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The burden of chronic kidney disease in systemic lupus erythematosus: A nationwide epidemiologic study

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Aberrant expression of alternative splicing variants in multiple sclerosis – a systematic review

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Running title: Alternative pre-mRNA splicing in MS

Highlights

- We identified 36 studies that investigated alternatively spliced gene isoforms in MS
- Splice variants were examined for 27 genes, e.g. immune-related and myelin genes
- An MS-associated skipping of *IL7R* exon 6 has been validated at mRNA and protein level
- Transcriptome data are increasingly utilized for the analysis of RNA splicing
- The individual clinical implications of aberrant splicing events remain to be explored

Abstract

OBJECTIVE: Alternative splicing is an important form of RNA processing that affects nearly all human genes. The differential expression of specific transcript and protein isoforms holds the potential of novel biomarkers for complex diseases. In this systematic review, we compiled the existing literature on aberrant alternative splicing events in multiple sclerosis (MS).

METHODS: A systematic literature search in the PubMed database was carried out and supplemented by screening the reference lists of the identified articles. We selected only MS-related original research studies which compared the levels of different isoforms of human protein-coding genes. A narrative synthesis of the research findings was conducted. Additionally, we performed a case-control analysis using high-density transcriptome microarray data to reevaluate the genes that were examined in the reviewed studies.

RESULTS: A total of 160 records were screened. Of those, 36 studies from the last two decades were included. Most commonly, peripheral blood samples were analyzed (32 studies), and PCR-based techniques were usually employed (27 studies) for measuring the expression of selected genes. Two studies used an exploratory genome-wide approach. Overall, 27 alternatively spliced genes were investigated. Nine of these genes appeared in at least two studies (*CD40*, *CFLAR*, *FOXP3*, *IFNAR2*, *IL7R*, *MOG*, *PTPRC*, *SP140* and *TNFRSF1A*). The microarray data analysis confirmed differential alternative pre-mRNA splicing for 19 genes.

CONCLUSIONS: An altered RNA processing of genes mediating immune signaling pathways has been repeatedly implicated in MS. The analysis of individual exon-level expression patterns is stimulated by the advancement of transcriptome profiling technologies. In particular, the examination of genes encoded in MS-associated genetic regions may provide important insights into the pathogenesis of the disease and help to identify new biomarkers.

Keywords: multiple sclerosis, alternative splicing, systematic review, RNA processing, gene isoforms, blood biomarkers, autoimmunity, transcriptomics, genetics, IL7R

1. Introduction

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS) that leads to demyelination, neuroaxonal degeneration, synaptic loss and gliosis [1]. Typical symptoms include vision problems, limb weakness, sensory loss, cognitive impairment, fatigue, pain and muscle spasms [1]. More than 2.3 million people worldwide suffer from MS [2]. The average age at diagnosis is between 20 and 40 years [3]. Different courses of MS are distinguished: Approximately 85% of the patients are diagnosed with relapsing-remitting MS (RRMS), and 15% have a primary progressive course of MS (PPMS) from onset [4, 5]. In about 60% of the RRMS patients, the disease turns after approximately 20 years into a secondary progressive MS (SPMS) [6].

The management of MS includes the treatment of acute relapses with glucocorticosteroids [7, 8] as well as symptomatic [9] and preventive therapies [10]. Within the past 20 years, more than 10 disease-modifying drugs (DMD) were approved for MS. The major mechanisms of action of DMD include immunomodulation (e.g. interferon- β and glatiramer acetate), alteration of cell trafficking (e.g. natalizumab and fingolimod) and immune cell depletion (e.g. alemtuzumab and ocrelizumab) [10]. A first-line therapy for RRMS is subcutaneously or intramuscularly administered interferon- β (IFN- β) [11]. A DMD for more active RRMS is fingolimod, an orally administered sphingosine-1-phosphate receptor modulator that prevents the migration of immune cells from lymph nodes into the peripheral blood, thereby reducing the annual relapse rate up to 69% [12]. More recently, intravenous infusions of ocrelizumab, a monoclonal antibody depleting CD20-expressing B cells, have been approved for the treatment of PPMS, given a 25% relative risk reduction in 24-week confirmed disability progression [13].

