time point comparisons. The graph shows the mean mRNA expression dynamics of 23 genes that were repeatedly found expressed at significantly higher or lower levels across at least two different time points compared to the pre-treatment levels. There were 18 up-regulated genes and 5 down-regulated genes (CD34, RPA4, HMGB1L4, RARS and BAZ2B). The MAID-score, a fold-change variant that is calculated from the data of all patients, is represented on the y-axis and the time points are represented on the x-axis.

GLMN and ITGAV. There were 22 genes that belonged to both of these GO terms.

There were several overrepresented GO terms, which are relevant to monocytes, for instance 'mononuclear cell proliferation' (GO:0032943, *P*-value = 5.76E-04), 'regulation of mononuclear cell proliferation' (GO:0032944, *P*-value = 5.44E-04) and 'leukocyte proliferation' (GO:0070661, *P*-value = 6.92E-04). These GO terms form a hierarchy where a GO term is part of a broader GO term, hence these GO terms share most of the genes. Genes found modulated in expression during GA treatment and belonging to all of these three terms are, for example, CD38, GLMN, IGHM, IL18, NCK2 and PTPRC. Other notable overrepresented GO terms were 'regulation of lymphocyte activation' and 'cytokine activity'.

Gene interaction network

The unified list of 463 genes was used as input for the Pathway Studio software to gather pair-wise interactions between them. The output resulted in 41 genes (= nodes) with 59 interactions (= edges). The interactions were visualized as a network (Figure 3). The edges vary according to their interaction type. There were 43 positive regulatory interactions, 14 inhibitory interactions and 2 binding interactions. The network revealed different interaction clusters. Seven of the genes, CXCL10, CXCL9, VCAM1, POMC, OXT, PTPRC and CD38, possess the majority of the interactions with the other genes in the network: except PTPRC, they all appeared as up-regulated during GA therapy.

POMC, a polypeptide hormone precursor, had 12 interactions and is therefore the most connected gene in the network. For instance, VCAM1, OXT, IL18 and ADCY6 (which were up-regulated) and IGFBP1 (which was down-regulated) are influenced by POMC according to the literature-based interaction network. The second cluster is based on VCAM1, a vascular cell adhesion protein, which had 11 interactions. It is regulated by IL18, POMC, ITGAV, CYP2C19, CXCL10, CXCL9, TRAF2, NOTCH4 and TIE1. Of these, ITGAV was down-regulated and all the other genes were up-regulated in response to GA treatment. The third cluster is formed by OXT, which regulates CD38, SLA5A5, GHRH and POMC. The fourth cluster is based on two up-regulated chemokines, CXCL9 and CXCL10, which together had 14 interactions. They have a feedback loop between them, and CXCL10 is further regulated by IL18, IL27 and XCL1, which were all up-regulated after the first week of GA therapy. The network also shows that PTPRC (= CD45) is linked with PTPRCAP, CD34, CD38, IL21 and VCAM1.

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Table 2 Analysis of gene functions

Term	GO accession	ExpCount	Count	Odds ratio	*P*-value
Extracellular region	GO:0005576 (CC)	42	76	2.06	2.27E-07
Cytokine activity	GO:0005125 (MF)	4	15	4.18	1.12E-05
Receptor binding	GO:0005102 (MF)	21	41	2.19	1.85E-05
Extracellular region part	GO:0044421 (CC)	22	42	2.06	5.17E-05
Immune response	GO:0006955 (BP)	17	35	2.19	6.74E-05
Extracellular space	GO:0005615 (CC)	17	34	2.17	9.00E-05
Immune system process	GO:0002376 (BP)	28	48	1.90	1.19E-04
Regulation of lymphocyte activation	GO:0051249 (BP)	5	14	3.30	2.19E-04
Defense response	GO:0006952 (BP)	18	33	2.01	4.38E-04
Regulation of lymphocyte proliferation	GO:0050670 (BP)	2	9	4.27	5.07E-04
Lymphocyte proliferation	GO:0046651 (BP)	3	10	3.89	5.08E-04
T-cell proliferation	GO:0042098 (BP)	2	8	4.75	5.37E-04
Regulation of mononuclear cell proliferation	GO:0032944 (BP)	2	9	4.23	5.44E-04
Cytokine receptor binding	GO:0005126 (MF)	4	12	3.33	5.48E-04
Mononuclear cell proliferation	GO:0032943 (BP)	3	10	3.82	5.76E-04
Regulation of leukocyte activation	GO:0002694 (BP)	5	14	2.97	5.90E-04
Regulation of leukocyte proliferation	GO:0070663 (BP)	2	9	4.14	6.26E-04
Leukocyte proliferation	GO:0070661 (BP)	3	10	3.72	6.92E-04
Regulation of cell activation	GO:0050865 (BP)	5	14	2.82	9.40E-04
Regulation of T-cell proliferation	GO:0042129 (BP)	2	7	4.93	9.56E-04

The top 20 Gene Ontology (GO) terms that were significantly overrepresented for the list of filtered genes (n = 463 genes) are shown in the table sorted by *P*-value. For example, in the second row, 'cytokine activity' is listed as an overrepresented GO term. 'GO:0005125' is the corresponding GO database accession. The third and fourth column give the expected number ('ExpCount', 4 genes) and the actual number ('Count', 15 genes) of genes in the filtering result that are associated with 'cytokine activity'. This led to an odds ratio of 4.18 and a *P*-value of 1.12E-05. The GO terms are classified into three major groups: biological process (BP), cellular component (CC) and molecular function (MF).

CD38 had six interactions: CD38 inhibits CD34, is regulated by IL21, and has feedback loops with OXT and PTPRC (Figure 3).

Discussion

This study focused on the *in vivo* effects of GA therapy on the gene expression of monocytes, which are the precursors of macrophages and dendritic cells. The monocytes were obtained from the blood of RRMS patients and we compared the transcript levels before and after the start of GA therapy. For each patient, the gene expression was measured at five time points up to two months into therapy. In these data, we identified 463 genes as up-regulated or down-regulated during therapy compared to pre-treatment levels. More than a hundred genes were filtered at early (after one day) and later (after two months) time points post treatment initiation. However, relatively few genes were repeatedly found to be differentially expressed in the course of time. This indicated that the gene regulatory effects of GA on monocytes are rather modest and no stable gene expression signature could be seen. Nevertheless, the mRNA changes of some genes might tell us something about GA's molecular mechanisms of action.

Compared to our study, in the gene expression study by Achiron *et al.*, only two time points were compared: before and after three months of GA treatment [52]. Therefore, the variability in the mRNA dynamics early during therapy possibly has been underestimated so far. Moreover, Achiron *et al.* studied the gene expression changes in PBMC of RRMS patients, whereas we studied monocytes. Using Affymetrix microarrays, they found 480 genes to be differentially expressed in response to GA administration, with the main effects being related to antigen-activated apoptosis, inflammation, adhesion, and MHC class I antigen presentation [52]. As in our study, there were more up-regulated than down-regulated genes. However, when comparing their gene list (n = 480) with ours (n = 463), only five genes (BAT1, ELOVL5, ETV7, MT1E and PCBD1) were in common. One explanation for that might be that GA possibly acts primarily on other subsets of circulating cells, for example, by altering the functional properties of (autoreactive) T-cells. Therefore, different gene regulatory effects might be seen in PBMC than in monocytes from GA-treated patients.

On the other hand, a recent cross-sectional study by Ottoboni *et al.* found no significant differences in the PBMC RNA profiles of untreated and GA-treated patients

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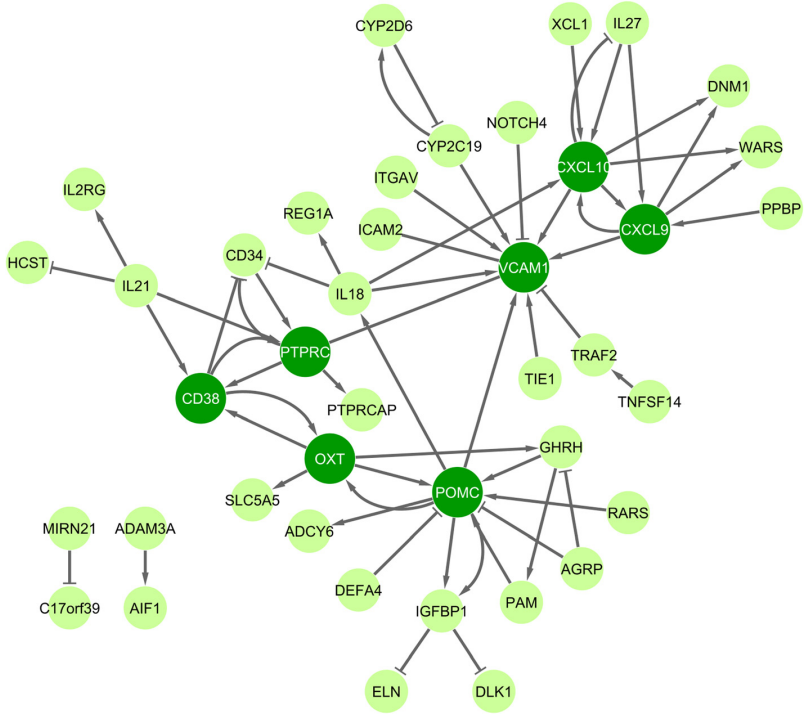

Figure 3 Interaction network of genes differentially expressed in response to glatiramer acetate (GA). A gene interaction network was retrieved for the 463 filtered genes using the Pathway Studio software. The software delivered a network of 41 genes (= nodes) with 59 interactions (= edges) between them. The other genes with no interaction are not shown in the figure. Edges are represented in different ways in the network. An arrow (→) means positive effect (n = 43), a 'T' (⊣) means negative effect (inhibition, n = 14) and a line (—) means binding (n = 2). The seven nodes, which are highlighted in dark green, are involved in the regulation of many other genes and thus may play an important role in the molecular mechanisms of action of GA. The network is available as a Cytoscape session file from the authors upon request.

[60]. In their study, they took into account the multiple testing by computing false discovery rates (FDR) [61]. In our data set, if we set the threshold for statistical significance at FDR < 0.05, also no gene remained as differentially expressed during GA therapy. Instead, we chose less conservative filtering criteria to detect even moderate shifts in the gene expression of monocytes, but, in consequence, also some weakly modulated and less expressed genes survived the filtering for GA-responsive genes (Additional file 2: Table S1). A subset of 23 genes was repeatedly identified to be up-regulated or down-regulated at different time points during therapy (Figure 2). These 23 genes might represent good candidates of molecular markers of GA activity. However, for confirmation, a larger independent study with more sensitive measurement techniques such as real-time PCR is needed. As another limitation of our study, we did not measure the transcript levels in the long-term after the first two months of treatment. Possibly, the full modulation of immunological processes by GA may require more time. It was also beyond the scope of the present study to examine whether the individual gene expression profiles are associated with the clinical data (for example, relapse rate, EDSS and MRI).

We performed a GO term enrichment analysis for the list of 463 filtered genes to classify them according to their functions and the biological processes they are involved in (Table 2). Overrepresented GO terms included 'lymphocyte proliferation' and 'regulation of T-cell proliferation'. These findings are consistent with earlier studies that showed that GA suppresses lymphocyte proliferation through modulation of monocytes and monocyte-derived dendritic cells, thereby reducing the number of autoreactive T-cells [38,62,63]. Members of these GO terms are, for example, cytokines such as IL18 and TNFSF14. Additionally, we searched for interactions between the genes and retrieved 59 interactions. Of note, these interactions were obtained by literature mining. Therefore, the gene network (Figure 3) shows direct as well as indirect regulatory effects on the transcript and protein level. Seven genes had several interactions (CXCL9, CXCL10, VCAM1, POMC, OXT, PTPRC and CD38).

The network contained different cytokines, for example, CXCL9 and CXCL10 whose expression was increased one day after the start of GA therapy. These two chemokines bind to the CXCR3 receptor and are involved in the recruitment of immune cells to sites of inflammation, principally acting on activated CD4+ Th1 cells, CD8+ T-cells and natural killer (NK) cells [64,65]. A previous study already showed that the transcription of CXCL10 is induced in PBMC after GA administration [66]. Both CXCL9 and CXCL10 were also described to be modulated in expression in blood during treatment with IFN-β [67]. Therefore, both drugs seem to affect the chemokine gradient between brain lesions and the peripheral immune compartment. The cytokine group further included interleukins (IL18, IL21, IL25 and IL27), which were all found to be up-regulated in our data set. Of those, IL18 and IL27 play important roles in the differentiation and expansion of naive CD4+ T-cells [68,69]. Moreover, TNFSF14, a member of the TNF cytokine family, was down-regulated one day after the first GA injection. TNFSF14 is known to function as a costimulatory factor regulating the activation of T-cells [70]. A single nucleotide polymorphism within an intron of the TNFSF14 gene is associated with MS susceptibility [71,72]. Another related member of the TNF family, LTB (lymphotoxin-β), was also filtered as differentially expressed. Both LTB and TNFSF14 bind to the LTBR receptor, and they provide communication links in innate and adaptive immune responses [73,74]. However, the transcriptional modulation of these cytokines was not stable over time and it thus remains unclear how these immunoregulatory effects may exactly contribute to the mechanisms of action of GA.

The interaction network of filtered genes also contains several cell adhesion receptors, for example, ITGAV, ICAM2 and VCAM1. ITGAV encodes the integrin αV, an integral membrane protein that can interact with a variety of extracellular matrix ligands. Integrins orchestrate monocyte differentiation into macrophages, and they play a role in macrophage adhesion, migration and tissue infiltration [75]. Moreover, ITGAV is known to mediate proinflammatory cytokine synthesis in human monocytes [76]. It was expressed at lower levels after the first injection of GA, which may reflect the previously described shift in the gene expression of monocytes towards an anti-inflammatory profile [34,39].

Other genes in the network are implicated in quite different biological processes, for example, RARS, WARS, PTPRC, PTPRCAP and MIRN21. RARS and WARS encode the arginyl- and tryptophanyl-tRNA synthetase, respectively. They catalyze the amino acid attachment to cognate tRNAs during protein synthesis. However, besides their role in protein translation, biologically active fragments of WARS were also discovered to be involved in angiogenesis signaling pathways [77]. PTPRC is a transmembrane glycoprotein associated with PTPRCAP. Both genes were found modulated in expression after one month of treatment compared to baseline. PTPRC functions as a regulator of cytokine receptor signaling and influences cellular processes such as cell proliferation [78]. Upon activation of monocytes, proteolytic processing of PTPRC results in a protein fragment, which is released and acts as an inhibitor of T-cell proliferation [79]. The microRNA gene MIRN21 was expressed at higher levels after two months compared to pre-treatment levels. MicroRNAs are involved in the post-transcriptional regulation of gene expression. The transcript MIRN21 harbors the mature microRNA hsa-miR-21, which has been shown to be up-regulated in active MS lesions [80] and to be higher expressed in PBMC of RRMS patients versus controls [81]. MicroRNAs in MS and therapy are worthy of being explored in more detail [82,83]. So far, there is only one study that has specifically investigated whether GA therapy affects the levels of mature microRNAs, but this study was limited to five selected microRNAs [84].

Other studies demonstrated that GA treatment leads to a change in the properties of monocytes from pro-inflammatory type I monocytes to anti-inflammatory type II monocytes [34]. However, although some cytokines were differentially expressed during GA therapy, the mRNA levels of TNF-α, TGF-β, IL10, IL12, IL1-β and IL1RA were not affected in our data set. Therefore, we could not observe a clear cytokine shift in monocytes in response to GA. One reason for that might be that in our study monocytes were isolated from peripheral blood samples of MS patients, whereas, in contrast, in the study by Weber *et al.* the monocytes were separated from the spleen of mice with EAE [34]. Moreover, our study was restricted to mRNA transcripts and we did not measure the amounts of the encoded proteins and their splice variants, whereas other groups analyzed the protein levels of monocytes in culture after *in vitro* stimulation [35,36,38]. Burger *et al.* studied the effects of GA on the transcription of two genes (IL1-β and IL1RA) in monocytes [39,40]. However, their results might not be reflected in our data since they used monocytes from blood donors and stimulated these cells *in vitro* with GA. In our study, we could neither identify a stable signature of differentially expressed genes nor a solid evidence of an increase of type II monocytes within the first two months of therapy. This finding cannot be explained by just the relatively small number of recruited patients. Therefore, we conclude that the *in vivo* effects of GA on monocytes in the peripheral blood are rather modest and variable. It is likely that most of the effects occur at the injection sites or in the draining lymph nodes where (MBP-specific) T-cells as well as monocytes and professional APC are confronted with GA peptides. Additionally, since GA is a mixture of randomly

synthesized peptides, the molecular effects might be somewhat different from injection to injection. All this makes it quite a challenge to study the drug's molecular mechanisms of action. Further studies are needed to better understand how GA modulates the immune system, also because new drugs similar to GA are currently tested for RRMS (for example, ATX-MS-1467, Apitope Technology Ltd., Bristol, UK).

Conclusions

There is a lack of transcriptome studies on the effects of GA in MS patients. Here, we presented the first genome-wide and cell type-specific analysis of the mRNA dynamics during GA therapy. Using microarrays, we longitudinally measured the gene expression of monocytes for a small patient group at five different time points. We identified 463 genes as differentially expressed within the first two months of GA treatment, the majority being associated with immunological processes (for example, cytokines). However, the changes in gene expression were not sustained over time, and most genes were seen up-regulated or down-regulated only once. Therefore, GA seems to have only little gene regulatory effects on monocytes. Our study nevertheless delivered some genes that are worth investigating in future studies regarding the molecular mechanisms of GA therapy in the peripheral blood of MS patients.

Additional files

Additional file 1: Figure S1. Analysis of the purity of the isolated monocytes. (A) We visualized the measured transcript levels of five selected genes, which are specifically expressed in different blood cell types, namely CD14 (monocytes), CD3D (T-cells), MS4A1 (for example, B-cells), KLRD1 (for example, NK cells) and HBD (erythrocytes). CD14 was expressed at very high levels (> 18,000) in all 37 samples of the microarray data set, whereas the other genes were expressed at very low levels (< 400). This demonstrates high purity of the monocytes isolated by MACS. (B) We used the Affymetrix microarray data by Novershtern *et al.* [59] to compare the mRNA levels of these genes in distinct human hematopoietic cell populations, for example, CD4+ and CD8+ T-cells, B-cells and monocytes. The preprocessed data were downloaded from the GEO database (accession number 'GSE24759'). The bar charts show the mean ± standard error of the expression values of the respective probe sets (given in brackets) in 14 different cell types. A limited purity of the isolated monocytes would be noticeable in figure A, because CD3D, MS4A1, KLRD1 and HBD are highly expressed in other cells of the blood.

Additional file 2: Table S1. Filtered differentially expressed genes. This Excel file contains four gene lists, which provide the genes that were identified as differentially expressed after one day (t1), one week (t2), one month (t3) or two months (t4) of GA therapy when compared to baseline levels (t0). For each gene, identifiers for the databases GeneCards, Entrez and HGNC (gene symbols) are provided together with their official full names. Additionally, the mean gene expression levels (averaged over the patients) and the respective standard deviations (SD) are given for all compared time points as well as the computed *t*-test *P*-values and MAID-scores. The column 'Regulation' denotes, if the gene was found up-regulated or down-regulated in response to GA relative to baseline.

Abbreviations

APC: Antigen-presenting cell; APL: Altered peptide ligand; BP: Biological process; CC: Cellular component; CDF: Chip definition file; CNS: Central nervous system; DGN: German society of neurology; EAE: Experimental autoimmune encephalomyelitis; EDSS: Expanded disability status scale; EDTA: Ethylenediaminetetraacetic acid; FDR: False discovery rate; GA: Glatiramer acetate; GEO: Gene expression omnibus; GO: Gene ontology; HSP: Heat shock protein; IFN: Interferon; IL: Interleukin; MACS: Magnetic-activated cell sorting; MAID: MA plot-based signal intensity-dependent fold-change criterion; MBP: Myelin basic protein; MF: Molecular function; MHC: Major histocompatibility complex; MRI: Magnetic resonance imaging; MS: Multiple sclerosis; NK: Natural killer; PBMC: Peripheral blood mononuclear cells; RIN: RNA integrity number; RRMS: Relapsing-remitting multiple sclerosis; Sc: Subcutaneous; SD: Standard deviation; TCR: T-cell receptor; Th: T helper cell; Treg: T regulatory cell.

