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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

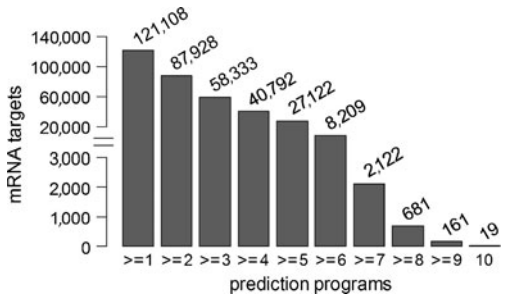
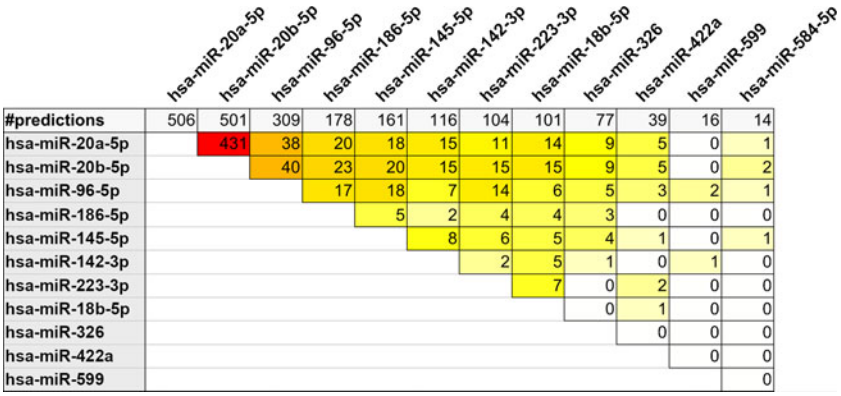


Fig. 1 Number of miRNA–mRNA interactions consistently predicted by multiple algorithms integrated within miRWalk. A total of 121,108 interactions determined by any of ten different algorithms could be found in the miRWalk database for the 16 MS-associated miRNAs. This number strongly decreases if we 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

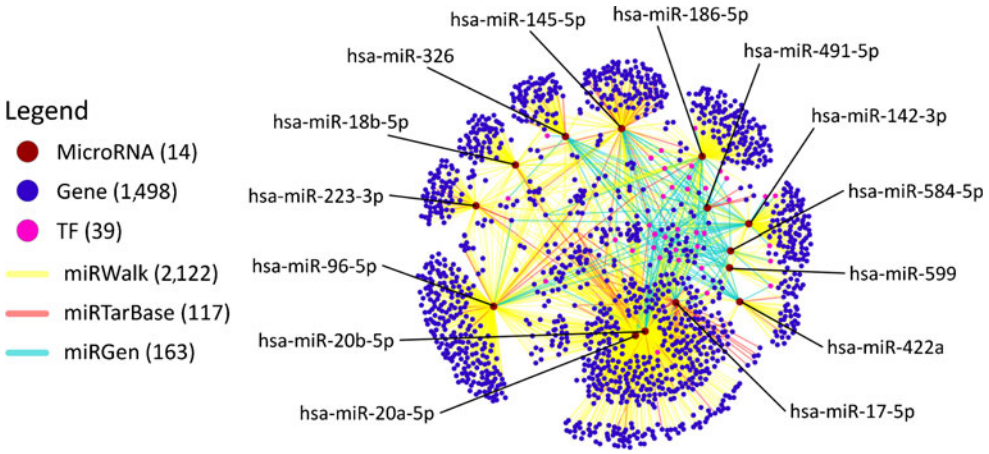


Fig. 3 Cytoscape network visualization of interactions between MS-associated miRNAs, target genes and TFs. TF-miRNA interactions were derived from the miRGen 2.0 database. Validated and predicted miRNA–mRNA interactions were derived from miRTarBase and miRWalk, respectively. hsa-miR-20a-5p and hsa-miR-20b-5p 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

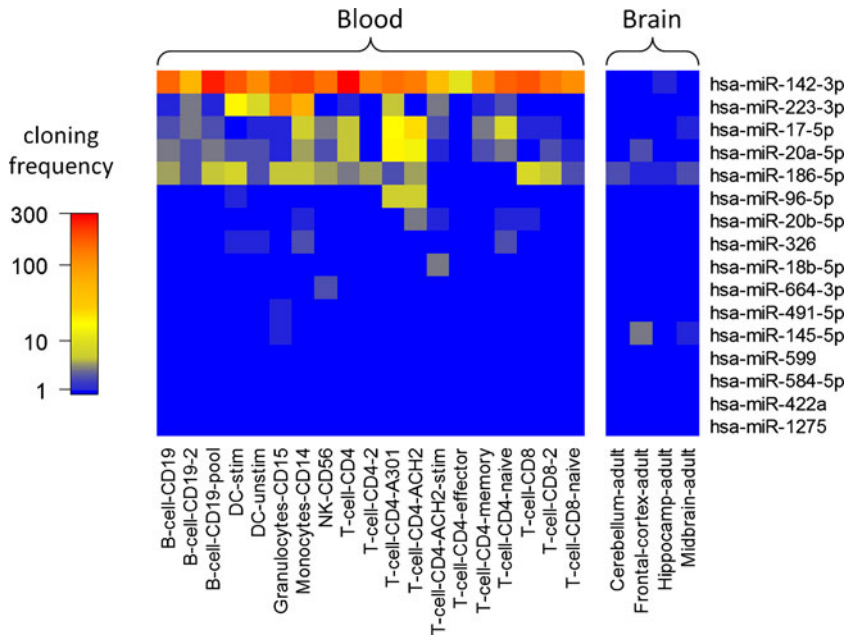


Fig. 4 Expression levels of 16 MS-associated miRNAs in 19 blood cell populations and 4 brain tissues. Cell type-specific expression data were downloaded from smirnaDB and visualized as a heatmap in the R software environment. hsa-miR-142-3p was highly expressed in all cell populations of the peripheral 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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Conflicts of interest The authors declare that they have no conflict of interest.

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