The etiology of MS is still not clear. Environmental factors like smoking, vitamin D deficiency and viral infections, for example with Epstein-Barr virus, have been associated with an increased susceptibility to MS [14-16]. In twin studies, a concordance rate for monozygotic twins of approximately 20% could be observed, suggesting a genetic component of MS [17, 18]. The largest genome-wide association study (GWAS) identified 233 genetic loci that are linked to MS risk [19]. More than 90% of the respective lead single-nucleotide polymorphisms

(SNP) are located in non-coding regions, with the underlying mechanisms being largely unknown. However, it is likely that intergenic and intronic MS-associated SNPs may have an effect on transcription and RNA processing, e.g. alternative splicing.

Splicing is a physiological process in the cell nucleus that coordinates the excision of non-coding regions (introns) and the assembly of expressed regions (exons) of RNA molecules by a large ribonucleoprotein complex, the spliceosome [20] (Figure 1A). *Cis*-regulatory elements like the 5' and 3' splice sites (donor and acceptor), the branchpoint sequence (BPS) and the polypyrimidine tract (PPT) represent important recognition sequences for the processing of RNA [21-23]. Furthermore, *trans*-acting splice factors such as SR proteins may have either enhancer or silencer functions [24]. The differential use of multiple splice sites offers the possibility to create different mRNAs from one pre-mRNA. Therefore, alternative splicing events (ASE), together with alternative transcription start and termination sites, lead to a great transcriptome diversity. The basic types of ASE are exon skipping, intron retention, alternative splice site selection and utilization of mutually exclusive exons (Figure 1B). Some genetic variants cause an alteration of splicing regulation and thereby lead to different diseases [25]. Splice-switching antisense oligonucleotides that are used in the treatment of monogenic neuromuscular disorders, e.g. spinal muscular atrophy and Duchenne muscular dystrophy, demonstrate that an effective intervention in alternative RNA splicing is possible [26].

Improved methods foster the elucidation of disease-specific ASE. It is now possible to profile the spliceo-transcriptome via microarrays and high-throughput sequencing as two important approaches for genome-wide analyses [27, 28]. While microarrays are based on short oligonucleotides derived for annotated transcripts, high-throughput sequencing produces short reads without prior knowledge on the sequence. Driven by advancements in technology and bioinformatics, the investigation of alternative splicing gained more and more attention in recent years [29, 30]. The characterization of ASE could support the search for biomarkers for the diagnosis, prognosis and monitoring of MS. The vast majority of research so far focused on the analysis of blood and cerebrospinal fluid (CSF) samples of MS patients with the aim to detect molecular biomarkers with high specificity and sensitivity [31, 32]. However, continued validation and testing is required before such biomarkers can be applied in clinical practice.

Here, we reviewed the literature for studies that investigated alternative splicing in the context of MS, and we show how transcriptome microarray data can provide insights at the exon level of gene transcripts. We discuss that a better understanding of the regulation of gene isoforms is helpful in elucidating the pathomechanisms of MS and in identifying novel biomarkers.

2. Material and methods

2.1. Search strategy and study selection

Studies for this systematic review were identified through the electronic database PubMed using the specific search terms "splicing" and "multiple sclerosis". The last update of the database search was performed on March 20, 2019. There was no restriction on the date of publication. Additionally, further articles, which were considered to be relevant from scanning the reference lists of the identified articles, were also included in the study selection process.

We considered the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) that were developed by an international group of researchers [33]. Accordingly, we applied the four steps "identification", "screening", "eligibility" and "inclusion". In the screening, only studies whose full text was published in English in a scientific journal were included. In the first part of the "eligibility" step, records were excluded if they presented no original research. Next, the articles were checked if at least two different splicing isoforms of a human protein-coding gene were measured experimentally. Finally, we screened the remaining articles for whether samples from MS patients were investigated. There was no restriction on the sample material, the examined cells or the used technologies. Moreover, no restrictions concerning disease duration, severity of disease, MS course or the treatment of patients were applied. Eligibility assessment was carried out by two reviewers (AR and EP) in accordance with the PRISMA workflow. Any discrepancies were resolved by discussion with a third reviewer (MH).