Competing interests

UKZ received research support as well as speaking fees from Teva, Biogen Idec, Bayer HealthCare, Novartis, Almirall, Merck Serono and Sanofi-Aventis. MH received speaking fees from Bayer HealthCare, Teva and Novartis. Meanwhile, RHG is a salaried employee of Teva, and he was a salaried employee of Novartis. MT, BKP, IS, DK and H-JT declare no potential conflict of interest.

Authors' contributions

UKZ and H-JT inspired and directed the work. IS was responsible for clinical documentation. The lab experiments were performed by RHG, DK, BKP and MT. MH and RHG carried out the analysis and interpretation of the data. MT and MH drafted the paper and prepared all tables and figures. H-JT and UKZ contributed to the writing of the manuscript. All authors read and approved the final manuscript.

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8.2 Manuscript - 2

MicroRNA expression changes during interferon-beta treatment in the peripheral blood of multiple sclerosis patients

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Article

MicroRNA Expression Changes during Interferon-Beta Treatment in the Peripheral Blood of Multiple Sclerosis Patients

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Abstract: MicroRNAs (miRNAs) are small non-coding RNA molecules acting as post-transcriptional regulators of gene expression. They are involved in many biological processes, and their dysregulation is implicated in various diseases, including multiple sclerosis (MS). Interferon-beta (IFN-beta) is widely used as a first-line immunomodulatory treatment of MS patients. Here, we present the first longitudinal study on the miRNA expression changes in response to IFN-beta therapy. Peripheral blood mononuclear cells (PBMC) were obtained before treatment initiation as well as after two days, four days, and one month, from patients with clinically isolated syndrome (CIS) and patients with relapsing-remitting MS (RRMS). We measured the expression of 651 mature miRNAs and about 19,000 mRNAs in parallel using real-time PCR arrays and Affymetrix microarrays.

We observed that the up-regulation of IFN-beta-responsive genes is accompanied by a down-regulation of several miRNAs, including members of the mir-29 family. These differentially expressed miRNAs were found to be associated with apoptotic processes and IFN feedback loops. A network of miRNA-mRNA target interactions was constructed by integrating the information from different databases. Our results suggest that miRNA-mediated regulation plays an important role in the mechanisms of action of IFN-beta, not only in the treatment of MS but also in normal immune responses. miRNA expression levels in the blood may serve as a biomarker of the biological effects of IFN-beta therapy that may predict individual disease activity and progression.

Keywords: interferon-beta; multiple sclerosis; peripheral blood; microRNA; gene expression

1. Introduction

Multiple sclerosis (MS) is a chronic disease of the central nervous system (CNS), which is characterized by multiple discrete areas of inflammatory demyelination, axonal degeneration, and glial scarring. The resulting loss of neurons and axons leads to diverse neurological symptoms, progressive disability, and a significant decrease in quality of life. The disease usually begins in early adulthood, and is more common in females. Different types of MS are distinguished: In about 85% of patients, the disease starts with a single demyelinating episode (clinically isolated syndrome, CIS) and progresses to a relapsing-remitting course (RRMS) with acute exacerbations and periods of remission [1–4].

A number of disease-modifying therapies for MS are available, and they are especially effective when applied in the early stages of the disease [4,5]. Injections of recombinant interferon-beta (IFN-beta) are considered a first-line option in the treatment of RRMS. IFN-beta has been shown to reduce the number of relapses and to suppress the accumulation of new inflammatory lesions in the brain. Three different preparations of IFN-beta are in clinical use. They differ in dose, route, and frequency of IFN-beta administration, but they are comparable regarding clinical efficacy [6].

IFN-beta has broad effects on the gene regulation of blood cells [7–11]. This has been shown by several studies that used microarray technology to analyze the gene expression dynamics in the peripheral blood of MS patients in response to IFN-beta therapy. In this way, more than a hundred genes have consistently been found differentially expressed during treatment [10]. The transcript levels of most of these genes are up-regulated within a few hours after IFN-beta injection, and they return to pre-treatment levels after a few days [12,13]. These IFN-beta-responsive genes are believed to mediate the beneficial effects of the treatment through immunomodulatory, antiproliferative, and antipathogenic processes [7–9].

While the therapeutic effects on the regulation of mRNAs have been extensively investigated, studies on the regulation of microRNAs (miRNAs) are lacking. miRNAs are a distinct class of small (~22 nt) non-coding RNA molecules [14]. They originate from precursor RNAs (pre-miRNAs) found in longer primary transcripts (pri-miRNAs), which often also contain the exons of an mRNA. Mature miRNAs act as post-transcriptional regulators. They repress gene expression via base-pairing with complementary sequences within the 3' untranslated regions (UTRs) of target mRNAs. This interaction

results in gene silencing by translational repression or target degradation. A miRNA can have hundreds of different mRNA targets, and a target might be regulated by a combination of multiple miRNAs [15]. The human genome encodes over 1000 miRNAs [16,17]. miRNAs are thus likely involved in most biological processes, and they play essential roles in the immune system and in the correct function of the CNS [18,19].

Dysregulated expression of miRNAs is associated with pathological conditions, including neurological diseases. Human MS studies showed altered miRNA expression in peripheral blood samples, lymphocyte subpopulations, and active CNS lesions from MS patients [20–22]. Studies with the animal model of MS, experimental autoimmune encephalomyelitis (EAE), also support the involvement of miRNAs in this disease [23,24]. These findings provided important insights into the pathophysiology of MS and opened a new avenue in biomarker research. If miRNA levels in the blood or brain of MS patients correlate with disease stage and progression of disability, they may also support early diagnosis and effective treatment in future [25].

In order to better understand the molecular mechanisms of action of IFN-beta therapy, it is important to investigate the miRNA expression dynamics during therapy. The regulation of miRNAs may contribute to the immunomodulatory and clinical effects of the treatment. Moreover, miRNAs might be markers for characterizing the biological response to IFN-beta. miRNA biomarkers for treatment monitoring could be useful in the individual management of disease activity. However, so far, there is only one study on the expression of miRNAs in MS patients during IFN-beta therapy: Waschbisch *et al.* obtained peripheral blood mononuclear cells (PBMC) from patients with RRMS, and analyzed the expression of five selected miRNAs by real-time PCR [26]. They compared the miRNA levels between treatment-naive patients (n = 36), IFN-beta-treated patients (n = 18), and patients treated with glatiramer acetate (GA, n = 20). As a result, none of the five miRNAs was differentially expressed in IFN-beta-treated patients, but *hsa-miR-146a-5p* and *hsa-miR-142-3p* were expressed at significantly lower levels in GA-treated patients [26]. Other researchers used microarrays to study the expression of hundreds of miRNAs in IFN-stimulated cells. In this way, O'Connell *et al.* observed that *hsa-miR-155-5p* is induced in primary murine macrophages after exposure to IFN-beta for 6 h [27]. Pedersen *et al.* studied the regulation of miRNAs in Huh7 cells and primary hepatocytes, which were stimulated with different concentrations of IFN-beta for up to 48 h [28]. They observed increased and reduced miRNA expression in response to IFN-beta, and showed that some of the IFN-beta-induced miRNAs mediate antiviral effects against hepatitis C virus. This provides an example of miRNAs as components of the innate immune response.

In this study, we used microarrays to investigate in parallel the expression dynamics of mRNAs and miRNAs in PBMC of patients with CIS or RRMS in response to therapy with subcutaneous (sc.) IFN-beta. The blood samples were obtained longitudinally from six patients at four time points in the early phase of therapy, namely before the first (baseline), second, and third IFN-beta injection as well as after one month of treatment. We then screened for significant changes in miRNA and mRNA expression, and identified several miRNAs as differentially expressed during therapy. Information of different databases was then integrated [22,29] to examine whether the expression of these miRNAs is cell type-specific and correlates with the levels of their target mRNAs. Predicted and experimentally verified miRNA-mRNA interactions were compiled to construct a network of IFN-beta-responsive

genes and miRNAs. To our knowledge, this is the first genome-wide miRNA profiling study on the *in vivo* effects of IFN-beta treatment in MS.

2. Results and Discussion

2.1. Study Population

Six female patients of Western European descent, and diagnosed with CIS (n = 2) or RRMS (n = 4), were recruited for this study (Pat1-6, mean age 37.5 years, Table 1). The patients were treatment-naive and started an immunomodulatory therapy with IFN-beta-1b (Betaferon, Bayer HealthCare) administered subcutaneously every other day. In the first weeks, the Betaferon titration pack was used, hence the patients started with a low dose (62.5 µg for the first three injections) that was gradually increased to the full dose (250 µg) after three weeks. All patients were continuously treated with IFN-beta-1b for at least one year. During follow-up, they were monitored for relapses and rated using the Expanded Disability Status Scale (EDSS). The individual disease activity during therapy was relatively low: Four of the patients (Pat1-4) were relapse-free and showed no disability progression within the first year of treatment (Table 1). The two patients with CIS (Pat1 and Pat5) did not convert to clinically definite MS in this period of time.

Table 1. Clinical data and demographic data of the patients.

Patient	Type	Age	Disease duration	EDSS (baseline)	EDSS (1 year)	Relapses (1 year)
Pat1	CIS	28	1	1.0	1.0	0
Pat2	RRMS	38	2	1.5	1.5	0
Pat3	RRMS	22	1	1.5	1.0	0
Pat4	RRMS	50	12	2.5	2.5	0
Pat5	CIS	60	2	1.5	2.5	0
Pat6	RRMS	27	2	2.0	1.0	2

Six female patients were recruited for the main cohort of this study. They were diagnosed with relapsing-remitting MS (RRMS) or clinically isolated syndrome (CIS) suggestive of MS. The age at study onset (in years) and the duration from the diagnosis to the start of IFN-beta-1b sc. therapy (in months) are shown. Additionally, the EDSS scores before treatment initiation (baseline) and after one year, as well as the number of relapses during the first year of clinical follow-up are given in the table.

Note that the patient group included only women. A differential hormonal regulation of immune system genes in blood cells has been described for different phases of the menstrual cycle [30]. Such differences in gene expression may have led to increased variance in the data. However, prior mRNA profiling studies observed no significant gender-specific differences in the gene expression signature in response to IFN-beta therapy [8,31], and this seems to be the case regarding the expression of miRNAs as well (see Section 2.5).

2.2. Parallel Measurement of mRNAs and MicroRNAs in Blood Cells

Patient blood samples were drawn immediately before first IFN-beta injection as well as two days, four days, and one month post therapy initiation. Total RNA of Ficoll-isolated PBMC from each sample was extracted to measure the levels of mRNAs and miRNAs with different platforms. We used

TaqMan Array Human MicroRNA cards (Applied Biosystems, Foster City, CA, USA) to quantify the expression of 651 mature miRNAs and Affymetrix HG-U133 Plus 2.0 microarrays (Affymetrix, Santa Clara, CA, USA) to quantify the expression of about 19,000 mRNAs. In this way, we obtained in parallel the mRNA and miRNA expression profiles from six patients (Pat1-6) within the first month of IFN-beta treatment.

The data were preprocessed as described in Section 3.6. Relatively low variation in the transcriptome profiles indicated high data quality, comparable to our previous microarray time course data sets [8,31,32]. In the miRNA data, systematic and stochastic variation was higher. For the TaqMan miRNA B-cards of Pat5, the raw threshold cycle (Ct) values were generally higher (Supplementary File 1) due to an unknown measurement bias. In the PBMC samples of the other five patients, approximately 400 miRNAs could be detected (Ct < 38) with the TaqMan miRNA arrays (Table 2). The raw TaqMan data were transformed to the linear scale, and coefficients of variation (*CV*) were calculated to assess the effects of data normalization. The normalization decreased the average *CV* over all 768 measured assays from 0.953 to 0.894. The *CV* for the housekeeping miRNA *hsa-miR-191-5p* [33] was 0.183. In comparison, assays for the non-coding RNAs *U6*, *U44* and *U48* had *CV*s of >0.35. The *CV* for the housekeeping mRNA GAPDH was 0.071.

Table 2. Numbers of microRNAs detected in the samples with the TaqMan cards set.

Patient	Baseline	~48 h	~96 h	1 month
Pat1	380	374	364	373
Pat2	375	407	392	362
Pat3	427	400	408	407
Pat4	390	387	388	389
Pat5	285	258	266	262
Pat6	431	451	446	391

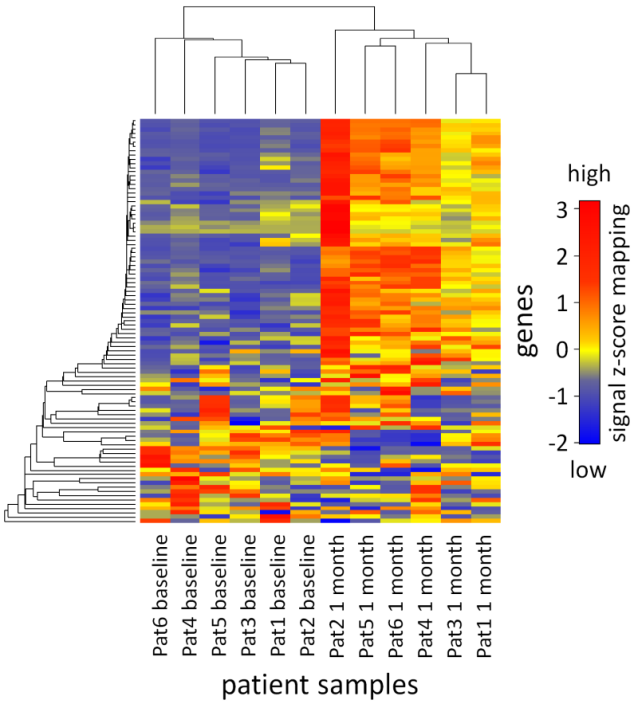
Approximately 400 miRNAs were found to be expressed (raw Ct value < 38) per sample. The numbers are lower for the PBMC samples of patient Pat5, where generally lower miRNA amounts were measured with the B-cards for all four time points (Supplementary File 1).

2.3. Analysis of mRNA Expression Dynamics

We filtered for mRNAs showing strong expression changes in response to IFN-beta therapy by comparing the baseline expression levels with the expression levels at the three time points during treatment. The MAID filtering method [34] was used to analyze the mRNA dynamics. As a result, 14, 34, and 66 genes were found to be expressed at higher or lower levels after two days, four days, and one month, respectively. In total, 95 genes were identified as up-regulated (n = 75) or down-regulated (n = 20) in the early course of the therapy (Supplementary File 2). The gene expression changes in the first month of subcutaneous IFN-beta-1b treatment are visualized in the heat map in Figure 1.

A permutation test (see Section 3.7) revealed that the number of 95 differentially expressed genes is significantly higher than would be expected by chance. In randomly permuted data sets, 29.6 genes on average were filtered. The number of filtered genes was below 95 in 98.8% of the permutations, which implies an empirical p-value of <0.05, demonstrating that most of the mRNA expression changes that we found are indeed due to the therapy.

Figure 1. Heat map visualization of the mRNA expression changes in response to IFN-beta. Shown are the baseline and one month transcript levels of the 95 genes that were identified as differentially expressed during IFN-beta therapy. The patient samples are represented in the columns, the genes are represented in the rows, and the gene expression levels were centered and scaled in row direction (z-scores). The clustering analysis clearly separated the PBMC samples obtained at baseline and after one month of therapy. The row labels of the heat map (*i.e.*, the respective genes) are given in Supplementary File 2. The upper half of the heat map contains most of the IFN-beta-induced genes.

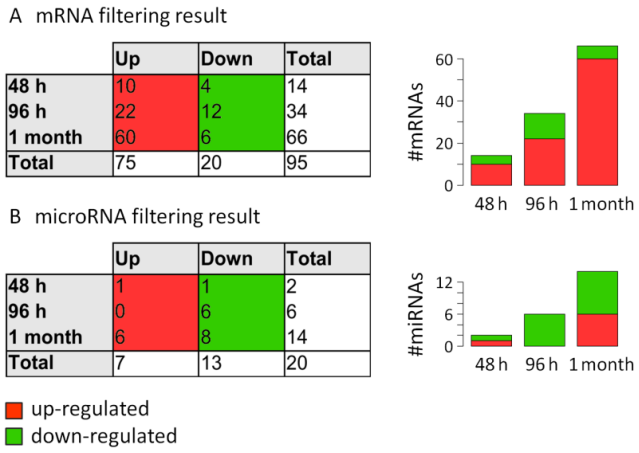


Despite the relatively small patient cohort (n = 6), the mRNA results were quite consistent with the literature. In another study, we already analyzed the PBMC gene expression profiles of a larger group of MS patients (n = 25) treated with IFN-beta-1b sc. [31]. In total, 63 of the 95 differentially expressed genes were also filtered in the previous study. Recently, we completed a similar microarray study on the effects of IFN-beta-1a sc. [8], in which 49 of the 95 genes were already identified as transcriptionally modulated (Supplementary File 2). Moreover, more up-regulated than down-regulated genes were filtered, which also confirms previous findings [10]. After the first IFN-beta injections, fewer genes were altered in expression than after one month (*cf.* Goertsches *et al.*, 2010 [31]). This can be explained by the fact that the patients started the first week of the therapy with a quarter of the full dose (Betaferon titration pack). Most of the filtered genes are part of an up-regulated type I IFN signature. For instance, *IFI6*, *IFI44L*, and *SIGLEC1* are known type I IFN-induced genes, which were up-regulated at all time points during therapy in comparison to baseline. In contrast, FCER1A was consistently down-regulated in response to therapy as has been described previously as well [8,31,32]. Regarding the functions of these genes, the reader is referred to the literature [7-9].

2.4. Analysis of MicroRNA Expression Dynamics

As for the mRNA data, the MAID filtering method [34] was used to identify miRNAs that are differentially expressed in PBMC within the first month of IFN-beta treatment. When we compared the expression levels at the three time points during therapy with the expression levels at baseline, 20 different miRNAs were filtered. Of these, seven miRNAs appeared as up-regulated and 13 miRNAs appeared as down-regulated in response to the therapy (Figure 2, Table 3 and Supplementary File 3). According to the permutation test, this is significantly more than expected by chance: In only 0.6% of the randomly permuted data sets, 20 (or more) miRNAs were filtered, and only 7.7 miRNAs were filtered on average.

Figure 2. Summary of the filtering of IFN-beta-responsive mRNAs and microRNAs. PBMC expression levels during therapy were compared to pre-treatment levels. The number (#) of differentially expressed mRNAs and miRNAs is depicted in the tables and bar plots. The row "Total" gives the union set over all three time point comparisons. (**A**) In the mRNA data, 95 genes were found to be modulated in expression in response to IFN-beta-1b treatment. As expected, most of them were up-regulated (n = 75) and known type I IFN-induced genes, and the strongest changes were observed at one month versus baseline; (**B**) In the miRNA data, the filtering method identified more down-regulated than up-regulated miRNAs during therapy, again with the strongest effects seen after one month.



Two of the 20 miRNAs (*hsa-miR-149-5p* and *hsa-miR-708-5p*) were filtered at two different time points. For the remaining miRNAs, the expression changes were not very stable in the course of therapy. This may be due to the small number of patients and the fact that the accuracy of miRNA measurements is in general limited. Therefore, our list of 20 miRNAs represents candidates that have to be validated in a larger patient cohort using, e.g., single real-time PCR assays.

Most of the miRNAs (n = 14) were filtered as up-regulated or down-regulated one month after IFN-beta-1b sc. treatment initiation. This corresponds to the results of the gene expression profiling, where the strongest changes in mRNA levels were also observed after one month (Figure 2). Previous studies demonstrated that the majority of IFN-beta-responsive genes can be seen at this time

point [8,31]. Therefore, we hypothesize that, similarly, the number of miRNAs that are modulated in expression is not much higher after long-term treatment. Instead, the development of neutralizing antibodies (NAb) to IFN-beta might impair the biological response to the drug in some patients [35]. However, further studies are needed to investigate the long-term regulation of miRNAs and the potential effects of NAb.

Table 3. Details of microRNAs differentially expressed during IFN-beta therapy.