2.2. Data extraction

To compare the included studies, a table providing detailed information for each study was created. For each study, we gathered the information which sample material was used (e.g. blood cells), which detection methods were used (e.g. polymerase chain reaction, PCR), whether genotypes were examined and whether a splicing reporter minigene assay was used [34]. Furthermore, we ascertained the study design (e.g. comparison of MS cases vs. controls or treated vs. untreated subjects) of the individual records and whether significant differences were reported between the groups.

For the genes and ASE that were investigated in the included studies, a second table was created. As some of the examined genes with alternative splice variants were named by aliases in the studies, e.g. CD45 for *PTPRC*, the official gene symbols of the HUGO Gene Nomenclature Committee (HGNC) were used for standardization. The GeneCards database Suite 4.9 was then used to retrieve integrated gene-centric information from several external databases [35]. For each gene, the identification number for the UniProt database release 2019_02 as well as the respective count of annotated protein isoforms were determined [36]. Additionally, we extracted the numbers of transcript variants and the gene identifiers according to Ensembl database release 95 [37].

2.3. Microarray datasets

To reanalyze at exon level the genes for which MS-specific ASE have been described in the literature, we used a transcriptome dataset that was previously published by our research group (GEO database SuperSeries accession number GSE73174) [38-41]. For this dataset, 150 high-density GeneChip Human Transcriptome Arrays (HTA) 2.0 with six million 25mer oligonucleotide probes per array were used to study gene expression shifts in response to fingolimod therapy in patients with MS. Peripheral blood samples were taken to isolate 5 different cell populations (CD4+, CD8+, CD14+, CD19+ and CD56+ cells) for 10 RRMS patients at 3 different time points (before the start of therapy, one day after the first dose of fingolimod as well as 3 months after treatment initiation). Details on extraction and processing of RNA as well as on hybridization and scanning of the Affymetrix HTA 2.0 microarrays are described elsewhere [41]. Here, we used a subset of 100 arrays and discarded the 3-month time point for which we observed strong therapy-induced transcriptome changes [40].

To realize a case-control analysis, we then compared our dataset for MS patients to public HTA 2.0 microarray datasets for healthy subjects. Four suitable datasets were found in the GEO database: GSE63379 (n=32 arrays for peripheral blood mononuclear cells, PBMC), GSE88887 [42] (n=60 arrays for whole blood samples) and two subsets of GSE111555 (n=84 arrays for whole blood samples and n=32 arrays for PBMC). In total, the 4 control datasets comprised 208 microarrays, yielding high-resolution transcriptome profiles of human blood cells.

2.4. Statistical evaluation

The HTA 2.0 microarrays are designed with 10 probes per exon fragment (PSR) and 4 probes per exon-exon splice junction (JUC), facilitating a deep view into alternative splicing patterns. The own and external raw data (308 CEL files in total) were processed in one run with the Transcriptome Analysis Console (TAC) 4.0.1 software, which is freely available from Thermo Fisher Scientific. The default settings of the program were used (e.g. SST-RMA normalization). For the ASE analysis, we employed the Splicing Index and the EventPointer algorithm [43] as implemented in the TAC software. By this means, the exon-level expression data of our MS patients (split into 5 immune cell populations) were compared against those of the 4 control groups. A gene was considered as differentially alternatively spliced if the previously reported ASE was consistently found with EventPointer p -value <0.0001 in all 4 comparisons.

3. Results

3.1. Study selection

Through PubMed database searching and reference list scanning, the literature search resulted in a sum of 160 articles. The flow diagram of the study selection process according to the PRISMA statement [33] is presented in Figure 2. Three of the 160 articles were excluded because the full texts were not published in the English language. A total of 121 articles were ineligible for the following reasons: no original research (e.g. reviews) (n=32), no investigation of at least two different splicing isoforms of human protein-coding genes (n=47) and no examination of sample material from MS patients (n=42). In the end, 36 studies were included in this systematic review [44-79].