Mature miRNA	Sequence	Expression change	Family	pre-miRNA	pri-miRNA	Location
hsa-let-7a-5p	UGAGGUAGUAGGUUGUAUAGUU	up-regulated	let-7	MIRLET7A1		chr9 (q22.32)
				MIRLET7A2	MIR100HG	chr11 (q24.1)
				MIRLET7A3	MIRLET7BHG	chr22 (q13.31)
hsa-let-7b-5p	UGAGGUAGUAGGUUGUGUGGUU	up-regulated	let-7	MIRLET7B	MIRLET7BHG	chr22 (q13.31)
hsa-miR-16-5p	UAGCAGCACGUAAAUAUUGGCG	up-regulated	mir-15	MIR16-1	DLEU2	chr13 (q14.2)
				MIR16-2	SMC4	chr3 (q25.33)
hsa-miR-27a-5p	AGGGCUUAGCUGCUUGUGAGCA	down-regulated	mir-27	MIR27A		chr19 (p13.13)
hsa-miR-29a-3p	UAGCACCAUCUGAAAUCGGUUA	down-regulated	mir-29	MIR29A		chr7 (q32.3)
hsa-miR-29b-1-5p	GCUGGUUUCAUAUGGUGGUUUAGA	down-regulated	mir-29	MIR29B1		chr7 (q32.3)
hsa-miR-29c-3p	UAGCACCAUUUGAAAUCGGUUA	down-regulated	mir-29	MIR29C		chr1 (q32.2)
hsa-miR-95	UUCAACGGGUAUUUAUUGAGCA	down-regulated	mir-95	MIR95	ABLIM2	chr4 (p16.1)
hsa-miR-149-5p	UCUGGCUCCGUGUCUUCACUCCC	down-regulated	mir-149	MIR149	GPC1	chr2 (q37.3)
hsa-miR-181c-3p	AACCAUCGACCGUUGAGUGGAC	down-regulated	mir-181	MIR181C		chr19 (p13.13)
hsa-miR-193a-3p	AACUGGCCUACAAAGUCCCAGU	down-regulated	mir-193	MIR193A		chr17 (q11.2)
hsa-miR-193a-5p	UGGGUCUUUGCGGGCGAGAUGA	down-regulated	mir-193	MIR193A		chr17 (q11.2)
hsa-miR-342-5p	AGGGGUGCUAUCUGUGAUUGA	up-regulated	mir-342	MIR342	EVL	chr14 (q32.2)
hsa-miR-346	UGUCUGCCCGCAUGCCUGCCUCU	up-regulated	mir-346	MIR346	GRID1	chr10 (q23.2)
hsa-miR-423-5p	UGAGGGGCAGAGAGCGAGACUUU	down-regulated	mir-423	MIR423	NSRP1	chr17 (q11.2)
hsa-miR-518b	CAAAGCGCUCCCCUUUAGAGGU	up-regulated	mir-515	MIR518B		chr19 (q13.42)
hsa-miR-532-5p	CAUGCCUUGAGUGUAGGACCGU	down-regulated	mir-188	MIR532	CLCN5	chrX (p11.23)
hsa-miR-708-5p	AAGGAGCUUACAAUCUAGCUGGG	down-regulated	mir-708	MIR708	TENM4	chr11 (q14.1)
hsa-miR-760	CGGCUCUGGGUCUGUGGGGA	up-regulated	mir-760	MIR760		chr1 (p22.1)
hsa-miR-874	CUGCCCUGGCCCGAGGGACCGA	down-regulated	mir-874	MIR874	KLHL3	chr5 (q31.2)

The table lists the 20 miRNAs found to be expressed at higher or lower levels in the PBMC of patients with CIS or MS in response to IFN-beta therapy. The base sequence, the gene regulatory effect of the treatment ("Expression change"), the miRNA family, the HGNC symbols of the precursor and primary miRNAs, as well as the genomic location are shown. Two of the mature miRNAs (*hsa-let-7a-5p* and *hsa-miR-16-5p*) are processed from more than one precursor miRNA. For 11 miRNAs the pri-miRNA transcript has been annotated. None of these pri-miRNAs appeared in the mRNA filtering result. Precursor miRNAs of *hsa-let-7a-5p* and *hsa-let-7b-5p*, and of *hsa-miR-29a-3p* and *hsa-miR-29b-1-5p* are clustered, *i.e.*, they share their transcription locus.

Apparently, there are more down-regulated than up-regulated miRNAs during therapy, which is the opposite of the mRNA results. This suggests that the induction of IFN-beta-responsive genes is paralleled by a preferential down-regulation of miRNAs, which is plausible given that miRNAs act as gene silencers. Therefore, we analyzed whether the miRNAs indeed participate in the regulation of the mRNA transcripts (see Section 2.7).

2.5. Validation of IFN-beta-Induced MicroRNA Expression Changes

We used Affymetrix miRNA microarrays to replicate the miRNA measurements of the PBMC samples from three patients (Pat1-3) before the start of IFN-beta therapy as well as after one month (see Section 3.4). These microarrays had a lower measurement range than the TaqMan miRNA arrays, though the comparability of the data was acceptable (Spearman's rho = 0.823). The statistical analysis of this additional data set is limited by the small number of patients. However, when we compared the mean miRNA expression levels before and one month after treatment initiation, 13 of the 20 filtered miRNAs showed the same trend of up-regulation or down-regulation (Supplementary File 3). At the significance threshold alpha = 0.10, four miRNAs (*hsa-miR-29a-3p*, *hsa-miR-29c-3p*, *hsa-miR-193a-3p*, and *hsa-miR-532-5p*) were confirmed to be down-regulated during treatment.

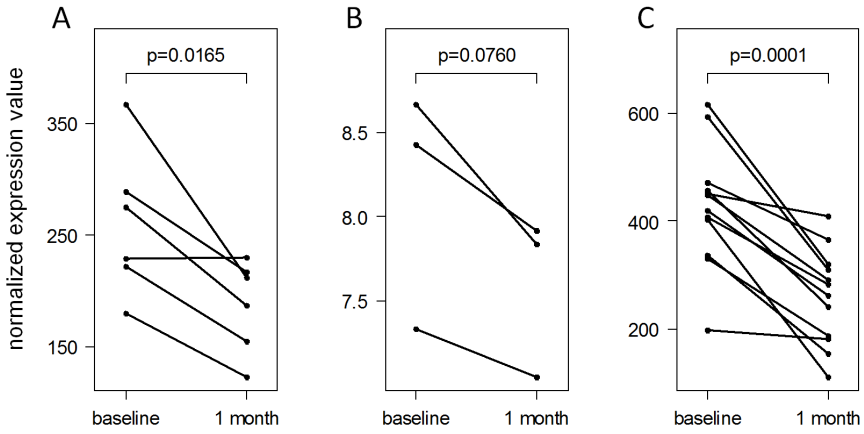
For further validation, we selected five of the 20 filtered miRNAs to quantify their expression in PBMC of an independent cohort of 12 patients using TaqMan single-tube assays (Supplementary File 4 and Supplementary File 5). These 12 patients (8 RRMS/4 CIS, 7 females/5 males, mean age 36.2 years) also started a therapy with IFN-beta-1b sc. The PBMC were obtained again in a longitudinal manner before the first drug injection and after one month of treatment. In this data set, *hsa-miR-29a-3p* and *hsa-miR-29c-3p* could be confirmed as differentially expressed in response to IFN-beta therapy (*t*-test *p*-values < 0.001). *hsa-miR-29c-3p* was expressed at lower levels during therapy in comparison to pre-treatment levels in all 12 patients, hence independent of disease stage (CIS or RRMS), age, and gender (Figure 3). Additionally, *hsa-miR-532-5p* was confirmed to be down-regulated (*p*-value = 0.048). *hsa-miR-16-5p* and *hsa-miR-149-5p* showed the same trend of expression change as in the other data sets (TaqMan miRNA arrays and Affymetrix miRNA arrays), but this was not statistically significant (*p*-values > 0.10, Supplementary File 3).

2.6. Functions of the MicroRNAs in the Context of Multiple Sclerosis

The filtered miRNAs (Table 3) affect diverse cellular functions and pathways, and some of them have been implicated in MS. In our data set, *hsa-let-7a-5p* and *hsa-let-7b-5p*, which belong to the let-7 family, were expressed at higher levels during therapy. Lehmann *et al.* showed that let-7 family members can activate TLR7 signaling in macrophages and microglia, thereby inducing neurodegeneration [36]. In CD4+ T cells, let-7 miRNAs reduce the expression of IL10, a cytokine with anti-inflammatory properties [37]. The up-regulation of *hsa-let-7b-5p* by IFN-beta has already been demonstrated *in vitro* in primary macrophages [38]. Interestingly, *hsa-let-7b-5p* is in turn capable of binding the endogenous IFN-beta transcript, forming a negative feedback loop for the regulation of IFN-beta protein [38]. Recently, Gandhi *et al.* found altered *hsa-let-7a-5p* levels in the blood plasma of patients with the secondary-progressive form of MS, but not of patients with RRMS [39].

The observed up-regulation of *hsa-miR-16-5p* in response to IFN-beta therapy may restore the aberrant expression of this miRNA in the disease. Two studies observed reduced levels of *hsa-miR-16-5p* in the blood of RRMS patients. One study compared the expression in PBMC and CD4+ T cells from untreated patients and healthy donors [40]. The other study showed that *hsa-miR-16-5p* is down-regulated in B cells as well [41].

Figure 3. Down-regulation of *hsa-miR-29c-3p* in response to IFN-beta therapy. The *hsa-miR-29c-3p* expression dynamics within the first month of IFN-beta treatment are presented. (**A**) TaqMan miRNA cards revealed reduced levels of this miRNA in the PBMC of 6 patients (Pat1-6, the main cohort); (**B**) Affymetrix miRNA arrays were then used to replicate the measurement for three of these patients (Pat1-3); (**C**) Finally, the down-regulation of *hsa-miR-29c-3p* was confirmed in an independent group of 12 patients (the validation cohort) using TaqMan single-tube assays. The Affymetrix analysis was based on hybridization of miRNA molecules to probes (probe set "*hsa-miR-29c_st*"), whereas the TaqMan analyses were based on real-time PCR. The TaqMan data are in linear scale, and the Affymetrix data are in log2 scale due to a different data preprocessing.



Three miRNAs of the mir-29 family were down-regulated one month after the start of therapy, and this was confirmed for two of them in an independent validation cohort of 12 patients (Figure 3). *hsa-miR-29a-3p* and *hsa-miR-29c-3p* were both expressed at relatively high levels (Supplementary File 3), and their mature sequences differ only in one base (Table 3). Therefore, it is likely that they play similar roles as post-transcriptional regulators. *hsa-miR-29a-3p* and *hsa-miR-29b-1-5p* belong to the same genomic cluster. Smith *et al.* demonstrated that IFN-gamma enhances the transcription of this *miR-29ab1* cluster, which acts in a negative feedback loop by regulating TBET and IFN-gamma [42]. Additionally, they showed decreased *hsa-miR-29b-3p* levels upon T cell activation in MS patients. This suggests a dysregulation of the feedback loop, which may bias T helper type 1 cell differentiation and may contribute to chronic inflammation [42]. Other studies also provided evidence that the members of the mir-29 family control innate and adaptive immune responses by targeting IFN-gamma [43,44]. The therapeutic down-modulation of mir-29 miRNAs might be mediated by NF-kappaB. The activation of NF-kappaB signaling, via ligation of Toll-like receptors, was shown to inhibit *miR-29ab1* expression [45]. Functionally, mir-29 promotes apoptosis, whereas repression of mir-29 levels is protective [45]. *hsa-miR-29a-3p* has been further shown to regulate myelin gene expression by Schwann cells [46].

hsa-miR-181c-3p was filtered as down-regulated during IFN-beta therapy. Several studies described the other strand of its pre-miRNA to be dysregulated in MS. Lower levels of *hsa-miR-181c-5p* were measured in PBMC [47] and in MS lesions [48] of patients in comparison to controls. On the other

hand, *hsa-miR-181c-5p* seems to be overabundant in the cerebrospinal fluid of patients with MS [49]. However, the biological processes that are influenced by the *hsa-mir-181c* miRNAs and their role in MS therapy remain largely unknown.

The expression of the mir-193 family members *hsa-miR-193a-3p* and *hsa-miR-193a-5p* was repressed during the therapy (Table 3). A study by Lindberg *et al.* demonstrated increased expression of *hsa-miR-193a-5p* in CD4+ T cells of RRMS patients compared to healthy subjects [50]. Otaegui *et al.* confirmed the potential relevance of this miRNA duplex in MS. Based on a co-expression network analysis, they postulated that *hsa-miR-193a-3p* is related to the remission stage of MS [51]. Moreover, the precursor molecule *hsa-mir-193a* was found to modulate apoptotic processes by promoting CASP3 activation induced by TNFSF10 signaling [52]. TNFSF10 (=TRAIL), in turn, is a known IFN-beta-induced gene and was transcriptionally up-regulated in the patients' PBMC (Supplementary File 2). The concomitant regulation of mir-193 miRNAs may thus contribute to the molecular mechanisms of action of IFN-beta.

Another miRNA caught our attention: *hsa-miR-223-3p* appeared to be the highest expressed miRNA in most PBMC samples (mean raw Ct value = 13.6). In a microarray study by Keller *et al.*, elevated levels of this miRNA were measured in the peripheral blood of RRMS patients as compared with healthy controls [53]. A significantly increased expression of *hsa-miR-223-3p* was later confirmed in PBMC from RRMS patients using real-time PCR [54]. Functionally, *hsa-miR-223-3p* modulates inflammatory responses by modulating the NF-kappaB pathway [55].

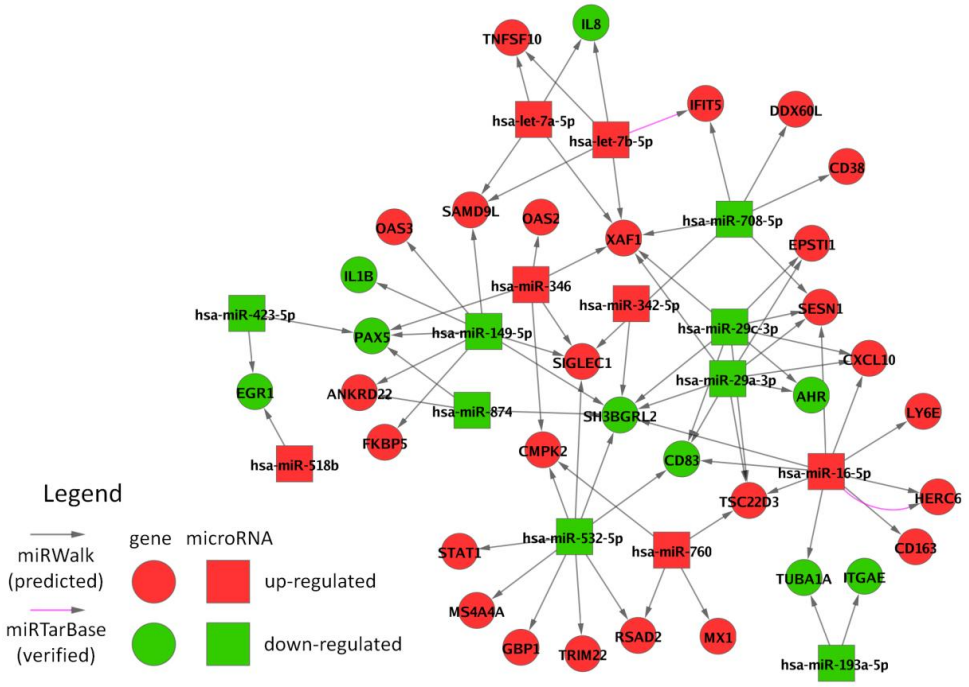
The remaining miRNAs identified in our study have so far not been mentioned in the context of MS, and their functions are poorly understood. miRNAs that have been repeatedly described to be differentially expressed in MS, e.g., *hsa-miR-142-3p*, *hsa-miR-146a-5p*, *hsa-miR-155-5p* and *hsa-miR-326* [22], were not contained in the filtering result, thus the therapy did not normalize their abnormal expression (*cf.* Waschbisch *et al.* [26]). Further studies are needed to decipher the immunological pathways involved, and to better understand the role of miRNA-dependent regulatory mechanisms in the immunopathogenesis of MS.

2.7. Interactions between Filtered MicroRNAs and mRNAs

Interactions between the 20 filtered miRNAs and the 95 filtered mRNAs were derived from two databases providing potential target genes of miRNAs. The miRWalk database [56] was used to obtain miRNA-mRNA interactions consistently predicted by multiple computational algorithms. This resulted in 74 potential interactions for 15 of the 20 filtered miRNAs. The miRTarBase database [57] was used to extract interactions with experimental evidence in the literature. This resulted in two verified interactions: *hsa-miR-16-5p* was identified as a post-transcriptional regulator of HERC6 in both databases. Moreover, there is an experimentally determined interaction between *hsa-let-7b* and IFIT5. IFIT5 is a known IFN-beta-induced gene. A recent study by Abbas *et al.* characterized IFIT5 as an innate immune effector molecule acting as a sensor of viral single-stranded RNAs [58]. This confers antiviral defense by inhibiting viral replication. However, IFIT5 also recognizes cellular RNAs, including tRNAs [59]. The full network of miRNA-mRNA target interactions is visualized in Figure 4. The most connected regulators in the network were *hsa-miR-16-5p* and *hsa-miR-532-5p* with

nine IFN-beta-responsive target genes each. *hsa-miR-29a-3p* and *hsa-miR-29c-3p*, which are closely related to each other, had eight predicted gene targets in common due to their similar mature sequences.

Figure 4. Verified and predicted interactions between IFN-beta-responsive microRNAs and mRNAs. The network of miRNA-target interactions between differentially expressed miRNAs and mRNAs was built using the databases miRWalk and miRTarBase. miRWalk reported results of 10 different prediction algorithms, and we only considered mRNA targets being computationally predicted by at least five of the 10 algorithms. miRTarBase provided experimentally validated miRNA-target interactions. In total, 15 of the filtered miRNAs were linked to 34 of the filtered genes by 74 predicted and two validated interactions. *hsa-miR-29a-3p* and *hsa-miR-29c-3p* (in the center-right) belong to the same miRNA family and are predicted to regulate eight target genes in common. The network is available as a Cytoscape session file (Supplementary File 6).



Down-regulated SH3BGRL2 and up-regulated XAF1 are simultaneously targeted by several ($n > 5$) miRNAs (miRNA target hubs [60]). Both genes have already been identified as differentially expressed in our previous studies on the effects of IFN-beta therapy [8,31] (Supplementary File 2). XAF1 is a critical mediator of IFN-beta-induced apoptosis. Its expression correlates with the cellular sensitivity to the pro-apoptotic actions of TNFSF10 [7,61]. However, while this supports the notion that miRNAs contribute to the mechanisms of action of IFN-beta, it should be noted that the majority of interactions in the network is predicted. For instance, the miRNA-mRNA interaction between *hsa-miR-16-5p* and SESN1 is predicted by nine out of 10 algorithms implemented in the miRWalk database, but has not yet been experimentally demonstrated. Another limitation is that we did not

analyze whether the miRNAs bind their target mRNAs at multiple sites. Such a cooperative regulation through repetitive elements in the 3' UTR can increase repression efficacy [62]. Additional studies are needed to validate the miRNA targets, e.g., by luciferase assays.

Opposing effects exist in the network since several mRNAs are targeted by down-regulated and up-regulated miRNAs (Figure 4). Moreover, the network does not include the effects of the transcription factors (TF), which are activated through the IFN-beta signaling pathway and which are known to regulate the expression of most of the genes [31,63]. Therefore, it is difficult to disentangle the effects of miRNA expression changes on the mRNA levels measured during therapy. Further studies are thus required, e.g., specific miRNA transfection experiments examining the impact on potential target genes at both the mRNA and protein level.

Our network analysis was limited to the set of filtered genes, and the many other potential target genes of the filtered miRNAs were out of scope. Moreover, we did not investigate whether the miRNAs target viral RNAs, which is also worth to be explored in detail [28]. Despite these limitations, we conclude that miRNA-mediated regulation plays an important role in the pleiotropic effects of IFN-beta in normal immune responses as well as in the treatment of MS.

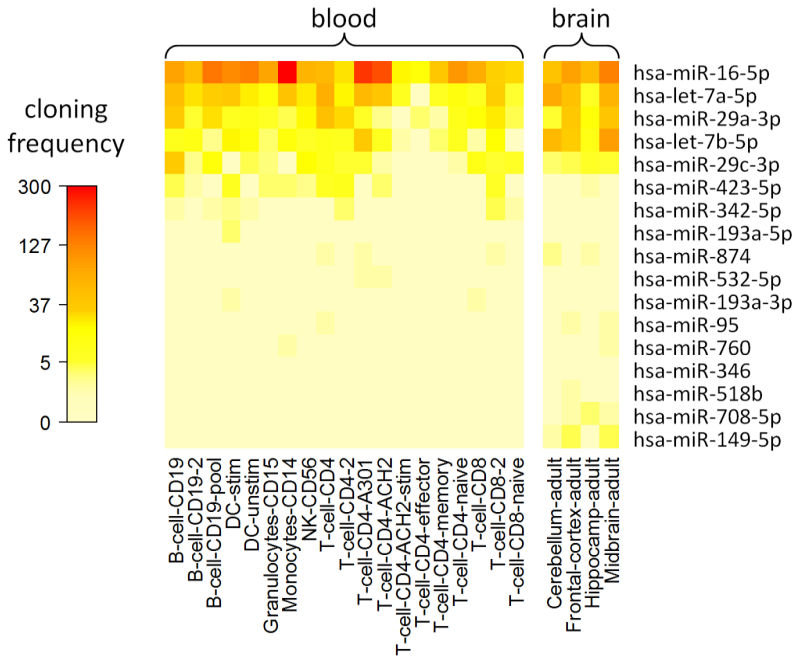
2.8. Cell Type-Specific Expression of IFN-beta-Responsive MicroRNAs

We analyzed whether the miRNA expression changes during therapy affect the gene expression in different cell populations involved in the disease. This was done by comparing the cell type-specific expression of the miRNAs using information from the smirnaDB database [64] (Figure 5). Of the filtered miRNAs, *hsa-miR-16-5p* is highly expressed among diverse blood cells, in particular monocytes and CD4+ T cell lines, whereas *hsa-miR-149-5p* is only detected in brain (Figure 5). Accordingly, in our time course data set, *hsa-miR-16-5p* was expressed at very high levels, and *hsa-miR-149-5p* was expressed at relatively low levels (Supplementary File 3). Several miRNAs, however, were not detected in some cell populations (clone count = 0) due to the limited sensitivity of the measurement technique used to generate these data [65]. More sensitive methods could be used to further analyze the cell type-specific roles of selected miRNAs in MS and therapy, e.g., *hsa-miR-29a-3p* and *hsa-miR-29c-3p*, which were expressed in B cells.

2.9. Final Remarks and Perspectives

The cellular regulation of the miRNAs is still not well understood. Many type I IFN-responsive genes harbor in their promoter region a specific sequence motif, the IFN-stimulated response element (ISRE), which is bound by IFN-activated TFs [7]. However, when we searched miRGen 2.0, a database of TF binding sites for miRNA transcripts [66], there was only one miRNA (*hsa-mir-203a*) that was predicted to have an ISRE located near the transcription start site. It is conceivable that some of the filtered miRNAs are regulated at the RNA processing level rather than at the transcriptional level. Several post-transcriptional mechanisms can affect mature miRNA biogenesis and stability [67]. Recent studies provided evidence that miRNAs can be suppressed by circular RNAs, which act as natural miRNA sponges [68]. On the other hand, the localization of the miRNAs might be altered, e.g., by microparticle shedding [69]. Additional research is needed to elucidate how the activity of miRNAs might be modulated during therapy.

Figure 5. Expression of 17 filtered miRNAs in different cell populations. The heat map visualizes the levels of IFN-beta-responsive miRNAs in 19 blood cell populations and four brain tissues. The data were downloaded from the smirnaDB database [64], which did not contain three of the 20 filtered miRNAs (*hsa-miR-27a-5p*, *hsa-miR-29b-1-5p* and *hsa-miR-181c-3p*). *hsa-let-7a-5p* and *hsa-miR-16-5p* are highly expressed in peripheral blood and brain. *hsa-let-7b-5p* and *hsa-miR-149-5p* are preferentially expressed in brain tissues. Several of the miRNAs (e.g., *hsa-miR-346*) were not detected in certain cell types [65].



Currently, there is a clear lack of studies investigating the changes in miRNA expression during MS therapy. Apart from the study by Waschbisch *et al.* [26] (see Section 1), there is only another study by Sievers *et al.*, who found differentially expressed miRNAs in B cells of patients treated with natalizumab [41]. Future miRNA profiling analyses should use a longitudinal design and address both the short-term and the long-term effects of the available treatments. Such studies may also help to understand why some patients continue to have clinical relapses, disability progression or active lesions despite therapy. Defining the individual response to treatment is difficult, but miRNAs may have the potential to be used as prognostic biomarkers, thereby facilitating improved patient care. The identification of miRNA biomarkers should be supported by functional studies on how miRNAs affect complex biological processes by targeting multiple genes in different cell types.

3. Experimental Section

3.1. Samples

Fifteen milliliters peripheral venous EDTA blood samples were taken from six patients (Table 1) immediately before first (baseline), second, and third IFN-beta injection as well as after one month.

For validation, further blood samples were taken from an independent cohort of 12 patients (Supplementary File 4) before and one month after the start of IFN-beta-1b sc. therapy. The samples were always processed within one hour. PBMC were separated by isopycnic centrifugation in Ficoll density gradients, and total RNA enriched with small RNAs was isolated using the mirVana miRNA isolation kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturers' protocols. The study was approved by the University of Rostock's ethics committee and carried out according to the Declaration of Helsinki. Written informed consent was obtained from all patients before study onset.

3.2. Gene Expression Profiling Using Microarrays

To quantify the mRNA levels, total RNA from each of the 24 PBMC samples of the main study cohort was labeled and hybridized to Affymetrix microarrays. Biotinylated cRNA were prepared according to the standard Affymetrix 3' IVT protocol from 200 ng total RNA (Expression Analysis Technical Manual; Affymetrix, Santa Clara, CA, USA). Following fragmentation, 15 µg of cRNA were hybridized for 16 h at 45 °C on Affymetrix GeneChip Human Genome U133 Plus 2.0 Arrays. The microarrays were washed and stained in the Affymetrix Fluidics Station 450, and scanned using the Affymetrix GeneChip Scanner 3000 (Affymetrix, Santa Clara, CA, USA).

3.3. MicroRNA Expression Analysis Using Real-Time PCR

To quantify the miRNA expression levels, we used the TaqMan Array Human MicroRNA A + B Cards Set v2.0 (Applied Biosystems, Foster City, CA, USA), which consists of two 384-well plates with TaqMan assay reagents. These plates contain 720 assays to measure 651 different human miRNAs. Moreover, there are 30 assays for positive controls, 2 assays for negative controls, and 16 assays were discarded as they link to dead miRNA entries in the miRBase database (release 17) [17]. Total RNA (120 ng) from each sample (n = 24) was reverse transcribed to cDNA using Megaplex RT Primers in combination with Megaplex PreAmp Primers (Life Technologies, Carlsbad, CA, USA). The real-time PCR measurements were then performed with predesigned primers and TaqMan probes with 45 cycles according to the manufacturer's instructions in a 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Raw Ct values were computed using the SDS 2.3 and RQ Manager 1.2 software (Applied Biosystems, Foster City, CA, USA), and undetermined data were set to Ct = 45.

3.4. Validation MicroRNA Analysis Using Microarrays

For validation, we replicated the miRNA expression measurements of the PBMC from three patients (Pat1-3) at two time points (before the first IFN-beta injection as well as after one month). Total RNA of these six samples was labeled and hybridized to Affymetrix GeneChip miRNA 2.0 arrays. Biotinylated RNA was prepared using the FlashTag Biotin HSR RNA labeling kit according to the standard Affymetrix protocol from 600 ng total RNA (Expression Analysis Technical Manual; Affymetrix). Following fragmentation, the biotin-labeled RNA was hybridized for 16 h at 45 °C on Affymetrix miRNA 2.0 arrays. The microarrays were washed and stained in the Affymetrix Fluidics Station 450, and scanned using the Affymetrix GeneChip Scanner 3000.

3.5. Validation MicroRNA Analysis Using Real-Time PCR

To verify the results in an independent cohort of patients, we measured 5 of the 20 filtered miRNAs in PBMC samples from 12 additional patients (Supplementary File 5). The blood samples were obtained before and after one month of IFN-beta-1b treatment. The miRNAs were selected based on a combination of different criteria, e.g., change of expression after one month according to both the TaqMan miRNA array data and the Affymetrix miRNA microarray data. The validation experiment was performed using TaqMan single-tube assays for *hsa-miR-16-5p* (Assay ID 000391), *hsa-miR-29a-3p* (Assay ID 002112), *hsa-miR-29c-3p* (Assay ID 000587), *hsa-miR-149-5p* (Assay ID 002255), and *hsa-miR-532-5p* (Assay ID 001518). Additionally, the housekeeping miRNA *hsa-miR-191-5p* (Assay ID 002299) was measured for normalization [33]. For each assay, 10 ng of total RNA from each sample (n = 24) were used to convert an individual miRNA to cDNA using an RT primer specific for the miRNA of interest (Applied Biosystems). The real-time PCR quantitation was performed in triplicates with predesigned primers and TaqMan probes according to the TaqMan MicroRNA Assay protocol with 45 cycles in a 7900HT Sequence Detection System (Applied Biosystems). An equivalent of 0.5 ng total RNA was used to obtain a single data point. Raw Ct values were computed using the SDS 2.3 and RQ Manager 1.2 software (Applied Biosystems).

3.6. Expression Data Preprocessing

In the case of the Affymetrix U133 Plus 2.0 gene expression microarrays, the raw probe-level signals were converted to expression values (signal intensities) using the MAS5.0 algorithm and custom GeneAnnot-based chip definition files (version 2.2.0) [70]. Data normalization was performed by loess normalization using the R package “affy”. Each Affymetrix GeneChip yielded mRNA levels of 19,204 human genes.

In case of the TaqMan miRNA cards, we first set the detection limit at Ct = 38 [71], and converted the raw Ct values to the linear scale using the equation $2^{-Ct} \times 10^{-9}$. After this step, a Ct value of 38 corresponds to an expression signal of 0.004, and a Ct value of 20 corresponds to an expression signal of 953. Systematic differences in the time course data were then corrected by loess normalization. This was done separately for each patient (n = 6) and each card (A and B). Finally, to reduce variation in the expression signals between the patients, we scaled the data of card A and of card B so that the respective 95% quantile was the same for each patient.

In case of the Affymetrix miRNA microarrays, the raw signals were converted to expression values using the RMA algorithm with quantile normalization. For each chip, this resulted in log-transformed expression levels for 20,706 probe sets interrogating small non-coding RNA transcripts. For each of the 20 filtered miRNAs (Table 3), there was one designated probe set.

In case of the TaqMan single-tube real-time PCR miRNA assays, we set the detection limit at Ct = 38 [71], calculated the mean Ct value of each triplicate, converted the raw Ct values to the linear scale using the equation 2^{-Ct}, normalized the results to the expression values of the housekeeping miRNA *hsa-miR-191-5p* [33], and scaled these ratios by a factor of 1000 for convenience.

The non-normalized and the normalized expression data of the six patients receiving IFN-beta therapy are available in the Gene Expression Omnibus (GEO) database via the SuperSeries record

GSE46293. This GEO entry links to all data from the Affymetrix U133 Plus 2.0 microarrays, the TaqMan MicroRNA Cards Sets v2.0, and the Affymetrix miRNA 2.0 microarrays.

3.7. Filtering of Differentially Expressed mRNAs and MicroRNAs

We filtered the Affymetrix U133 Plus 2.0 and TaqMan miRNA cards data sets for IFN-beta-responsive genes and miRNAs by comparing the PBMC expression levels immediately before treatment initiation (baseline) with the expression levels two days, four days, and one month after the start of IFN-beta therapy. Up-regulation and down-regulation of genes and miRNAs were quantified using signal intensity-dependent fold-changes (MAID-scores) as described in our previous studies [8,34] (see also http://www.hki-jena.de/index.php/0/2/490). MAID-scores represent adjusted fold-changes, where a higher fold-change (*i.e.*, relative change in expression) is required for genes expressed at low levels than for genes expressed at high levels. This is realized by an exponential function (MAID regression curve) that is fitted to the signal intensity-dependent variation in the data. For each time point comparison and each type of array, we computed the MAID-score for all patients and for all measured genes and miRNAs. We then selected the genes and miRNAs being up-regulated (MAID-score above the cutoff C) or being down-regulated (MAID-score $< -C$) in at least four of the six patients. We chose $C = 2$ for the gene expression data set, and $C = 1$ for the miRNA expression data set. For the latter, a lower MAID-score cutoff was chosen because the larger variation in the miRNA data already leads to a higher MAID regression curve.

To provide an estimate of the number of genes and miRNAs passing the MAID filtering by chance, a permutation test was performed. The data sets were permuted 1000 times by randomly rearranging the temporal sequence of the data of each patient. The same filtering criteria as described above were then applied to each permutation.

As an alternative filtering criterion, we statistically compared the PBMC expression levels of mRNAs and miRNAs before and during treatment using paired *t*-tests (Supplementary File 2 and Supplementary File 3). However, considering the relatively small number of patients in our study, the MAID filtering method is thought to be more robust to the variation in the data.

3.8. Visualization of the mRNA Expression Data

A heat map was used to visualize the expression changes of the 95 filtered mRNAs (Figure 1). The heat map displays the respective data of all 6 patients before the start of therapy and after one month. To reorder the rows and columns of the data matrix, hierarchical clustering was performed with the single linkage method and Pearson's correlation coefficient as a measure of similarity. For visualization purposes, the expression values (signal intensities) were centered and scaled row-wise (resulting in z-scores) with the standard R heat map function.

3.9. Evaluation of the MicroRNA Validation Data

Spearman's rank correlation coefficient (rho) was calculated to evaluate whether the TaqMan miRNA array data correlate with the Affymetrix miRNA microarray data (120 data pairs: 20 miRNAs, three patients, and two time points). Paired *t*-tests were computed for assessing the difference in

expression after one month of IFN-beta-1b sc. therapy versus baseline in the preprocessed and normalized data of the Affymetrix microarrays (Pat1-3) and of the TaqMan single-tube assays (n = 12 additional patients).

3.10. Interaction Network Analysis

We studied the regulatory interactions between the miRNAs and their target mRNAs by integrating the information from two different databases. Experimentally verified and computationally predicted target genes of the 20 IFN-beta-responsive miRNAs were extracted from the databases miRTarBase (version 3.5) [57] and miRWalk (April 2013) [56], respectively. miRWalk contained predictions for all 20 filtered miRNAs, and miRTarBase contained interactions for 9 of the 20 miRNAs. For the prediction of targets with miRWalk, we applied the option of the web server to run the calculations with 10 different prediction algorithms on 3' UTRs of all human genes, and then gathered only the miRNA-mRNA interactions that were predicted by at least 5 of the 10 algorithms. Finally, interactions being associated with the 95 filtered genes were visualized as a network using the Cytoscape software (version 2.8.0) [72].

3.11. MicroRNA Expression in Different Cell Populations

To investigate the expression of the filtered miRNAs in different peripheral blood cell types and brain regions, we used the smirnaDB database, which provides expression levels of 692 human miRNAs for 170 cell populations and tissues [64]. This miRNA expression atlas is based on sequence analysis of small RNA clone libraries [65]. The relative cloning frequencies of miRNAs represent a measure of miRNA expression. However, in this data set, many miRNAs were identified at very low clone counts (*cf.* Landgraf *et al.* [65]). The data for 19 blood cell types (including three CD4+ T cell lines) and four brain tissues were downloaded from smirnaDB and visualized as a heat map in the R software environment.

4. Conclusions

To our knowledge, this is the first longitudinal genome-wide study examining the *in vivo* effects of IFN-beta treatment on miRNA expression in blood cells of patients with CIS or RRMS. The strongest changes in mRNA and miRNA expression were detected one month after the start of IFN-beta-1b sc. treatment. We observed that the induction of IFN-beta-responsive genes is paralleled by a preferential down-regulation of miRNAs. This suggests that the regulation of miRNAs contributes to the molecular mechanisms of action of IFN-beta in protective immune responses as well as in MS therapy. We confirmed the down-regulation of *hsa-miR-29a-3p*, *hsa-miR-29c-3p*, and *hsa-miR-532-5p* in an independent cohort of patients. We further analyzed the interactions between differentially expressed miRNAs and mRNAs. The largest number of predicted interactions to IFN-responsive genes was found for *hsa-miR-532-5p* and *hsa-miR-16-5p*. Up-regulated *hsa-miR-16-5p* was expressed at very high levels in different cell types of the blood, in particular monocytes. However, unraveling the complex gene regulatory interactions between TFs, miRNAs and genes remains a big challenge for the future. Functionally, some of the 20 filtered miRNAs (e.g., members of the mir-29 family) are

associated with apoptosis and are involved in IFN signaling feedback loops. miRNA expression profiles in blood cells may provide biomarkers for monitoring the biological response to therapy to predict individual disease activity and progression. They may also help to better understand the pathogenetic mechanisms and to optimize the treatment of MS. Our results provide a rationale for subsequent studies in larger MS cohorts.

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Conflict of Interest

U.K. Zettl received research support as well as speaking fees from Bayer HealthCare, Biogen Idec, Merck Serono, Novartis, Sanofi, Almirall and Teva. M. Hecker received speaking fees from Bayer HealthCare, Novartis and Teva. M. Thamilarasan, D. Koczan, I. Schröder, K. Flechtner, S. Freiesleben, G. Füllen and H.-J. Thiesen declare no potential conflicts of interest.

Supplementary Information

Supplementary File 1 (TIFF image): Distribution of the raw Ct values measured with the TaqMan miRNA arrays. The data of the A-cards (A) and the B-cards (B) are shown for all six patients and all four time points. The detection limit was set at Ct = 38.

Supplementary File 2 (XLS Excel spreadsheet): mRNA filtering result. This table provides the 95 genes that were up-regulated or down-regulated in the PBMC of the patients two days, four days or one month after the initiation of subcutaneous IFN-beta-1b therapy. Different types of information are given for each gene, e.g., gene symbol, official full name, Entrez Gene identifier, and the MAID filtering results.

Supplementary File 3 (XLS Excel spreadsheet): microRNA filtering result and validation. In comparison to pre-treatment levels, 20 miRNAs were found to be expressed at higher or lower levels in PBMC during IFN-beta treatment. The table provides, e.g., the TaqMan Detector identifiers, the MAID filtering outputs as well as the results from the validation analyses.

Supplementary File 4 (XLS Excel spreadsheet): Clinical data and demographic data of the patients in the validation cohort. Twelve patients were recruited to confirm the observed expression changes of five selected miRNAs within the first month of IFN-beta-1b sc. treatment.

Supplementary File 5 (XLS Excel spreadsheet): Validation real-time PCR data set. TaqMan single-tube assays were used to quantify the expression of five selected miRNAs before the start of IFN-beta therapy and after one month in PBMC of an independent cohort of 12 patients

(Supplementary File 4). This table contains the raw Ct values of these five miRNAs and the housekeeping miRNA *hsa-miR-191-5p* [33]. The measurements were done in triplicates.

Supplementary File 6 (CYS Cytoscape session file): miRNA-mRNA interaction network. This Cytoscape file (http://www.cytoscape.org) [72] contains computationally predicted and experimentally determined interactions between IFN-beta-responsive miRNAs and mRNAs. The interactions were obtained from the databases miRWalk [56] and miRTarBase [57]. A visualization of the network is shown in Figure 4.

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8.3 Manuscript - 3

Integration of microRNA databases to study microRNAs associated with multiple sclerosis

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Integration of MicroRNA Databases to Study MicroRNAs Associated with Multiple Sclerosis

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Abstract MicroRNAs (miRNAs) are small non-coding RNAs which regulate many genes post-transcriptionally. In various contexts of medical science, miRNAs gained increasing attention over the last few years. Analyzing the functions, interactions and cellular effects of miRNAs is a very complex and challenging task. Many miRNA databases with diverse data contents have been developed. Here, we demonstrate how to integrate their information in a reasonable way on a set of miRNAs that were found to be dysregulated in the blood of patients with multiple sclerosis (MS). Using the miR2Disease database, we retrieved 16 miRNAs associated with MS according to four different studies. We studied the predicted and experimentally validated target genes of these miRNAs, their expression profiles in different blood cell types and brain tissues, the pathways and biological processes affected by these miRNAs as well as their regulation by transcription factors. Only miRNA–mRNA interactions that were predicted by at least seven different prediction algorithms were considered. This resulted in a network of 1,498 target genes. In this network, the MS-associated miRNAs hsa-miR-20a-5p and hsa-miR-20b-5p occurred as central hubs regulating about 500 genes each. Strikingly, many of the putative target genes play a role in T cell activation and signaling, and many have transcription factor activity. The latter suggests that miRNAs often act as regulators of regulators with many secondary effects on gene expression. Our present work provides a guideline on how information of different databases can be integrated in the analysis of miRNAs. Future investigations of miRNAs shall help to better understand the mechanisms underlying different diseases and their treatments.