3.2. Assessment of the included studies

For the qualitative synthesis of the 36 selected articles, we extracted information on the investigated genes, the type of sample material, the laboratory methods and the study design (Table 1). Almost all studies (n=34) evaluated only 1-3 different genes. The exceptions were the microarray investigation by Tian *et al.*, in which 92 different alternatively spliced genes were identified [67], and the recent RNA sequencing analysis by Cardamone *et al.*, in which 957 alternatively spliced genes (1114 ASE) were identified [78]. The first articles in this research field were published in the year 2000 [50, 53]. While only 10 articles were published until 2007, the number of published articles increased by a factor of >3 within the last years. The studies explored disease mechanisms and the informative value of ASE with regard to the diagnosis and therapy of MS. The comparison of MS cases and controls was the most common study design (n=31). Accordingly, the aberrant expression of specific mRNA splicing isoforms has been discussed as potential disease-specific biomarkers. Several studies (n=20) investigated genotype-dependent ASE, and 7 studies examined the effect of IFN- β treatment on gene isoform levels in blood cells. Two of these studies additionally included blood samples from patients treated with immunosuppressive agents and glatiramer acetate, respectively [59, 68]. The research groups by Gilli *et al.* and López-Gómez *et al.* analyzed the long-term effect of IFN- β injections by comparing RNA isoform levels in blood cells from MS patients before and

up to 2 years after the start of IFN- β treatment [69, 74]. The size of the analyzed MS cohorts varied from 5 patients to more than 1000 patients [44, 48].

Most commonly, PCR assays were utilized for the measurement of gene isoforms (n=27 studies). Cardamone *et al.* additionally performed RNA sequencing using an Illumina NextSeq 500 platform to determine ASE that are modulated by MALAT1, a non-coding RNA that is expressed at higher levels in MS patients and involved in the regulation of the pre-mRNA splicing machinery [78]. Tian *et al.* was the only research group that used Affymetrix GeneChip Exon 1.0 ST Arrays to identify significant differences in the alternative splicing of transcripts distinguishing untreated MS patients and healthy controls [67]. Of the 957 genes filtered by Cardamone *et al.* [78] and the 92 genes filtered by Tian *et al.* [67], only 3 genes (*DDX39B*, *IFNAR2* and *NFAT5*) were examined in the other included studies. Six studies included experiments with a splicing reporter minigene spanning at least one exon and the flanking intronic sequences. The study by Gregory *et al.*, for instance, was the first demonstrating via transfections of minigene constructs that the MS-associated SNP *rs6897932* affects the inclusion of exon 6 of *IL7R* [44].

Blood cells, e.g. whole blood samples or PBMC, have been examined in 32 studies and, therefore, they were the most widely used sample material. In 24 of these studies, transcript isoform expression was examined with PCR-based methods or microarrays. Protein isoform levels, on the other hand, were determined in 19 of these studies, e.g. with flow cytometry, enzyme-linked immunosorbent assays (ELISA) or immunoblots. Specific immune cell types, e.g. T cells, B cells or dendritic cells, were analyzed in a total of 16 studies. Taking into account that MS is an inflammatory disease of the CNS, we found that a total of 5 studies used post-mortem brain tissues or CSF samples for the analysis. Ten studies compared alternative splicing expression patterns among different cells and tissues. For instance, Boyle *et al.* explored the subcellular localization of *MOG* protein isoforms using confocal microscopy and the expression of the respective RNA variants in brain and other tissues using PCR followed by gel electrophoresis [55]. All studies that investigated ASE in cell culture conditions (n=15) employed human cell lines, e.g. HEK 293, HeLa or Jurkat.

A subset of 3 studies compared gene isoform levels in sample material from patients with different MS courses [45, 50, 52]. McKay *et al.* determined the ratio of soluble to full-length *IL7R* mRNA in the blood of patients with PPMS and RRMS [45]. In two studies by Sharief *et al.*, the levels of the long and short protein isoforms of *CFLAR* were investigated in the blood of RRMS and SPMS patients [50, 52]. In another study by this group, *CFLAR* isoform expression was compared between clinically active and clinically stable RRMS patients [51].