Keywords Multiple sclerosis · MicroRNA · Databases · Gene regulatory networks

Abbreviations

CIS	Clinically isolated syndrome
CNS	Central nervous system
EAE	Experimental autoimmune encephalomyelitis
GO	Gene Ontology
miRNA	MicroRNA
MS	Multiple sclerosis
NGS	Next generation sequencing
PBMC	Peripheral blood mononuclear cells
pre-miRNA	Precursor-miRNA
pri-miRNA	Primary-miRNA
PWM	Position weight matrix
RISC	RNA-induced silencing complex
RRMS	Relapsing-remitting multiple sclerosis
SNP	Single nucleotide polymorphism
TF	Transcription factor
TFBS	Transcription factor binding site
Treg cell	T regulatory cell

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Introduction

Multiple sclerosis (MS) is a chronic, immune-mediated disease leading to demyelination and axonal loss in the central nervous system (CNS). The inflammations are transient, but post-inflammatory gliosis and neurodegeneration, causing multifocal sclerotic plaques primarily in the white matter of the CNS, can lead to functional deficits and permanent disability. The course of this disease is unpredictable, and relapsing forms are as well known as progressive courses. The definite pathogenesis of MS remains unclear, but environmental factors, immune dysregulation, and genetic predispositions play significant roles [1–3]. Many researchers studied the genomics, transcriptomics, proteomics, and metabolomics of this disorder to unravel potential pathological mechanisms. One experimental approach is the analysis of the patients' individual gene expression in the course of the disease and during therapy to reconstruct and better understand the underlying molecular networks [4, 5]. In 1993, a new class of RNAs called microRNAs (miRNAs) was detected in *Caenorhabditis elegans* [6] and also later in humans [7]. Meanwhile, alterations in the cellular expression levels of miRNAs were linked to the pathogenesis of a variety of diseases [8, 9] and several groups have identified miRNAs associated with MS [10–13].

miRNAs are small (~22 nt) RNA molecules that post-transcriptionally regulate gene expression. They are transcribed usually by RNA polymerase II with protein-coding or non-coding genes [14]. It is assumed that approximately 35 % of mammalian miRNA loci overlap with annotated protein-coding genes, with 90 % of these being located in the introns [15]. The initial transcript, called the primary-miRNA (pri-miRNA), can be thousands of nucleotides in length and contains one or more stem-loop structures that harbor the mature miRNAs. In the nucleus, the ribonuclease III (RNaseIII) Drosha processes the pri-miRNAs to hairpin-structured precursor-miRNAs (pre-miRNAs), which are typically 70–110 nt in length. After a pre-miRNA is actively transported to the cytoplasm, the RNaseIII Dicer develops a miRNA–miRNA* duplex (~20 bp) out of this pre-miRNA and links one strand (the future mature miRNA) to an RNA-induced silencing complex (RISC). The other strand (miRNA*) either degrades or sometimes becomes a second mature miRNA [14, 16–18]. Finally, binding usually the 3′ UTR of a target mRNA, mature miRNAs lead to the cleavage or to the translational repression of the mRNA, depending on the degree of sequence complementary [14]. This post-transcriptional process contributes to the regulation of many protein-coding genes with diverse biological functions and thus has implications for various diseases, [9].

It is assumed that one miRNA is able to regulate hundreds of mRNA targets [19]. However, up to the present, only few microRNA–mRNA interactions have been experimentally validated, e.g., by luciferase reporter assays. Hence, computational tools are commonly used to predict putative target genes [20–22]. miRNAs contain a special region (the seed region) located in the 5′ end of the strand (2nd–8th nt) that plays an important role for target recognition. This fact is widely used in bioinformatic target prediction algorithms. Other frequently used properties for target prediction are the thermodynamics of the microRNA–mRNA interaction and the evolutionary conservation of the target sites [20–22]. In the last few years, due to the growing interest in miRNAs, there has been a great development of databases collecting, organizing, and providing specific miRNA information. Several different types of databases exist, e.g., databases with general information on miRNAs (such as sequence and genome position), databases with information on potential mRNA targets, and databases associating miRNAs with specific diseases (Table 1) [23–42].

In this review, we provide an insight into the different existing miRNA databases and illustrate how to integrate their information for examining (a) the network-like interactions of miRNAs with mRNA targets, (b) the effects of miRNAs on cellular functions, and (c) their regulation by transcription factors (TFs) leading to tissue-specific expression. As a case study, we will focus on miRNAs that have been mentioned in the context of MS.

MicroRNA Databases

One of the central public databases with general information on miRNAs is **miRBase**. In version 18, miRBase contains 1,527 different human pre-miRNAs with 1,921 different mature miRNA entries. It offers details about current miRNA nomenclatures, sequences, genomic locations, precursor forms, and literature references [28, 33]. Potential target genes of miRNAs are not contained in miRBase, but other databases have been implemented to provide such information (Table 1). Some databases collect miRNA–mRNA interactions from the current literature in PubMed and list them as experimentally validated targets, e.g., **miRTarBase** [31] and **TarBase** [39]. miRTarBase version 2.5 contains 285 human miRNAs, having 2,860 manually curated interactions with 1,721 different genes. It has more entries and is updated more regularly than TarBase.

Since the amount of validated targets is relatively small, many other databases use computational algorithms for target prediction. Seed matches, conservation of the sequence and thermodynamics of the miRNA–mRNA interaction are widely used for predicting potential targets [20–22]. The disadvantage of this approach is the enormous number of possible targets, and therefore results may contain a lot of

Table 1 Overview of different types of microRNA databases

Content	Database	Reference	Last update	Web address
General information	**miRBase**	[28]	Nov 2011	http://www.mirbase.org
Experimentally verified mRNA targets	miRecords	[42]	Nov 2010	http://mirecords.biolead.org
	miRTarBase	[31]	Oct 2011	http://mirtarbase.mbc.nctu.edu.tw
	TarBase	[39]	Jun 2008	http://diana.cslab.ece.ntua.gr/tarbase/
Computationally predicted mRNA targets	DIANA-microT	[38]	Jul 2009	http://diana.cslab.ece.ntua.gr/microT/
	MicroCosm	[28]	Oct 2007	http://www.ebi.ac.uk/enright-srv/microcosm/
	microRNA.org	[24]	Aug 2010	http://www.microrna.org
	miRWalk	[25]	Mar 2011	http://www.ma.uni-heidelberg.de/apps/zmf/mirwalk/
	PicTar	[34, 35]	Mar 2007	http://pictar.mdc-berlin.de
	TargetScan	[36]	Mar 2012	http://www.targetscan.org
Transcription factor binding sites	**miRGen**	[23]	Nov 2009	http://diana.cslab.ece.ntua.gr/mirgen/
	CircuitsDB	[26]	Jan 2011	http://biocluster.di.unito.it/circuits/
Single nucleotide polymorphisms	**miRNASNP**	[27]	Aug 2011	http://www.bioguo.org/miRNASNP/
Expression data sets	miRDB	[41]	Jan 2012	http://www.mirdb.org/wiki/index.php5
	smirnaDB	[30]	May 2009	http://www.mirz.unibas.ch/cloningprofiles/
Association to diseases	**miR2Disease**	[32]	Mar 2011	http://www.mir2disease.org
	HMDD	[37]	Mar 2012	http://cmbi.bjmu.edu.cn/hmdd
	PhenomiR	[40]	Feb 2011	http://mips.helmholtz-muenchen.de/phenomir/

As of version 18, miRBase contains 1,921 distinct human mature miRNA entries. It is a central source of diverse types of general information including miRNA nomenclatures, sequences, genomic locations and families. Numerous databases provide experimentally verified miRNA targets and computational algorithms for target prediction. The table gives only selected examples of such databases. Further databases offer information about miRNA expression in different tissues and cell types, literature-derived associations of miRNAs with diseases, and potential transcription factor binding sites for investigating the transcriptional regulation of miRNAs. Databases that were used for the analyses presented in this review are written in bold

false positives. **TargetScan** [19, 36], **DIANA-microT** [38], **PicTar** [29, 34, 35], **MicroCosm** [28], and **microRNA.org** [24] are the most established and commonly used miRNA–mRNA interaction databases. Of these, MicroCosm and microRNA.org are based on the same algorithm called miRanda [43]. There are also databases collecting and providing both, validated targets and predicted targets of other databases, showing the differences between the predictions, e.g., **miRecords** [42] and **miRWalk** [25]. miRWalk is a database with its own target prediction algorithm, but also allows to simply compare the results with nine other prediction algorithms. Demanding a target to be predicted by several algorithms can be very useful to reduce the high number of putative and maybe false positive targets. Furthermore, miRWalk is so far the only database offering target predictions outside the 3′ UTR as well. This option may be useful since recent studies have shown that miRNAs in some cases regulate also via 5′ UTR regions, promoter regions, or amino acid coding regions [44–46]. It is important to note that the information of miRNA–mRNA interaction databases can be used in two ways: (a) to retrieve potential gene targets of miRNAs and (b) to identify for a list of genes those miRNAs that are assumed to regulate them. For the latter analysis, web tools such as FatiGO of the Babelomics platform exist [47, 48].

Another way of investigating miRNAs is to look up potential connections to human diseases. For this purpose, databases such as **miR2Disease** [32] and **PhenomiR** [40] deliver curated information from the literature in PubMed about associations of miRNAs with disorders. Some other databases provide tissue- and cell type-specific expression profiles of miRNAs, which might be useful to elucidate cellular functions of miRNAs. **miRDB** uses the miRNA expression data set by Liang et al. [49] on 40 normal human tissues and the results (miRNA copies per cell) can be shown in a table, besides other general information [41]. Another expression profile providing database can be found on the MirZ web server [30], which contains the expression atlas **smirnaDB** that is based on the data by Landgraf et al. [50]. In contrast to miRDB, smirnaDB even provides miRNA expression levels of pathological tissues and cell types [30]. **miRGen 2.0** [23] is a database that also

integrates the expression data of Landgraf et al. [50]. Furthermore, miRGen 2.0 lists single nucleotide polymorphisms (SNPs) at the pre-miRNA location and transcription factor binding sites (TFBS) in the promoter region of pri-miRNA transcripts. For the determination of potential TFBS, miRGen 2.0 relies on the tool MatchTM [51], utilizing the public library of TF position weight matrices (PWMs) from the Transfac database (version 6.0) [52]. By uncovering which TFs are likely involved in the transcription process of miRNAs, miRGen 2.0 helps revealing the mutual regulatory relationships between miRNAs and TFs. Finally, **miRNASNP** [27] provides a resource of SNPs in pre-miRNAs and their flanking regions based on the dbSNP database build 132. SNPs can affect the miRNA-mediated regulatory functions and, as a consequence, can be related to immune-mediated diseases such as MS.

MicroRNAs in Multiple Sclerosis

In miR2Disease (as of March 2012), 16 different miRNAs are listed to be associated with MS according to four different studies (Table 2) [53–57].

Keller et al. [56] examined the differences in the miRNA expression profiles in peripheral blood cells between relapsing-remitting MS (RRMS) patients and healthy controls with the help of oligonucleotide microarrays. In their study, ten miRNAs were found to be significantly dysregulated in the MS group (hsa-miR-20b-5p, hsa-miR-142-3p, hsa-miR-145-5p, hsa-miR-186-5p, hsa-miR-223-3p, hsa-miR-422a, hsa-miR-491-5p, hsa-miR-584-5p, hsa-miR-664-3p, and hsa-miR-1275). Five of these miRNAs have already been associated with other diseases [55].

Otaegui et al. [57] focused on the relevance of miRNAs in the relapse stage of MS patients. Peripheral blood mononuclear cells (PBMC) were taken from patients during relapse and during remission as well as from healthy subjects, and the expression levels of miRNAs were measured with the help of TaqMan real-time PCR arrays. Their data analysis revealed increased expression levels of hsa-miR-18b-5p and hsa-miR-599 in the relapsing patient group compared to the healthy group. Additionally, elevated levels of hsa-miR-96-5p were described as characteristic of the remitting phase of the disease [57].

Du et al. [54] analyzed peripheral blood leukocyte samples of RRMS patients with real-time PCR. They found particularly in the CD4+ T cell population an increased expression of hsa-miR-326 in relapsing patients, but not in remitting patients. In the same study, they also investigated this miRNA in mice with experimental autoimmune encephalomyelitis (EAE), an animal model of MS. Overexpression of hsa-miR-326 resulted in severe EAE with many T helper 17 (Th17) cells, while to the contrary, in vivo silencing of hsa-miR-326 resulted in a milder form of EAE and less Th17 cells. In this way, Du et al. [54] could show an influence of hsa-miR-326 on Th17 cell differentiation with a potential contribution to the pathogenesis of MS.

Cox et al. [53] analyzed about 700 miRNAs in whole blood samples of therapy-naïve patients with different MS subtypes and healthy controls with an Illumina Sentrix array matrix. In all subtypes of MS, hsa-miR-17-5p and hsa-miR-20a-5p were significantly underexpressed in comparison to the healthy group. Additionally, Cox et al. [53] confirmed these results with real-time PCR and provided evidence that hsa-miR-17-5p and hsa-miR-20a-5p are regulators of genes involved in T cell activation.

We analyzed the genomic location of the 16 MS-associated mature miRNAs with the help of miRBase and noticed that 5 of the 16 miRNAs are located intronically (hsa-miR-186-5p, hsa-miR-326, hsa-miR-491-5p, hsa-miR-584-5p, hsa-miR-664-3p). This reflects the fact that roughly one third of miRNA loci overlaps with introns of protein-coding genes [15]. The other 11 miRNAs are located in non-coding genes, and none of the miRNAs has multiple genomic loci, i.e., each miRNA is produced from only one pre-miRNA. The pri-miRNA is known for only 8 of the 16 miRNAs (Table 2). pri-miRNAs are often not well annotated because they are short-lived and present only inside the nucleus, which makes it difficult to characterize them experimentally [58]. Some of the 16 miRNAs are organized in clusters, which means that they are transcribed with the same pri-miRNA. The two miRNAs, hsa-miR-17-5p and hsa-miR-20a-5p, belong to the miR-17~92 cluster [59], and hsa-miR-18b-5p and hsa-miR-20b-5p belong to the miR-106a~363 cluster [60]. Interestingly, the miR-106a~363 cluster is highly homologous to the miR-17~92 cluster. Both clusters contain similar and even identical miRNAs, which are subsumed in miRBase as one family of miRNAs with similar target genes and functions [59–61]. We found for 5 of the 16 miRNAs at least one SNP in the genomic region of the pre-miRNA in the miRNASNP database [27]. The SNP described for hsa-miR-664-3p (rs113256801) is even in the region of the mature miRNA (Table 2).

Although miR2Disease is useful for gaining an insight into MS-associated miRNAs, it has to be mentioned that not all current studies dealing with miRNAs in MS are already included in this database. In particular, studies published after the year 2010 are missing in miR2Disease. We shortly introduce some of the additional miRNAs that have been related to MS, although we excluded them from our further investigations, focusing on the 16 miRNAs listed in miR2Disease. In the study by Junker et al. [62], miRNA profiles of active and inactive CNS lesions of MS patients were analyzed and compared to control specimens. This led to the detection of different up-regulated miRNAs (e.g., hsa-miR-34a-5p, hsa-miR-155-5p, and hsa-miR-326) in active MS lesions. Recently, Noorbakhsh et al. [63] observed

Table 2 Details of miRNAs associated with MS according to miR2Disease

Mature miRNA Symbol	Mature miRNA Sequence	Reference	Pattern MS vs Ctr	miRNA family	pre-miRNA Symbol	pri-miRNA Symbol	Category	pre-miRNA SNPs
hsa-miR-17-5p	CAAAGUGCUUACAGUGCAGGUAG	Cox et al. [53]	Lower	mir-17	MIR17	MIR17HG	RNA gene	
hsa-miR-18b-5p	UAAGGUGCAUCUAGUGCAGUUAG	Otaegui et al. [57]	Higher	mir-17	MIR18B		RNA gene	
hsa-miR-20a-5p	UAAAGUGCUUAUAGUGCAGGUAG	Cox et al. [53]	Lower	mir-17	MIR20A	MIR17HG	RNA gene	
hsa-miR-20b-5p	CAAAGUGCUCAUAGUGCAGGUAG	Keller et al. [55]	Lower	mir-17	MIR20B		RNA gene	
hsa-miR-96-5p	UUUGGCACUAGCACAUUUUUGCU	Otaegui et al. [57]	Higher	mir-96	MIR96		RNA gene	rs41274239, rs73159662
hsa-miR-142-3p	UGUAGUGUUUCCUACUUUAUGGA	Keller et al. [55]	Higher	mir-142	MIR142		RNA gene	
hsa-miR-145-5p	GUCCAGUUUUCCCAGGAAUCCCU	Keller et al. [55]	Higher	mir-145	MIR145	LOC728264	RNA gene	
hsa-miR-186-5p	CAAAGAAUUCUCCUUUUGGGCU	Keller et al. [55]	Higher	mir-186	MIR186	ZRANB2	Intronic	
hsa-miR-223-3p	UGUCAGUUUGUCAAAUACCCCA	Keller et al. [55]	Higher	mir-223	MIR223		RNA gene	rs34952329
hsa-miR-326	CCUCUGGGCCCUUCCUCCAG	Du et al. [54]	Higher	mir-326	MIR326	ARRB1	Intronic	rs72561778
hsa-miR-422a	ACUGGACUUAGGGUCAGAAGGC	Keller et al. [55]	Higher	mir-422	MIR422A		RNA gene	
hsa-miR-491-5p	AGUGGGGAACCCUUCCAUGAGG	Keller et al. [55]	Higher	mir-491	MIR491	KIAA1797	Intronic	
hsa-miR-584-5p	UUAUGGUUUGCCUGGGACUGAG	Keller et al. [55]	Higher	mir-584	MIR584	SH3TC2	Intronic	
hsa-miR-599	GUUGUGUCAGUUUAUCAAAC	Otaegui et al. [57]	Higher	mir-599	MIR599		RNA gene	
hsa-miR-664-3p	UAUUCAUUUAUCCCCAGCCUACA	Keller et al. [55]	Higher	mir-664	MIR664	RAB3GAP2	Intronic	rs113256801
hsa-miR-1275	GUGGGGGAGAGGCUGUC	Keller et al. [55]	Higher	mir-1275	MIR1275		RNA gene	rs76156362, rs77821659

As of March 2012, miR2Disease lists 4 different studies that identified 13 miRNAs as higher expressed and 3 miRNAs as lower expressed in blood cells of MS patients compared to controls. Each of the 16 mature miRNAs is processed from one single precursor miRNA. Five miRNAs are located within introns of protein-coding genes, while the remaining are located within non-coding genes. For 8 miRNAs, the pri-miRNA transcript has not been annotated. Five of the miRNAs have at least one SNP within the genome region of the pre-miRNA (as of dbSNP build 132). hsa-miR-17-5p and hsa-miR-20a-5p belong to the miR-17~92 cluster [59], and hsa-miR-18b-5p and hsa-miR-20b-5p belong to the miR-106a~363 cluster [60]. *Ctr*=healthy controls, *MS*=multiple sclerosis, *SNP*=single nucleotide polymorphism

increased levels of hsa-miR-155-5p, hsa-miR-338-5p, and hsa-miR-491-3p in the cerebral white matter of patients with MS. Various other groups examined the miRNA expression in peripheral blood from MS patients compared to healthy controls and found further miRNAs being dysregulated in MS patients, for instance miRNAs of the miR-106~25 cluster [64], hsa-miR-1, hsa-miR-126-3p, hsa-miR-193a-3p and hsa-miR-497-5p [65], hsa-miR-21-5p, hsa-miR-146a-5p and hsa-miR-146b-5p [66], hsa-miR-15a-5p [67], hsa-miR-614 and hsa-miR-572 [68], hsa-miR-155-5p [69], hsa-let-7g-5p and hsa-miR-150-5p [70], hsa-miR-27b-3p, hsa-miR-128, and hsa-miR-340-5p [71]. Waschbisch et al. [72] confirmed the relevance of hsa-miR-146a-5p and hsa-miR-155-5p in MS and its treatment. Three of the 16 miRNAs listed in miR2Disease (hsa-miR-17-5p, hsa-miR-142-3p, and hsa-miR-326) were independently verified to be significantly modulated in expression in peripheral blood cells of MS patients [65, 72].