3.3. Investigated alternative splicing events related to MS

Altogether, 27 different genes have been explicitly studied in the 36 articles (Table 2). The most studied gene is the cytokine receptor *IL7R* that plays an important role in the differentiation and proliferation of human T cells. A soluble form of *IL7R* results from an alternative splicing of exon 6. In 2007, Gregory *et al.* discovered the C allele of SNP *rs6897932* in exon 6 as a significant risk factor for MS susceptibility in four independent family-based and case-control datasets [44]. They cloned the two allelic versions of this SNP in splicing reporter minigenes and observed a twofold increase of exon 6 skipping for the MS-associated C allele compared with the T allele. In a follow-up study from 2017, the same research group elucidated *cis*-acting elements and *trans*-acting factors controlling splicing of this exon [49]. They showed that the knockdown of the RNA helicase *DDX39B* leads to a significantly higher level of exon 6 skipping and an increased secretion of soluble *IL7R*. The study by Hoe *et al.* is the only one that examined *IL7R* isoforms at the protein level in sera from MS patients and controls [47]. They confirmed that carriers of the T allele of SNP *rs6897932* have significantly lower levels of soluble *IL7R*, and there was a non-significant trend toward higher levels in the MS patient group compared with healthy controls.

For the reason that *MOG* is a target in inflammatory demyelinating diseases [80], Jensen *et al.* used brain tissue specimens to determine relative levels of *MOG* transcript variants for different haplotypes [56]. They observed a significant expression difference of transcripts resulting from two alternative splice acceptor sites of the last exon 10, which was related to the alleles of SNP *rs2857766*. While this study was focused on the influence of genetic variants on splicing, Boyle *et al.* evaluated 6 different *MOG* isoforms with regard to their cellular localization by cloning

them into mammalian expression vectors [55]. They showed that while the two full-length isoforms are expressed at the cell surface, three alternatively spliced isoforms characterized by exon 8 skipping have a more intracellular distribution. Another *MOG* isoform, an alternative last exon variant which lacks a transmembrane domain, is preferentially secreted. All six isoforms were detectable in normal appearing white matter from MS patients, but the *MOG* splicing pattern was not different compared to brain tissue from normal controls.

Most of the genes for which ASE have been investigated in the MS literature are involved in immune responses. Some of them encode cytokine receptors. Beyond *IL7R*, these are *IFNAR2*, *CSF1R* and members of the TNF receptor superfamily. The functional consequence of an MS-associated genetic variant in *TNFRSF1A* was investigated by two research groups [59, 60]. They showed that the putative causal risk allele G of SNP *rs1800693* promotes exon 6 skipping and thus directs the expression of a soluble form that lacks the transmembrane and cytoplasmic domains and that can perturb TNF- α signaling. Splicing isoforms of the type I interferon receptor *IFNAR2* modulate the responsiveness to both endogenous and recombinant IFN- β . Gilli *et al.* demonstrated that the transcript levels of the soluble form (exon 8 skipped) are significantly upregulated in PBMC of MS patients over the first year of IFN- β therapy [69]. *CSF1R* is the receptor for CSF1, a cytokine which regulates monocyte/macrophage differentiation. In the study by Spurlock *et al.*, a decreased transcript abundance of *CSF1R* exon 1-11 was observed in the blood of MS patients compared to healthy controls [65].

Several of the alternatively spliced genes control transcriptional programs. For instance, *SP140* regulates the expression of immune-related genes that are linked to MS and other autoimmune diseases [81]. GWAS have identified the *SP140* gene locus to be associated with the risk of developing MS [19], and Matesanz *et al.* could show that the T allele of SNP *rs28445040* causes skipping of exon 7 in *SP140* [64]. *FOXP3* and *NFAT5* also encode proteins that influence transcription. Matarese *et al.* found that regulatory T cells (Treg) cells from patients with RRMS express lower amounts of the 44- and 47-kDa *FOXP3* isoforms (resulting from exon 3 skipping/inclusion) as compared with Treg from controls, and they uncovered a link between glycolysis and *FOXP3* pre-mRNA splicing via ENO1, a mechanism that may affect the induction and suppressive function of Treg [57, 58]. *NFAT5* regulates osmotic stress-related

genes and is involved in high salt-induced Th17 cell development [82]. A splicing pattern analysis by Paraboschi *et al.* revealed that the levels of an exon 2-skipped *NFAT5* isoform are significantly increased in MS cases vs. controls [66]. The inclusion of exon 2 depends on the genotype of SNP *rs12599391*, which is located within an intronic splicing enhancer (ISE). Interestingly, *NFAT5* is activated by the Th17-specific protein kinase *PRKCA* [83], for which an MS-associated genetic polymorphism (*rs35476409*) was shown to affect exon 4 inclusion [63].