Regulatory MicroRNA Interactions

We extracted potential mRNA targets of the 16 miRNAs from the databases miRTarBase version 2.4 [31] and miRWalk [25] (August 2011). This information was then used to visualize a network of miRNA–mRNA interactions in the Cytoscape software [73]. For the target prediction with miRWalk, we used the option of the web server to calculate with all ten offered prediction algorithms. In total, 121,108 target predictions were obtained from miRWalk. To reduce the huge number of predicted targets, we only used the miRNA–mRNA interactions being predicted by at least 7 of the 10 algorithms. This resulted in 2,122 potential interactions (Fig. 1). The overlap of predicted target genes between pairs of miRNAs is shown in Fig. 2. As our filtering

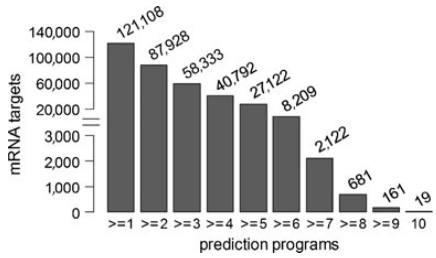
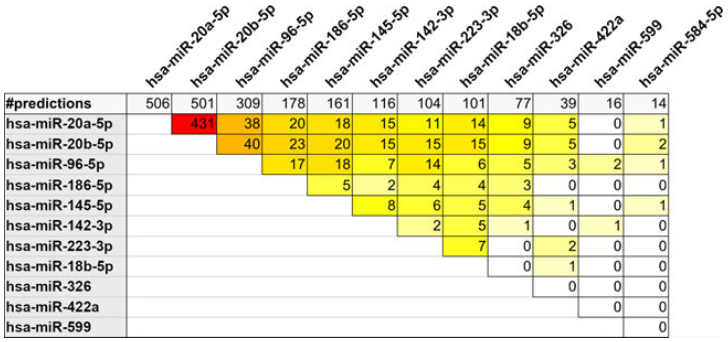


Fig. 1 Number of miRNA–mRNA interactions consistently predicted by multiple algorithms integrated within miRWalk. A total of 121,108 interactions determined by any of ten different algorithms could be found in the miRWalk database for the 16 MS-associated miRNAs. This number strongly decreases if we filter for interactions that are consistently predicted by several programs. Only 8,209 interactions were predicted by more than five different algorithms, and the intersection of all ten offered algorithms resulted in 19 miRNA–mRNA interactions. This rather small overlap indicates the diversity of strategies used by these prediction programs and the disparity of their results

of interactions requested consistent results across 7 or more algorithms, no interaction remained for 4 of the 16 miRNAs (hsa-miR-17-5p, hsa-miR-491-5p, hsa-miR-664-3p, and hsa-miR-1275). However, this does not mean that these four miRNAs have no targets—it rather illustrates the trade-off when analyzing predicted miRNA–mRNA interactions: a more stringent filtering could result in the loss of useful information, while a more tolerant filtering leads to increased numbers of possibly false-positive predictions making subsequent analyses more difficult. On the other hand, limitations of the prediction programs might have lead to inconsistent results for these four miRNAs. Many algorithms miss organism-specific target sites when they demand sequence conservation [74], and it is usually neglected that miRNA targeting requires structural accessibility [75], involves also other molecules than RNAs, and is not always restricted to 3′ UTR regions [44–46]. To otherwise increase the accuracy of miRNA–mRNA interactions, one might favor target genes having multiple predicted binding sites for the same miRNA, and respective information is provided by, e.g., TargetScan, DIANA-microT, and miRDB. Besides, known interactions from the literature and from curated databases of experimentally verified targets should be integrated.

Using miRTarBase, we found several interactions for hsa-miR-17-5p and hsa-miR-491-5p, for which there was no predicted target gene in the filtered miRWalk result. In total, miRTarBase contained 12 of the 16 MS-associated miRNAs (all but hsa-miR-142-3p, hsa-miR-599, hsa-miR-664-3p, and hsa-miR-1275), and we could retrieve 117 validated miRNA–mRNA interactions for these miRNAs (Table 3). Thirty of these interactions occurred also in the miRWalk-derived interaction list. We finally ended up with 1,498 target genes that were connected to 14 different miRNAs (hsa-miR-664-3p and hsa-miR-1275 were not existent in miRTarBase and did not show relevant results in miRWalk) by 2,239 predicted or validated interactions (Fig. 3, Online Resource 1). The two most connected miRNAs in the network are hsa-miR-20a-5p (525 interactions) and hsa-miR-20b-5p (513 interactions). Both miRNAs share many mRNA targets (n=434), hinting to similar functions.

In addition to the investigation of putative target genes, we analyzed for the 16 miRNAs, which TF potentially bind to the promoter regions of their corresponding pri-miRNA transcripts, thus regulating their expression. Predicted TFBS were derived from miRGen 2.0 [23]. This database comprises 9,322 TFBS predictions for the whole human genome. miRGen 2.0 contained 12 of the 16 miRNAs (all but hsa-miR-18b-5p, hsa-miR-223-3p, hsa-miR-664-3p, and hsa-miR-1275), and 221 TFBS predictions associated with 74 Transfac PWMs were obtained for these 12 miRNAs. To take into account the inherent redundancy of Transfac entries, we consolidated very similar or identical sequence motifs by using the web tool STAMP [76]. STAMP



	hsa-miR-20a-5p	hsa-miR-20b-5p	hsa-miR-96-5p	hsa-miR-186-5p	hsa-miR-145-5p	hsa-miR-142-3p	hsa-miR-223-3p	hsa-miR-18b-5p	hsa-miR-326	hsa-miR-422a	hsa-miR-599	hsa-miR-584-5p
#predictions	506	501	309	178	161	116	104	101	77	39	16	14
hsa-miR-20a-5p		431	38	20	18	15	11	14	9	5	0	1
hsa-miR-20b-5p			40	23	20	15	15	15	9	5	0	2
hsa-miR-96-5p				17	18	7	14	6	5	3	2	1
hsa-miR-186-5p					5	2	4	4	3	0	0	0
hsa-miR-145-5p						8	6	5	4	1	0	1
hsa-miR-142-3p							2	5	1	0	1	0
hsa-miR-223-3p								7	0	2	0	0
hsa-miR-18b-5p									0	1	0	0
hsa-miR-326										0	0	0
hsa-miR-422a											0	0
hsa-miR-599												0

Fig. 2 Number and pairwise overlap of target genes of MS-associated miRNAs predicted using miRWalk. Only 12 of the 16 MS-associated miRNAs are listed, since four miRNAs (hsa-miR-17-5p, hsa-miR-491-5p, hsa-miR-664-3p, and hsa-miR-1275) had no interactions that were predicted by at least seven out of ten algorithms in miRWalk. The most target genes were retrieved for hsa-miR-20a-5p and hsa-miR-20b-5p. Since their mature sequences differ only in two bases, they have many common mRNA targets (n=431). Therefore, they may play crucial and similar roles as post-transcriptional regulators in MS

computes a motif tree, which allows to group related motifs. Nine of the 74 Transfac PWMs were removed a priori since they do not belong to TFs in human, mouse, or rat. The remaining 65 PWMs were reduced to 39 distinct DNA-binding patterns (Online Resource 2). For instance, TFBS predictions for OCT1 (POU2F1) exist for three different PWMs (Transfac identifiers V$OCT1_02, V$OCT1_06, and V$OCT1_Q6), and, therefore, they were pooled. We integrated the condensed TFBS information into the Cytoscape network by adding 39 TF nodes that are connected to 12 miRNAs by 163 edges (Fig. 3, Online Resource 1). Interestingly, 11 genes of these 39 TF nodes have been specified as target genes by miRWalk or miRTarbase (e.g., FOXJ2 and MYC), demonstrating the presence of regulatory feedback loops.

Next, we took a closer look at the genes that are highly connected in the network, since they may play crucial roles in MS or related immune disorders. MS is characterized by the involvement of a variety of immune cells like T helper 1 (Th1) cells, T helper 17 (Th17) cells, T regulatory (Treg) cells and B cells [77]. We found that genes being targeted by several MS-associated miRNAs are in many cases TFs or key players involved in the regulation of these cells. **CDKN1A** (p21) is the gene with the most interactions in

Table 3 Validated target genes of the 16 MS-associated miRNAs provided by miRTarBase

MicroRNA	#Targets	Target gene symbols
hsa-miR-17-5p	31	APP, BCL2, BCL2L11, BMPR2, CCL1, CCND1, CDKN1A, DNAJC27, E2F1, FBXO31, GPR137B, JAK1, MAP3K12, MAPK9, MEF2D, MUC17, MYC, NCOA3, NPAT, OBFC2A, PKD2, PTEN, PTPRO, RUNX1, SMAD4, TGFBR2, THBS1, TNFSF12, VEGFA, YES1, ZNFX1
hsa-miR-18b-5p	1	ESR1
hsa-miR-20a-5p	19	APP, BCL2, BMPR2, BNIP2, CCND1, CDKN1A, E2F1, HIF1A, MAP3K12, MEF2D, MUC17, MYC, NRAS, PTEN, RUNX1, SMAD4, TGFBR2, THBS1, VEGFA
hsa-miR-20b-5p	12	ARID4B, BAMBI, CDKN1A, CRIM1, ESR1, HIF1A, HIPK3, MUC17, MYLIP, PPARG, STAT3, VEGFA
hsa-miR-96-5p	8	ADCY6, CDKN1A, FOXO1, FOXO3, HTR1B, KRAS, MITF, PRMT5
hsa-miR-145-5p	24	BNIP3, CBFB, CDKN1A, CLINT1, DFFA, FLI1, FSCN1, HOXA9, IFNB1, IGF1R, IRS1, KLF4, KRT7, MUC1, MYC, MYO6, PARP8, POU5F1, PPP3CA, SOX2, STAT1, TIRAP, TMOD3, YES1
hsa-miR-186-5p	2	FOXO1, P2RX7
hsa-miR-223-3p	8	CHUK, E2F1, LMO2, MEF2C, NFIA, NFIX, RHOB, STMN1
hsa-miR-326	6	GLI1, MSH3, NOTCH1, NOTCH2, PKM2, SMO
hsa-miR-422a	2	CYP7A1, CYP8B1
hsa-miR-491-5p	3	BCL2L1, CHD4, TAF10
hsa-miR-584-5p	1	ROCK1

Twelve of the 16 MS-associated miRNAs were contained in miRTarBase version 2.4. Overall, 117 interactions have been extracted. Repeatedly appearing genes are underlined, e.g., CDKN1A, which is targeted by five of these miRNAs. For 31 genes, there is experimental evidence in the literature that they are regulated by hsa-miR-17-5p. Sixteen of these genes are also a target of hsa-miR-20a-5p

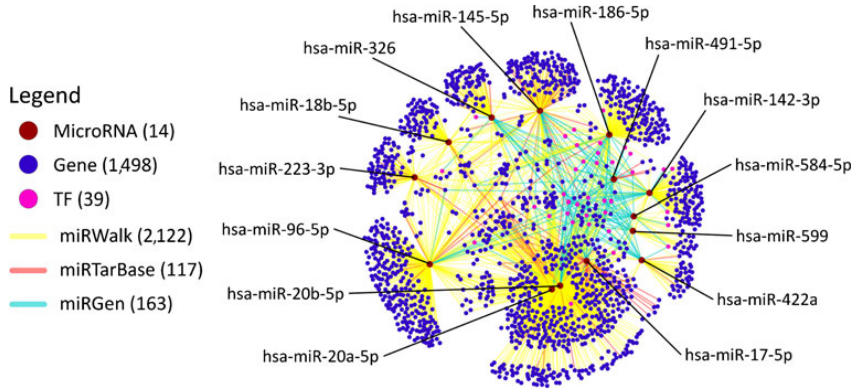


Fig. 3 Cytoscape network visualization of interactions between MS-associated miRNAs, target genes and TFs. TF-miRNA interactions were derived from the miRGen 2.0 database. Validated and predicted miRNA–mRNA interactions were derived from miRTarBase and miRWalk, respectively. hsa-miR-20a-5p and hsa-miR-20b-5p are closely related to each other and are predicted to regulate many target genes in common. They are also in close vicinity to hsa-miR-17-5p, whose targets were obtained from miRTarBase only and which belongs to the same miRNA cluster as hsa-miR-20a-5p. There are several feedback loops in the network, e.g., SOX9 is both a transcriptional regulator and a target of hsa-miR-145-5p. The network visualization is available as a Cytoscape session file as Online Resource 1. *TF*= transcription factor

our network. Five of the MS-associated miRNAs are predicted to regulate the expression of this gene, e.g., hsa-miR-20a-5p and hsa-miR-20b-5p are linked to CDKN1A according to both miRTarBase and miRWalk. CDKN1A is known to block the S-phase induction of T cells, and dysregulation of this gene can contribute to autoimmune processes [78]. Interestingly, de Santis et al. [64] showed that also hsa-miR-25-3p and hsa-miR-106b-5p act on CDKN1A expression, thereby modulating the TGFbeta pathway and influencing maturation and differentiation of Treg cells in MS patients. **RUNX1**, associated with four miRNAs in the network, regulates the development and function of T cells. It is a central factor in the differentiation of Th17 cells with a dual effect on IL17 transcription. Inducing and building complexes with RORgammaT, RUNX1 enhances IL17 transcription. However, in the presence of FOXP3 in Th17 cells, it has a negative effect on RORgammaT-mediated transcription, building complexes with FOXP3 [79, 80]. **FOXO1** and **FOXO3** (with five and three edges in the network, respectively) belong to the forkhead family of TFs. FOXO1 is a suppressor of T cell proliferation, activation, and differentiation. It was shown that the down-regulation of FOXO1, for instance by hsa-miR-182-5p, is necessary for the clonal expansion of T cells [81]. The deletion of FOXO1 in mice results in inflammatory diseases and reduced Treg cell differentiation and additional deletion of FOXO3 exacerbates these effects [82]. The TF **E2F1** is targeted by four miRNAs in the network. It was reported that E2F1 helps to regulate the threshold for antigen-stimulated activation of T cells as well as their negative selection in the thymus [83]. Iglesias et al. [84] observed up-regulated E2F1-dependent genes in PBMC of RRMS patients and showed that E2F1-deficient mice develop a less disabling form of EAE. Another interesting target gene is **STAT3**, which is regulated by two miRNAs in our network. STAT3 influences the balance of Th17 and Treg cells. In Th17 cells, it works downstream of pathways activated by IL6, IL21, or IL23, thus mediating inflammatory responses. On the other hand, STAT3 can bind to regulatory elements of IL10 in Treg cells and enhance the expression of this anti-inflammatory cytokine [85, 86]. Liu et al. [87] observed resistance to EAE development in STAT3 knock-out mice. A study by Frisullo et al. [88] showed evidence that in patients with clinically isolated syndrome (CIS), higher levels of phosphorylated STAT3 in CD4+ T cells favor an early conversion to clinical definite MS.

We observed that some of the TFs, which are predicted to regulate the transcription of the MS-associated miRNAs by miRGen 2.0, play important roles in the immune system as well. For instance, the TFs **AP1** and **NFkappaB** are linked to 4 and 6 of the 14 miRNAs in the network, respectively. AP1, together with NFAT, NFkappaB, and OCT1, is involved in T cell activation, particularly through enhancing the expression of IL2, whose dysregulation may promote autoimmunity [89–91]. NFkappaB and AP1 have overlapping functions in the regulation of pro-inflammatory genes, of which some, e.g., TNFalpha and MMP9, have been previously associated with MS or EAE [92, 93]. Hilliard et al. [94] reported that NFkappaB1-deficient mice show decreased T cell proliferation, reduced incidence, and a less severe course of EAE. The importance of NFkappaB in the differentiation of autoimmune T cells was confirmed by various other groups [95–97]. Bonetti et al. [98] found higher levels of AP1 and NFkappaB in oligodendrocytes

of MS lesions in the CNS, further emphasizing the roles of both TFs in the pathology of MS. **OCT1**, another highly connected TF in the network, belongs to the octamer TF proteins and is linked to 12 miRNAs. OCT1 stimulates the expression of IL2 in tight association with AP1 [91, 99]. Additionally, OCT1 can enhance IL3 transcription [100] and is suggested to be involved in the inhibition of IL4, IL5, and IL8 transcription [101, 102]. In a recent work by Riveros et al. [103], OCT1 was counted to the 25 most important MS-associated TFs, regulating genes involved in T cell specification.

Expression and Function of the MicroRNAs

MS is driven by different immune cells (Th1, Th17, Treg, and B cells) [77], which circulate in the peripheral blood and enter the brain when the blood–brain barrier is damaged. To investigate the expression of the MS-associated miRNAs in different peripheral blood cell types and brain regions, we used the smirnaDB database, which provides expression levels of 692 human miRNAs for 170 different cell types and tissues [30]. For the 16 miRNAs that have been identified as potentially MS-specific markers in the blood (Table 2), there was data for 19 blood cell populations and 4 brain regions (Fig. 4). hsa-miR-142-3p was the highest expressed miRNA in all cell populations of the peripheral blood. Another miRNA, hsa-miR-223-3p, was highly expressed in dendritic cells, monocytes, and granulocytes but was expressed at relatively low levels in other cell types of the blood and brain. In brain tissues, the expression of all 16 MS-associated miRNAs seemed to be very low, although hsa-miR-186-5p was somewhat higher expressed than the others. Some miRNAs like hsa-miR-599 were not detected at all, likely because of the limited sensitivity of the measurement technique that was used to generate these data (cloning and sequencing of small RNA libraries) [50]. Still, this analysis demonstrates how the smirnaDB database can be applied to link certain miRNAs to specific cell types. In release 18 of miRBase, also useful links to miRNA expression data (deep sequencing reads) are provided [33]. Another source of data is, of course, the GEO database [104]. Here, e.g., the microarray data set of Cox et al. [53] on 733 microRNAs measured in 96 samples including 59 whole blood samples of MS patients (accession GSE21079) and the data set of Keller et al. [56] on 863 microRNAs measured in 454 samples including 23 whole blood samples of MS patients (accession GSE31568) can be found. However, we did not use these data for further analyses.

To investigate potential functions of miRNAs, one can simply examine the functions of their target genes. Different databases exist that define functional groups of genes. For instance, KEGG [105] and Gene Ontology (GO) [106] provide an assignment of genes to certain pathways and biological processes. The idea to deduce the functions of miRNAs from their targets has been implemented in the web servers DIANA-miRPath [107] and MMIA [108]. These tools facilitate, e.g., to identify the molecular pathways being affected by miRNAs based on a KEGG enrichment analysis, where it is tested whether genes of a particular pathway are overrepresented ("enriched") in the list of target genes. To gain insights into the main functions of the 1,498 predicted and validated target genes and thus to reveal the potential cellular roles of the MS-associated miRNAs, we

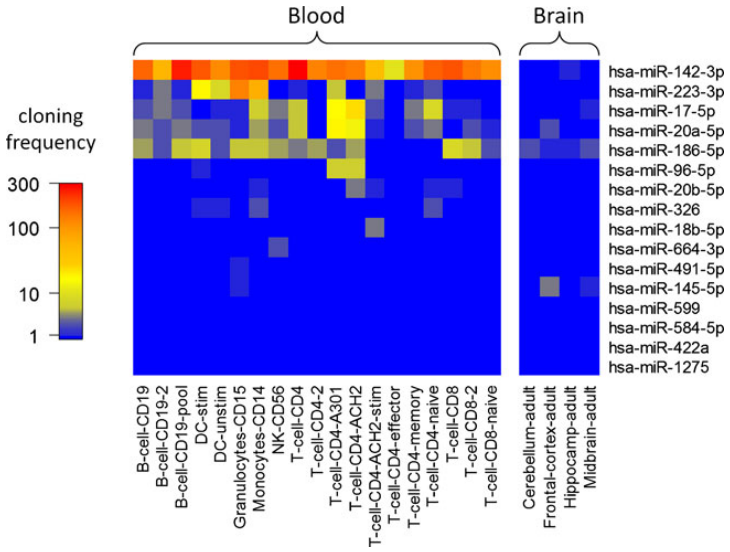


Fig. 4 Expression levels of 16 MS-associated miRNAs in 19 blood cell populations and 4 brain tissues. Cell type-specific expression data were downloaded from smirnaDB and visualized as a heatmap in the R software environment. hsa-miR-142-3p was highly expressed in all cell populations of the peripheral blood, but not in brain sections. hsa-miR-223-3p was expressed at high levels primarily in dendritic cells, monocytes, and granulocytes. In brain sections, several MS-associated miRNAs were not detected with the experimental methods that were used to obtain these data (clone count=0) [50]

performed a gene enrichment analysis with the KEGG pathways and the GO terms of the molecular function category. We compared the functions of the target genes to the functions of a reference gene list (all genes listed in the GeneCards database version 3.04) using the R package GOstats [109]. The top 15 functional terms and pathways of this enrichment analysis are shown in Table 4.