3.4. Detection of alternative splicing events in transcriptome data

The 36 included articles distinguished splicing variants of 27 different human protein-coding genes. The lack of validation studies prompted us to reevaluate the ASE in huge Affymetrix HTA 2.0 microarray datasets of blood cells from MS patients and healthy subjects. In this analysis, a subset of 19 genes was found to be differentially alternatively spliced (EventPointer *p*-value <0.0001 for each comparison against the 4 control datasets) in at least one of the 5 examined cell populations (CD4+, CD8+, CD14+, CD19+ and CD56+ cells). For 7 genes, the reported ASE reached statistical significance even in all case-control comparisons (Table 2).

As an example, we visualized the exon-level data of *IL7R*, which has been repeatedly described in the literature to be implicated in MS (Figure 3). It has been shown that skipping of exon 6 is augmented by reduced expression of the activator *DDX39B* and by strengthening of an exonic splicing silencer (ESS) by the MS risk allele C of SNP *rs6897932* [44, 49]. Moreover, CPSF1 has been identified to interfere with spliceosome binding of *IL7R* pre-mRNA [84]. As a consequence, the amount of soluble and membrane-bound protein isoforms is influenced. On HTA 2.0 microarrays, *IL7R* is interrogated by 245 oligonucleotide probes (probe set TC05000159.hg.1). In our data, the highest *IL7R* mRNA levels were seen in CD4+ and CD8+ cells. A closer inspection of the signal intensities for exon fragments and splice junctions clearly demonstrated the more frequent skipping of exon 6 in patients with MS. More specifically, we detected significantly lower average levels for exon 6 and higher levels for the junctions spanning the exon boundaries 5-7 (exon 6 skipping) and 4-7 (exon 5-6 skipping) in the MS patient group as compared to the healthy control group (Figure 3).

4. Discussion

While GWAS have provided important insights into the genetic contribution to MS susceptibility [19], molecular biomarkers with high sensitivity and specificity for MS are still not available. It is likely that several MS-associated genetic variants affect regulatory regions for pre-mRNA splicing. Alterations in alternative splicing have already been demonstrated for a variety of diseases [25, 85]. Here, we performed an electronic literature search for studies related to MS, and we reassessed the expression of splicing variants in transcriptome data of MS patients and healthy controls to further explore the biomarker potential of ASE. The article selection process was conducted according to the PRISMA guidelines [33]. For this systematic review, we screened a total of 160 articles, and we summarized the results of 36 articles that were identified as relevant to the topic. Most of the studies focused on genes that participate in inflammatory pathways or genes that encode CNS myelin proteins.

The majority of the reviewed studies examined blood cells. Blood sampling is less invasive for patients and less extensive for the medical staff compared to the collection of CSF by lumbar puncture. Nevertheless, CSF samples may be more suitable for the detection of biomarkers, as inflammatory lesions in MS selectively affect the CNS [31]. The lack of studies using CSF for the investigation of disease-specific ASE can be explained by the fact that the analysis of specimens with low cell counts was not very sensitive until a few years ago. The rapid technological development in recent years, however, enabled reliable measurements of even small amounts of RNA or protein [86-88]. Because of these advancements, it is intriguing to speculate that future studies may identify splicing products in CSF that correlate with inflammation and degeneration in MS. So far, four studies examined the expression of alternatively spliced human genes in post-mortem human brain tissue in order to better understand the pathophysiological processes in MS. For instance, different *MOG* isoforms have been analyzed in brain samples [55, 56]. The findings of such studies can support and stimulate investigations of other tissues or body fluids.

The reviewed studies in the field of MS measured different RNA and protein isoforms that arise by alternative splicing and by usage of alternative transcription starts and ends. Out of 27

reported genes, 18 genes were investigated in only one study. The currently most comprehensively studied gene is *IL7R* whose isoform expression was analyzed in 8 articles. These studies revealed that the MS risk allele C of SNP *rs6897932* leads to a more frequent skipping of exon 6, resulting in an increased production of transcripts that encode a soluble form of the protein [44]. Other GWAS with cohorts of primary biliary cholangitis and type 1 diabetes patients also showed an association for the *rs6897932* SNP [89, 90]. Similarly, genetic variants in *GSDMB* appeared in GWAS on rheumatoid arthritis and ulcerative colitis [91, 92]. Furthermore, *SP140* and *TNFRSF1A* have been implicated as risk factors in Crohn's disease and ankylosing spondylitis, respectively [93, 94]. This illustrates that the same functional mechanisms may play a role in different immune-mediated conditions that share the same genetic susceptibility loci. Further research is thus warranted to pinpoint the causal disease risk variants and to elucidate their biological consequences such as ASE.

Genotype-dependent variation in splicing regulatory elements plays a crucial role for individual splicing pattern differences, which may influence onset and outcome of chronic illnesses. Diverse bioinformatic approaches have been developed to predict such elements *in silico* [22, 95, 96], and splicing reporter assays can be used to evaluate the effect of genetic polymorphisms and cofactors of the spliceosome machinery in cell culture [34]. As an example, Cardamone *et al.* cloned *IL7R* exon 6 and *SP140* exon 7 with different SNP alleles and with the respective intronic flanking sequences into minigene plasmids. The *IL7R/SP140* minigene constructs were then cotransfected either with a MALAT1-expressing plasmid or with an empty vector into HEK 293 cells. By subsequent PCR analysis, MALAT1 overexpression was found to significantly increase skipping of *IL7R* exon 6 and *SP140* exon 7 in a genotype-independent manner, demonstrating that this non-coding RNA affects the repertoire of alternatively spliced transcripts in MS [78]. Minigene assays have been also employed to study *de novo* and polymorphic Alu insertions [97]. RNA abundance and splicing are modulated by such transposable elements via RNA-protein interactions [98]. The current research on MS is still at the beginning to gain new pathomechanistic insights by assessing splicing-affecting variants and the resulting defects. Future studies should consider nucleotide diversity in larger sequence environments to better understand the context dependence of splicing regulation.

Aberrant expression levels of alternative splicing isoforms are not only of interest for diagnostic purposes but also for monitoring therapeutic interventions in MS. Serum levels of soluble *TNFRSF1A* have been shown to be significantly reduced in MS patients treated with glatiramer acetate relative to untreated patients [59], and expression changes of *IFNAR2* transcript isoforms in PBMC were found to be an indicator of the biological responsiveness to IFN- β therapy [69]. A recent study by Bedri *et al.* explored the potential role of cytokine receptors as biomarkers for treatment effects [99]. In plasma samples from MS patients, they observed a decline in soluble *IL7R* protein levels during natalizumab therapy, while subsequent fingolimod therapy led to increased levels. This is of relevance as soluble *IL7R* diminishes excessive IL7 signaling in human T cells and increases IL7 bioavailability [100]. Therefore, cytokines continue to be attractive therapeutic targets for neuroinflammatory disorders [101]. On the other hand, ASE may yield biomarkers of the individual clinical response to immunomodulatory treatments. However, further studies are needed to confirm and extend these findings.