It was striking that many target genes seem to participate in different T cell pathways, e.g., the MAPK signaling pathway. In total, 54 of the miRNA targets belong to this pathway, which is significantly more than was expected from the reference gene list (p value=1.5×10^{-10}). The MAPK pathway, mediated by the three different protein kinases p38, ERK, and JNK [110], is involved in different regulation processes of T cells and supports the production of cytokines such as IFNgamma, IL10, IL17, and IL23. Due to the regulation of specific genes in T cells, the MAPK pathway may have also an influence on the pathogenesis of MS and EAE, which was examined in different studies [111–113]. Another central pathway in T cell differentiation is the TGFbeta signaling pathway. Genes of this pathway were significantly overrepresented in the target gene list as well (p value=2.5×10^{-8}). TGFbeta has a dual effect on naïve T cells, depending on the presence of different cytokines. In the absence of IL6, TGFbeta promotes Treg cell development and inhibits Th1/Th2 responses, supporting anti-inflammatory processes. On the other hand, in the presence of IL6, it favors the differentiation of Th17 cells, leading to increased inflammatory responses [114, 115]. Consequently, evidence is accumulating that TGFbeta is a crucial factor in the pathophysiology of MS [116, 117]. Besides, it was remarkable that the enrichment analysis revealed that many mRNA targets code for proteins with TF activity. Therefore, the MS-associated miRNAs can be regarded as the regulators of the regulators. This means, they post-transcriptionally regulate the expression of TFs, which themselves enhance or inhibit gene expression, leading to an additional and indirect effect of miRNAs on transcript levels in cells. Zinc finger proteins, a major family of human TFs, were also significantly enriched in the list of predicted and validated target genes (GO:0008270, p value=0.0003, not shown in Table 4). Zinc finger genes typically contain sequence repeats in their coding regions, and it was recently shown that some miRNAs bind to target sites in these regions outside the 3′ UTR, effectively regulating the expression of these TFs [46].

While the presented way of analyzing the functions and pathways affected by miRNAs is quiet simple, several open issues should be realized. Firstly, the accuracy of the gene enrichment analysis depends on the accuracy of miRNA–mRNA interactions. Results will be distorted if the predictions of interactions contain many false-positives. Secondly, the effect of miRNAs on the expression of a particular gene is sometimes difficult to estimate. If a miRNA regulates a gene's expression through degradation, increased levels of the miRNA can lead to decreased levels of the target mRNA. However, in our example, some miRNAs were described as higher and some as lower expressed in MS

Table 4 Enrichment analysis of target gene functions using KEGG pathways and the GO molecular function ontology

GO term/KEGG pathway	Accession	Expected count	Count	Odds ratio	P value
Protein binding	GO:0005515	598	821	2.16	6.4×10^{-39}
Binding	GO:0005488	1,011	1,149	2.36	1.5×10^{-25}
Nucleic acid binding TF activity	GO:0001071	82	160	2.31	2.3×10^{-17}
Sequence-specific DNA binding TF activity	GO:0003700	82	159	2.29	4.9×10^{-17}
Enzyme binding	GO:0019899	61	128	2.48	2.2×10^{-16}
TF binding	GO:0008134	25	63	2.98	8.0×10^{-12}
MAPK signaling pathway	KEGG:04010	21	54	3.00	1.5×10^{-10}
Protein kinase activity	GO:0004672	50	97	2.20	1.6×10^{-10}
Protein serine/threonine kinase activity	GO:0004674	36	76	2.42	3.5×10^{-10}
Sequence-specific DNA binding	GO:0043565	57	104	2.08	5.6×10^{-10}
Pathways in cancer	KEGG:05200	26	60	2.65	1.0×10^{-9}
Transcription activator activity	GO:0016563	27	60	2.58	2.8×10^{-9}
Axon guidance	KEGG:04360	10	31	3.71	1.9×10^{-8}
TGF-beta signaling pathway	KEGG:04350	7	24	4.64	2.5×10^{-8}
Protein domain specific binding	GO:0019904	37	71	2.18	4.6×10^{-8}

Shown are the top 15 overrepresented terms of the GO molecular function ontology and KEGG pathways with odds ratio>2. The functional terms and pathways are sorted according to the p values of the hypergeometric test calculated by GOstats [109]. As an example, 160 of the 1,498 target genes belong to the category "nucleic acid binding TF activity", which is significantly more than expected by chance (expected count=82). *GO*=Gene Ontology, *TF*=transcription factor

(Table 2). In that case, opposing effects exist if a mRNA is simultaneously targeted by down- and up-regulated miRNAs. Thirdly, the net effect of dysregulated miRNAs on a particular pathway is hard to quantify for the same reason. Note that the function of a gene within a pathway is critical as well, since it might be an activator or inhibitor. Finally, the mutual regulatory interactions (including feedback loops) over time lead to diverse secondary effects, which makes it difficult to unravel causes and effects. All this hampers the study of miRNAs. A separate functional analysis of up- and down-regulated miRNAs might be helpful but should be interpreted with caution. The association of the levels of miRNAs and mRNAs to the functions of cells remains largely a challenge for the future.

Concluding Remarks

A major topic in miRNA studies is the investigation of miRNA gene targets. Several different computational algorithms have been developed and implemented in public databases to predict interactions between miRNAs and target mRNAs with perfect or imperfect sequence complementarity (Table 1). The databases differ in their information content and up-to-dateness. The huge amount of potential target genes is associated with a considerable number of possibly false positive predictions, making it difficult to interpret the results. Therefore, it is reasonable to utilize target genes that have been described in experimental studies and that are listed in databases like miRTarBase [31]. However, validated interactions are usually integrated in such databases with some delay, and their quantity is still relatively low, even though high-throughput techniques such as CLIP-Seq and Degradome-Seq promise to fill this gap in the future [118]. Another way to reduce the enormous number of predicted target genes to ease further analysis and interpretation is to use multiple algorithms of different databases and to focus on miRNA–mRNA interactions being consistently predicted by several algorithms (ensemble strategy). We showed in this review how this can be done using meta-resources like miRWalk [25]. Further interaction information can be obtained by a non-curated literature search with text mining tools. Such information is delivered by, e.g., the "validated targets module" of the miRWalk database and more elaborated commercial software products like Pathway Studio [119]. Finally, the mutual interactions between miRNAs and genes can be visualized in networks using software platforms like Cytoscape [73]. The architecture of such networks can then be further analyzed, e.g., to identify network modules or regulatory feedback loops.

Some databases like miR2Disease provide insights into studies of miRNAs associated with human disorders. This can be very useful, but it has to be considered that the information in such databases is not complete, and the latest studies can be missing. So far, 13 different studies have associated miRNA expression patterns in blood with MS [53–57, 64–72]. hsa-miR-142-3p, hsa-miR-146a-5p, hsa-miR-155-5p, and hsa-miR-326 have been repeatedly described as higher expressed in the blood, while two independent studies found elevated levels of hsa-miR-155-5p also in brain tissues of MS patients [62, 63, 120]. Several other miRNAs have been proposed as further potential disease biomarkers. Differences in the results can be ascribed to differences of the studies in the analyzed cell populations, measurement technology [10], and data analysis as well as in the clinical and demographic characteristics of the patients and their treatment status. Additional confirmation is needed to assess the specificity and clinical value of these so far nominated miRNAs. Certainly, future studies will examine specific issues in more detail, e.g., compare miRNA expression profiles in blood or brain cells between different subtypes of MS (CIS, RRMS, secondary progressive MS) or between different phases of disease activity (remission vs. relapse). To date, only few groups like Waschbisch et al. [72] have investigated the effects of immune-modulating MS therapies on miRNAs. Such studies are needed to analyze whether a therapy can influence the dysregulation of miRNAs and to better understand the therapeutic mechanisms of action in a miRNA-gene network context. miRNAs may improve the diagnosis, monitoring, and management of MS and could lead to new therapies which specifically target miRNAs and the biological processes they control. They may even serve as prognostic biomarkers for MS, helping to predict individual courses of the disease and to distinguish between therapy responders and non-responders. Although this is a very active field of research, we still just begin to anticipate the biological significance and therapeutic potential of miRNAs.

Enrichment analyses with KEGG pathways and GO functional terms allow to reveal the different molecular processes affected by miRNAs. In our study, we found that many target genes of the MS-associated miRNAs participate in T cell activation and differentiation, which has also been described by Junker [11]. Our results support the hypothesis that miRNAs dysregulated in MS favor the development of pro-inflammatory T cell phenotypes and potentially regulate Treg functions. Due to their modulating role in both neuronal and immune system processes, miRNAs are likely important players in the pathogenesis of neurological, immune-mediated disorders [121]. Moreover, it was striking that the miRNAs seem to play a crucial role in regulating TFs, hence having an additional indirect influence on gene regulation. This may be a general phenomenon since previous studies have also found that TFs prevail among miRNA targets [122]. A work flow how to analyze the primary and secondary targets of microRNAs (with secondary targets

being target genes of miRNA-regulated TFs) was presented in the study by Tu et al. [122]. They obtained the secondary targets using TFBS predictions provided by the tfbsConsSites track of the UCSC genome browser. This track is based on PWMs from the public version of the Transfac database. A drawback of this approach is that for many TFs no well-defined PWMs exists. Nevertheless, an analysis of the secondary targets can be very meaningful, because while miRNAs often act by reducing the mRNA level of its targets through destabilization [123], they can also act through translational repression [14]. Therefore, even if the target gene expression is not necessarily affected, a miRNA might still have significant influences on the expression of genes downstream of a TF or pathway it is targeting.

The more researchers are sharing their miRNA data in public databases like GEO and ArrayExpress, the more important it is to make use of this rich source of information. For instance, one can compare the expression levels of specific miRNAs between different cell types, tissues, and disease conditions. In the last few years, many different experimental platforms have been developed to generate miRNA data sets [10]. New techniques allow the measurement of a greater variety of miRNAs with higher sensitivity. This enables researchers to investigate specific cell types and specimens where miRNAs are present at very low amounts, e.g., microparticles in plasma [124–127]. The era of next generation sequencing (NGS) will deliver new results regarding the expression of miRNAs and their target genes. An RNA-seq analysis with NGS provides the opportunity to sequence all RNA transcripts in a sample simultaneously without specific assays and prior knowledge, and may help, e.g., to find new transcript variants, detect miRNA modifications [127], and investigate SNPs in miRNAs. Despite difficulties in handling the generated data, such technologies bear the potential to better elucidate the complex processes of cellular gene regulation and with that the pathomechanisms of diseases such as MS.

In this review, we presented different kinds of databases in the context of miRNAs, their contents, and utility. We showed how to use various miRNA databases and how to integrate the heterogeneous information to investigate functions and interactions of miRNAs. This may serve as a guideline for similar studies on different issues or diseases.

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8.4 Manuscript - 4

MicroRNAs in multiple sclerosis and experimental autoimmune encephalomyelitis

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Review

MicroRNAs in multiple sclerosis and experimental autoimmune encephalomyelitis

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ABSTRACT

MicroRNA (miRNA) are small non-coding RNA molecules about 21–25 nucleotides long. They control gene regulation by translational repression and cleavage. Several studies have shown that many miRNA are associated with the etiology of different diseases. Recent developments in diverse miRNA profiling platforms like microarray and quantitative real-time PCR may enable the identification of specific miRNA as novel diagnostic and predictive markers for various diseases. MiRNAs could even be used as therapeutic drug targets. Multiple sclerosis (MS) is a chronic autoimmune disease affecting the central nervous system. Dysregulated immune system processes result in demyelination of neurons and consequently, electrical impulses that travel along the nerves are disrupted resulting in the impairment of organs. In the past three years, there has been an increased interest in establishing miRNA-based biomarkers for MS. So far, there are six studies on miRNA expression in MS patients in which first miRNAs were discovered as potential disease markers. For instance, one study showed that blood levels of miR-145 can discriminate MS patients from healthy controls, and another showed that active lesions in the brain are characterized by a strong up-regulation of miR-155. Studies on experimental autoimmune encephalomyelitis (EAE), the animal model of MS, further support the significance of miRNA as e.g. mice with miR-155 deletion are highly resistant to EAE. Such investigations help to understand the molecular processes involved in the disease. The identification of miRNA markers that are associated with type of MS, individual disease activity or clinical progression under treatment may open new avenues for early diagnosis and optimized therapy of MS.

Contents

Abbreviations: BBB, blood-brain barrier; CNS, central nervous system; EAE, experimental autoimmune encephalomyelitis; HV, healthy volunteers; miRNA, microribonucleic acid; MS, multiple sclerosis; PBMC, peripheral blood mononuclear cells; PPMS, primary progressive multiple sclerosis; RISC, RNA induced silencing complex; RRMS, relapsing–remitting multiple sclerosis; RT-PCR, reverse transcription-polymerase chain reaction; SPMS, secondary progressive multiple sclerosis; Th, T helper cell; TCR, T cell receptor; TGF-β, transforming growth factor beta; T-reg, regulatory T-cell; CDKN1A, cyclin-dependent kinase inhibitor 1A; TLR, toll like receptor.

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Table 1
MiRNA target prediction databases.

MicroRNA target prediction database	Uniform resource locator
MicroCosm	http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/
miR2Disease Base	http://www.mir2disease.org/
miRBase	http://mirbase.org/
miRecords	http://mirecords.biolead.org/
PMRD: plant microRNA database	http://bioinformatics.cau.edu.cn/PMRD/
TargetScan	http://www.targetscan.org/
microRNA	http://www.microrna.org/microrna/home.do
TarBase	http://diana.cslab.ece.ntua.gr/tarbase/
miRBD	http://mirdb.org/miRDB/
miRGator	http://genome.ewha.ac.kr/miRGator/miRGator.html
miRGen	http://www.diana.pcbi.upenn.edu/miRGen.html
miRNAMap	http://mirnamap.mbc.nctu.edu.tw/
Vir-Mir	http://alk.ibms.sinica.edu.tw/cgi-bin/miRNA/miRNA.cgi
ViTa	http://vita.mbc.nctu.edu.tw/
DIANA MicroTest	http://www.diana.pcbi.upenn.edu/cgi-bin/micro_t.cgi
microInspector	http://bioinfo.uni-plovdiv.bg/microinspector/
NBmiRTar	http://wotan.wistar.upenn.edu/NBmiRTar/login.php
PicTar	http://pictar.mdc-berlin.de/
PITA	http://genie.weizmann.ac.il/
RNA22	http://cbcsrv.watson.ibm.com/rna22.html
RNAhybrid	http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/
miRWalk	http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/

1. Introduction

MicroRNAs (miRNA) are small endogenous non-coding RNA molecules ~21–25 nucleotides in length. They regulate gene expression of target genes in a sequence-specific manner [1]. Down-regulation of RNA transcript targets is facilitated either by translational repression (imperfect sequence match) or by mRNA cleavage (perfect match). MiRNAs have emerged as one of the key players in regulation of gene expression in various cell types.

Although the first miRNA was identified over fifteen years ago, we just started to appreciate their involvement in various disease pathogenesis. Recently numerous, expression profiling studies have detected specific miRNA 'signatures' for a variety of diseases, including multiple sclerosis (MS) (see Section 6). MiRNA genes are often located at genomic regions associated with pathogenesis, which suggests that miRNA molecules may be involved in crucial cellular changes [2]. There are about 1100 miRNAs reported in humans and most of them have predicted targets from various databases (Table 1).

lin-4 in *C. elegans* was the first miRNA to be discovered by Lee, Feinbaum and Ambros in the year 1993 [3]. The *lin-4* gene did not code for a protein but instead for a short RNA transcript which regulates the timing of the larvae development. The mechanism involved was that *lin-4* miRNAs translationally represses lin-14 mRNAs, which codes for a nuclear protein [3].

MS is primarily an inflammatory disorder of the central nervous system (CNS). It is characterized by the infiltration of lymphocytes into the CNS leading to axonal demyelination of neurons [4]. MS is heterogeneous with respect to clinical manifestations and responses to therapy. It is widely considered that MS is related to environmental factors in individuals with complex genetic-risk profiles [4,5]. MS leads to chronic disability through deficits of sensation and of motor, autonomic and neurocognitive function. There are several studies conforming the presence of inflammatory cells and their products in the brain lesions of MS patients, in addition to reports from animal models experiments. These studies led to the generally accepted hypothesis that MS is mediated by pathogenic T-cell responses against myelin antigens, followed by a more complex neurodegenerative process [4]. The auto-reactive T-cells migrate across the blood-brain barrier (BBB) and mediate damage against the neurons and their protective myelin sheaths and axons. This leads to a signal conduction block or decrease at the site of demyelination [6]. The development of high-throughput functional genomics platforms has made it possible to identify novel relationships between various molecular mechanisms and the pathogenesis of MS [7]. There is growing evidence that the differential down-regulation and up-regulation of selective miRNAs may be an important mechanism for maintaining disease progression (Table 2). Identification of biomarkers appears desirable for an improved diagnosis of MS as well as for monitoring disease activity and individual treatment responses.

2. Biogenesis of miRNAs

First, RNA polymerase II transcribes the primary miRNA (pri-miRNA) transcripts in human and animals [8]. The following miRNA maturation is a two-step process involving two enzymes with ribonuclease III (RNase III) activity, Drosha and Dicer. Drosha and its partner protein DGCR8 (DiGeorge syndrome critical region 8) process the nuclear pri-miRNA into a ~70 nucleotide precursor miRNA (pre-miRNA) [9]. The pre-miRNA is then exported from the nucleus to the cytoplasm by Exportin-5/RanGTP which specifically recognizes the structure of pre-miRNA molecules [10]. In the cytoplasm, Dicer together with its partner protein TRBP (trans-activator RNA binding protein) digests the pre-miRNA into a 21–25 nucleotide miRNA duplex. In a complex process that is not yet clearly understood one strand of this duplex is recruited to be loaded into the RNA-induced silencing complex (RISC) [11]. RISC will bind to its target mRNA in the 3′ UTR resulting in degradation or translational repression. The translational repression process may adapt several mechanisms including co-translational protein degradation, inhibition of translational elongation, premature termination of translation, and inhibition of translation initiation [12] (Fig. 1).

Table 2
MiRNA involved in multiple sclerosis. Gene loci refer to chromosome position.

MiRNA	Gene loci	Cell type	Statement	Reference
miR-106b	7q22.1	CD4 + CD25+	modulates the TGF-β signaling pathway	De Santis et al. [38]
miR-17	13q31.3	CD4+, CD8+, B	upregulated in CD4+ cells from MS patients	Lindberg et al. [7]
miR-18b	Xq26.2	PBMC	maybe involved in remission in MS patients	Otaegui et al. [32]
miR-20a	13q31.4	CD4+	significantly under-expressed in MS compared to controls	Cox et al. [33]
miR-25	7q22.2	CD4 + CD25+	modulates the TGF-β signaling pathway	De Santis et al. [38]
miR-326	11q13.4	PBMC	regulates Th-17 differentiation and is associated with the pathogenesis of MS	Du et al. [40]
miR-34a	1p36.22	Brain tissue	significantly expressed in lesions of MS patients and astrocytes of EAE mice	Junker et al. [37]
miR-497	17p13.1	CD4+, CD8+, B	down-regulated in CD4+ cells of patients with MS	Lindberg et al. [7]
miR-599	8q22.2	PBMC	higher expressed in patients experiencing a relapse versus controls	Otaegui et al. [30]
miR-96	7q32.2	PBMC	highly expressed in remitting and less in relapse status	Otaegui et al. [30]

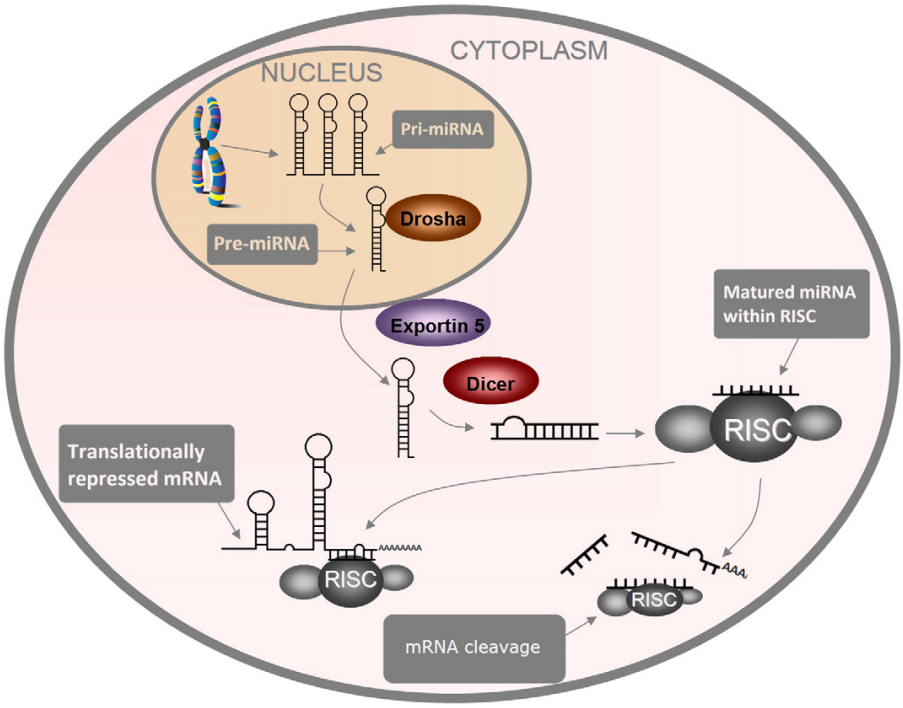

Fig. 1. MicroRNA biogenesis and mode of action. The microRNA pathway steps in mammalian cells are shown: A miRNA gene is transcribed into a primary miRNA transcript called the pri-miRNA. Pri-miRNA is cleaved by drosha to a hairpin pre-miRNA. Pre-miRNA is transported out of the nucleus by Exportin-5. Pre-miRNA is cleaved by dicer to form a short double-stranded mature miRNA duplex. MiRNA duplex separates into two single-stranded mature miRNAs and complexes with a RISC-like structure. The miRNA/RISC complex binds to mRNA, which is translationally repressed and cleaved.

3. MiRNA profiling platforms

Patterns of differentially expressed genes detected by high-throughput platforms provide insights into a disease's physiology and can also be used as biomarkers [13,14]. MiRNA microarrays enable the study of miRNA expression dynamics in various biological samples. RNA samples used for miRNA microarray hybridization need to be enriched for small RNAs. The first step of a miRNA microarray experiment is therefore the isolation of total RNA and the enrichment or direct isolation of small RNAs. In the next step, miRNAs are labelled and cleaned-up, followed by miRNA hybridization to arrays spotted with miRNA probes. Then the matrix is washed and scanned. Subsequent bioinformatic analysis may identify miRNAs differentially expressed between the samples (e.g. disease versus healthy). miRNA microarray results can be validated by, northern blot, quantitative reverse transcription-polymerase chain reaction (RT-PCR), or other analytical methods. Recent advancements of highly efficient labelling and novel microarray probe design enable direct labelling as low as 120 ng of total RNA using Cy3 or Cy5, without fractionation or amplification, to produce accurate measurements that span a linear dynamic range from 0.2 amol to 2 fmol of input miRNA [15].

Table 3
MiRNA in the context of immunoregulation.

MicroRNA	Cell type	Statement	Reference
miR-150	B cell	Critical for B-cell differentiation	Zhou et al. [50]
miR-181b	B cell	Involved in class switch recombination in activated B-cells	de Yébenes et al. [49]
miR-23a	B cell	miR-23a family members can inhibit B-cell development	Kong et al. [51]
miR-146a	T cell	Controls T-reg cell-mediated regulation of Th1 responses	Lu et al. [47]
miR-146a	T cell	Expressed in interleukin-17 producing T-cells in rheumatoid arthritis patients	Niimoto et al. [48]
miR-155	T cell	Promotes autoimmune inflammation by enhancing inflammatory T-cell development	O'Connell et al. [41]
miR-181a	T cell	Intrinsic modulator of T-cell sensitivity and selection	Li et al. [45]
miR-182	T cell	Induced by IL-2, promotes clonal expansion of activated helper T lymphocytes	Stittrich et al. [46]

4. MiRNAs and the immune system

It is widely accepted that miRNAs are involved in immune system development and regulation (Table 3). T-cell specific deletion of Dicer results in a reduced number of T-cells in the thymus and periphery [16,17]. Dicer deficiency in B-lymphocytes diminishes B-cell survival and as consequence of that reduces antibody production [18]. MiR-155 plays a role in immune system responses to infections. The importance of a proper regulation of miR-155 expression is also apparent by its much higher expression in several types of hematopoietic malignancies [19]. Microarray analysis of miR-146a targets after miR-146a over-expression in Akata B-cell line has demonstrated miR-146a-mediated inhibition of a group of interferon-responsive genes [20]. MiR-150 has a dynamic

expression profile during lymphocyte development, it is highly expressed in matured B-cells and T-cells but not in their progenitors [21]. The proposed role of miR-150 in B-cell development involves the transcriptional regulation of transcription factor c-Myb [21]. *In vivo* studies have shown that decreased expression of c-Myb results in fewer matured B-cells and can block the transition from pro-B-cell to pre-B-cell, which can be observed in miR-150 transgenic mice as well [21].

MiR-181a is mostly expressed in bone marrow, spleen and thymus. B-cells have the highest expression of miR-181a. When miR-181a was over-expressed *in vitro* it induced a two to three fold increase in $CD19^+$ B-cell populations. Moreover, miR-181a is involved in T-cell differentiation and function [22,23], and is assumed to regulate T-cell receptor (TCR) signal strength by down-regulating expression of several protein tyrosine phosphatases and by activation of two TCR signalling molecules, lymphocyte-specific protein tyrosine kinase (Lck) and extracellular signal-regulated kinase (Erk).

Mice with miR-17-92 cluster deletion die within minutes of birth, most likely because of the underdevelopment of the heart and lungs [24]. BCL-2-interacting mediator of cell death (Bim), a pro-apoptotic member of the anti-apoptotic BCL-2 family, has been identified as a target of miR-17–92 cluster members. MiR-17–92 mediates regulation of the expression of tumour suppressor PTEN [25]. Another miRNA, miR-223 is expressed specifically in cells of the granulocytic lineage. Its expression changes during maturation, becoming incrementally higher as granulocytes mature [26]. MiR-223 mutant mice spontaneously develop inflammatory lung pathology and exhibit exaggerated tissue destruction after endotoxin challenge [26].

5. MiRNA and the nervous system

MiRNAs are abundant in the brain and are essential for efficient brain function. For instance, miR-134 has been shown to inhibit the translation of an mRNA encoding a protein kinase, LIM domain kinase 1 (Limk1), which controls spine development. Exposure of neurons to extracellular stimuli such as brain-derived neurotrophic factor relieves miR-134 inhibition of Limk1 translation and in this way may contribute to synaptic development, maturation and plasticity [27].

Expression of miRNA gene cluster miR379–410, which comprises up to 50 microRNA genes, is induced by neuronal depolarization and has an important function in dendritic development in cultured neurons [28]. MiR-134, a member of this cluster, was earlier shown to be localized to dendrites of cultured hippocampal neurons, where it regulates dendritic spine morphology by its reversible inhibitory effect on Limk1 mRNA in response to brain-derived neurotrophic factor [28].

MiR-124a is a non-neuronal cell-specific miRNA, which decreases the levels of hundreds of non-neuronal transcripts. The transcriptional repressor RE1 silencing transcription factor (REST) has a reciprocal activity, inhibiting the expression of neuronal genes in non-neuronal cells [29]. REST regulates the expression of a family of miRNAs, including brain-specific miR-124a. In non-neuronal cells and neural progenitors, REST inhibits miR-124a expression, allowing the persistence of non-neuronal transcripts. As progenitors differentiate into mature neurons, REST leaves the miR-124a gene loci, and non-neuronal transcripts are degraded selectively [29].

6. MiRNAs in multiple sclerosis

MS is a complex genetic disease associated with inflammation in the CNS white matter mediated by auto-reactive T-cells. Many mRNA expression profiling data sets have been generated to better understand the disorder and the effects of particular treatments. For instance, in peripheral blood mononuclear cells (PBMC) from MS patients distinct expression patterns have been identified between relapse and remission disease state [30,31]. As of November 2010, six studies exist that investigated miRNAs in the context of MS. They are summarized in the following.

In the first study [32], PBMC were obtained from MS patients in relapse status ($n=4$) and in remission status ($n=9$) as well as from healthy controls ($n=8$). RT-PCR with Taqman low-density array was used to profile the expression levels of 364 different miRNAs. The authors could show differential expression of three miRNA (hsa-mir-18b, hsa-mir-493 and hsa-mir-599) between MS patients experiencing a relapse and healthy controls.

In the second study [33], the miRNA transcriptome in whole blood samples from 59 treatment-naïve MS patients were analyzed: 18 patients were diagnosed with primary progressive multiple sclerosis (PPMS), 17 were diagnosed with secondary progressive multiple sclerosis (SPMS) and 24 patients had relapsing–remitting multiple sclerosis (RRMS). For comparison, expression levels were examined in 37 healthy controls. The platform used here was the Illumina Sentrix array matrix. In this study miR-17 and miR-20a were significantly down-regulated in all types of MS. miR-17 and miR-20a are known to regulate various genes involved in T-cell activation.

In the third study [34], whole blood from 20 RRMS patients and 19 healthy controls was analyzed using the Geniom real-time analyzer platform. This enabled the measurement of 866 miRNAs. The best single disease-specific miRNA marker was miR-145. According to this study, higher levels of miR-145 clearly discriminate MS patients from controls with a specificity of 89.5% and a sensitivity of 90.0%.

In the fourth study [7], 365 miRNAs were analyzed in lymphocytes of RRMS patients. The study showed the first evidence for distinct miRNA expression profiles in CD4, CD8 and B-cells of MS patients ($n=8$) compared with those of healthy volunteers ($n=10$). MiR-17-5p, which is involved in auto-immunity, was up-regulated in CD4 cells of MS patients. This correlated with alterations in the expression of potential target genes of miR-17-5p, phosphatase and tensin homology and phosphatidyl-inositol-3-kinase regulatory subunit 1, which were down-regulated upon stimulation of CD41 cells with anti-CD3/CD28 *in vitro*.

Recently, the same group started two different studies. In one study, they analyzed miRNA expression patterns in serum samples from RRMS, SPMS and PPMS patients and healthy volunteers, five from each group. They found 46, 26 and 48 differentially expressed miRNAs in RRMS, SPMS and PPMS compared to the healthy volunteers. Nine miRNAs were redundant in all the three comparisons. MiR-21 and miR-106b were up-regulated in all MS courses. MiR-106b belongs to the miR-17–92 cluster [35]. In the other study, B-lymphocytes were isolated from peripheral blood of 19 RRMS patients in remission (11 untreated 8 treated with natalizumab) and 10 healthy volunteers. The miR-17–92 cluster members (miR-17-5p, 19a/b, 20a, 92a) were found to be down-regulated in the B-lymphocytes of the MS patients. Moreover, natalizumab seems to have some unique effects on miRNA expression in B-lymphocytes of RRMS patients [36].

In the fifth study [37], miRNAs in MS lesions were profiled. Three microRNAs, miR-34a, miR-155 and miR-326, were found up-regulated in active compared to inactive MS lesions. These miRNAs target the 3′ UTR of CD47. The study therefore suggests that deregulated miRNAs in MS lesions reduce CD47 in brain resident cells, this leads to the release of macrophages from inhibitory controls. Ten microRNAs that were most strongly up-regulated in active multiple sclerosis lesions, were also found in astrocytes. One of those, miR-155, is known to modulate immune responses in different ways but so far had not been assigned to CNS resident cells.

In the sixth study [38], $CD4^+$ $CD25^{high}$ T-cells from 12 RRMS patients in stable condition and 14 healthy controls were analyzed. The platform used here was the Agilent human miRNA microarray v.2 (#G4470B, Agilent technologies). A total of 723 human miRNAs were detected and 23 of them were identified as differentially expressed in $CD4^+$ $CD25^{high}$

cells from MS samples compared to healthy controls. This was the first study that investigated miRNA expression profiles in $CD4^+$ $CD25^{high}$ T-regulatory cells (T-regs) isolated from peripheral blood of MS patients. In a preliminary sub-study, the authors compared $CD4^+CD25^{high}CD127^{low}$ T-regs and $CD4^+$ $CD25^{high}CD127^+$ T-effector cells, and found a significantly higher miR-19b T-reg/T-effector cell ratio in MS versus HV. The respective ratio of miR-106b and miR-25 at least tended to be higher in MS. Over-expression of the miR-106b-25 cluster, in particular miR-25 and miR-106b, can silence two important effectors of the TGF-β (transforming growth factor beta) signalling pathway: CDKN1A/p21 (cyclin-dependent kinase inhibitor 1A) and BCL2L11/Bim. TGF-β is involved in differentiation and maturation of T-reg cells. Therefore, the dysregulation of this miRNA cluster may alter T-reg cell activity in the course of MS, by altering TGF-β functions.

7. MiRNAs in experimental autoimmune encephalomyelitis (EAE)

Experimental autoimmune encephalomyelitis (EAE) is an animal model of brain inflammation. EAE induced in rodents is widely used as a model of MS. The disease appears in exacerbation and remission and is characterized by loss of nerve conduction and chronic progression of disability. The two main components involved are macrophages and T-lymphocytes mediating the destruction of myelin sheaths surrounding the nerves. Several studies have shown that actively induced EAE models can reproduce the typical temporal maturation of MS lesions from inflammation with or without deposition of immunoglobulin through demyelination and axonal damage to gliosis and partial remyelination [39].

There are growing evidences that interleukin 17 (IL-17) producing T helper cells (Th-17 cells) are key players in various autoimmune diseases, including MS. In a recent study, miR-326 expression was shown to correlate with disease severity in patients with MS and mice with EAE [40]. *In vivo* silencing of miR-326 resulted in fewer Th-17 cells and a milder EAE, while in contrast over-expression resulted in more Th-17 cells and a severe EAE. Mice with miR-155 deletions ($Mir155^{-/-}$) where highly resistant to EAE.

The function of miR-155 is to promote inflammatory properties of T-cells including Th-17 and Th-1 cell subsets [41]. MiR-155 is one of the first miRNAs connected to inflammation just because of its potent up-regulation in multiple immune cell lineages by toll-like receptor (TLR) ligands, inflammatory cytokines, and specific antigens [42–44].

8. Conclusion and perspectives

Since their discovery, the field of miRNA research has grown tremendously, and we presently know that these molecules are important regulators of gene expression and can influence immune system development. The relatively small number of miRNAs as well as the possibility of using standardized, fast, and inexpensive detection methods suitable for high-throughput analysis, make miRNAs promising candidates for *ex vivo* detection biomarkers for MS. Recent studies have shown that microRNAs may play a key role in many aspects of MS pathogenesis and that alteration of their expression can have profound effects on MS phenotypes. The translation of these findings to *in vivo* models and clinical studies will unquestionably lead to greater insights into their utility in clinical settings. MiRNA expression profiles introduce a newly appreciated layer of complexity to the diverse physiology and pathology of MS. Regarding existence of several hundred mammalian miRNAs along with many other putative noncoding RNAs, it is likely that the results reviewed here are just the tip of the iceberg in terms of the involvement of regulatory RNAs in orchestrating development and function of the immune system regarding to MS.

Disclosure statement

All authors declare that they have no competing interest in connection with this paper.

Take-home messages

- MiRNAs are one of the key regulators of the gene expression.
- MiRNAs can significantly alter the response of the immune system.
- MiRNAs play a critical role in the biogenesis of MS.
- MiRNAs can evolve as potential drug targets for the treatment of MS.
- Studies so far have proved the importance of miRNAs as a worthy biomarker candidate.

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Recent clinical trials suggest that tacrolimus is safe and effective as an induction or maintenance therapy of lupus nephritis

Tacrolimus is a potent immunosuppressive and immunomodulatory agent used to prevent rejection in organ transplantation, which has been recently proposed as effective for the treatment of nephritis in SLE patients. The article by Lee et al. (**Lupus 2011;20:636-40**) is a systematic review of the literature reporting the results of clinical trials on the efficacy and safety of tacrolimus as an induction or maintenance therapy for lupus nephritis (LN). Up to July 2010, seven clinical trials, which met the review inclusion criteria, were considered, examining a total of 115 patients with biopsy-proven LN and 54 controls, during a follow-up period of 6-24 months. Both case-control and open-label studies were evaluated. Unpublished reports or retrospective studies were not included. Tacrolimus was compared with placebo or standard treatments, i.e. glucocorticoids and/or cyclophosphamide or azatioprine, in case-control studies, or evaluated as a single therapeutic regimen in open-label prospective studies.

In general, tacrolimus therapy was well tolerated and did not produced serious adverse effects in all the studies.

Although the evaluated studies were few and clinically heterogeneous with respect to study design, LN class, tacrolimus dosage and follow-up duration, they were almost concordant in assessing the efficacy and safety of tacrolimus for induction and maintenance therapy of LN, thus suggesting tacrolimus as a promising alternative to conventional immunosuppressive agents.

Anna Ghirardello

9. Personal declaration

I hereby declare that this Ph.D. thesis 'MicroRNA and MessengerRNA Expression Changes during Interferon-beta and Glatiramer Acetate Treatment in Peripheral Blood cells of Multiple Sclerosis Patients' has been compiled by me under the supervision of Prof. Dr. Uwe Klaus Zettl (Department of Neurology, University of Rostock). The dissertation is the product of my own work and has not been previously submitted for the award of any degree, diploma or examination to any other University or Institution. All the sources I have used or quoted have been indicated and acknowledged as complete references.

Place: Rostock, Germany

Date: 13.03.2014

Madhan Thamilarasan

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11. Curriculum vitae

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PhD student, University of Rostock, Germany
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"Neuroscience - Transcriptomics of Multiple Sclerosis"
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Junior research fellow, Indian Institute of Science, Bangalore, India
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"To find a novel diagnostic marker for glioma using genomic and proteomic approaches"
Experience: Molecular biology techniques, Brain tumor samples handling, Cell culture

Senior Demonstrator, Christian Medical Collage hospital, Vellore, India
January 2007 - July 2007
"Gene regulation in breast cancer prognosis"
Experience: Real time PCR, DNA microarray, Data analysis, Microsoft office tools

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"Targeted drug delivery using peptides"
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Master of Science (M.Sc.) in Microbial gene technology
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Honors and Awards

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Johannes-Sayk-Forschungspreis für Klinische Neuroimmunologie - 2010
Second prize for poster presentation at the 8th-Ostseesymposium. Title: "Detection and quantification of microRNA expression in human peripheral blood microvesicles from multiple sclerosis patients treated with Interferon-beta-1b"

ECTRIMS 2011 travel grant
The European Committee for Treatment and Research in Multiple Sclerosis (ECTRIMS)

CSIR Junior Research Fellowship - 2008
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Certifications

Good Clinical Practice (GCP) - participation certificate
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Statistical aspects of genomics research
United Europeans for the development of PHArmacogenomics in Multiple Sclerosis (UEPHA*MS program – 2010)

Real Time PCR training course
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Publications

Glatiramer acetate treatment effects on gene expression in monocytes of multiple sclerosis patients
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Springer Publishing Company January 2012 (book article)
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Conferences & Poster presentations

European Committee for Treatment and Research in Multiple Sclerosis. ECTRIMS-2011 (19 – 22 October), Amsterdam, Netherlands &, ECTRIMS-2010 (13-16 October), Gothenburg, Sweden.

2011: Topic: MicroRNA detection and quantification in human peripheral blood microvesicles from multiple sclerosis patients treated with interferon-beta-1b

2010: Topic: Quantification of microRNA expression in peripheral blood microvesicles from multiple sclerosis patients treated with subcutaneous Interferon-beta-1b

Die Deutsche Gesellschaft für Neurologie e.V. DGN-2011 (28 September-1 October), Wiesbaden, Germany.

2011: Topic: Quantification of microRNA Expression in Human Peripheral Blood Microvesicles from Multiple Sclerosis Patients treated with Interferon-beta-1b

International Congress of Neuroimmunology. ISNI-2010 (26-30 October) Sitges, Spain.

2010: Topic: Quantification of microRNA Expression in Peripheral Blood Microvesicles from Multiple Sclerosis Patients treated with subcutaneous Interferon-beta-1b

American Academy of Neurology. AAN-2012 (21-27 April) New Orleans, Louisiana, USA & AAN-2010 (10-17 April) Toronto, Ontario, Canada)

2012: Topic: MicroRNA Expression Changes in Response to Interferon-beta-1b Therapy in Blood Cells of Patients with Multiple Sclerosis

2010: Topic: Time Dependent *Ex Vivo* RNA Expression Analysis of Monocytes Derived from Glatiramer Acetate Treated Multiple Sclerosis Patients

2010: Topic: Quantification of microRNA Expression in Human Peripheral Blood Microvesicles from Multiple Sclerosis Patients treated with Interferon-beta-1b

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