One of our study eligibility criteria was the investigation of different isoforms of a human protein-coding gene. However, non-coding RNAs also contribute to transcriptomic complexity, although their regulation, spliceosomal processing and function has not been sufficiently researched so far. Several spliced transcripts can originate from human endogenous retrovirus (HERV) elements. A connection of HERV with MS has been discussed for years, but the consequences resulting from reactivated expression of retroviral sequences, which usually lack protein-coding capacity, are still unclear [102, 103]. Christensen *et al.* used RNA from blood cells to detect transcripts related to the HERV-H subfamily [104]. They observed that two different HERV-H splice variants occurred together in about 40% of the MS patients but only in 10% of the controls. Another phenomenon potentially interfering with alternative splicing is represented by backsplicing of immature RNA. By this means, some spliced introns can escape degradation and generate circular RNAs [105-107]. A dysregulation of circular RNAs has been reported in MS patient blood. Cardamone *et al.* detected a significantly higher level of a circular RNA consisting of exons 4 and 5 of the *GSDMB* gene in PBMC from patients with MS compared to controls [61]. Moreover, Iparraguirre *et al.* carried out a microarray analysis of whole blood samples and found 406 circular RNAs to be differentially expressed in MS [108].

We performed a case-control reanalysis using high-density microarray datasets, which affirmed significant ASE for 19 out of the 27 examined genes. By this means, we showed that modern transcriptome profiling technology and computational biology facilitate the large-scale analysis of ASE [43, 109]. Population transcriptome studies have fostered the identification of naturally occurring genetic variants that modulate alternative splicing [110, 111], but several challenges remain: For instance, still little is known on the contribution of RNA processing on transcript diversity of disease-associated genes of the human major histocompatibility complex (MHC) [112]. Moreover, microarray and RNA sequencing data are usually based on short sequences, thus missing information about the combination of exon junctions in individual transcripts. Emerging long-read RNA sequencing methods, however, should provide such information [113]. Individual cells can also exhibit differences in gene expression and splicing. Therefore, single-cell transcriptomics may be employed to investigate this heterogeneity [114]. Exploring the causal impacts of specific SNPs on splicing can be cumbersome. To address this issue in a high-throughput manner, massively parallel reporter assays have been designed [110]. Finally, novel proteomic profiling solutions should be incorporated to complement the characterization of dysregulated alternative splice variants [115]. The elucidation of splicing-altering disease susceptibility alleles has the potential to advance individual genomic medicine [30]. However, continued efforts are needed to improve our understanding of how ASE may affect disease onset, course and activity and the effects of immunomodulatory treatments in MS.

To conclude, we here provide an overview of published research about the expression of alternatively spliced gene isoforms in MS. Our systematic literature search identified a total of 36 studies from the last two decades, with a rising trend in recent years. Most studies used PCR techniques to measure selected genes, but first attempts have been made to incorporate transcriptome data from high-resolution microarrays and RNA sequencing. Splice variants were assessed for 27 genes, including cytokine receptors, transcription factors and myelin genes. Further studies are warranted that link genetic, RNA and protein data in a context- and cell type-specific manner to uncover splicing regulatory mechanisms. This will set the stage to gain new insights into the immunopathology of MS and to apply this knowledge in clinical practice in terms of biomarkers for disease diagnosis and prognosis as well as therapeutic decisions.

Abbreviations

ASE	alternative splicing event
BPS	branchpoint sequence
CNS	central nervous system
CSF	cerebrospinal fluid
DMD	disease-modifying drug
ELISA	enzyme-linked immunosorbent assay
ESE	exonic splicing enhancer
ESS	exonic splicing silencer
GA	glatiramer acetate
GEO	Gene Expression Omnibus
GWAS	genome-wide association study
HERV	human endogenous retrovirus
HGNC	HUGO Gene Nomenclature Committee
HTA	Human Transcriptome Array
ID	identifier
IFN- β	interferon-beta
IMSUP	immunosuppressor
ISE	intronic splicing enhancer
ISS	intronic splicing silencer
JUC	junction probe set
MHC	major histocompatibility complex
mRNA	messenger ribonucleic acid
MS	multiple sclerosis
n.a.	not available
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
PPMS	primary progressive multiple sclerosis
PPT	polypyrimidine tract
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
PSR	probe selection region probe set
RNA	ribonucleic acid
RRMS	relapsing-remitting multiple sclerosis

SNP	single-nucleotide polymorphism
SPMS	secondary progressive multiple sclerosis
SST-RMA	signal space transformation robust multi-array average
TAC	Transcriptome Analysis Console
Th cell	T helper cell
Treg	regulatory T cell

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