Biological reflections in body fluids of multiple sclerosis progression and multiple sclerosis-related fatigue

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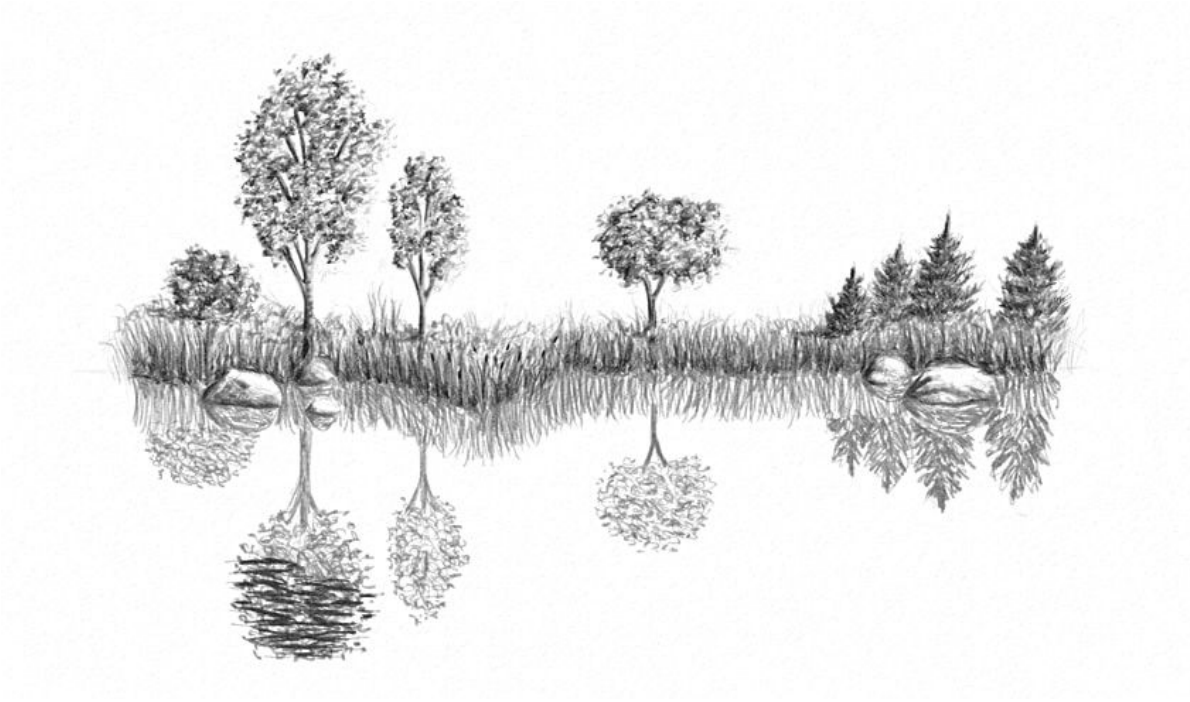
Part 1. Chapter 1.

Recent progress in omics-driven analysis of MS to unravel pathological mechanisms



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Abstract

At present the pathophysiology and specific biological markers reflecting pathology of multiple sclerosis (MS) remain undetermined. The risk of developing MS is considered to depend on genetic susceptibility and environmental factors. The interaction of environmental factors with epigenetic mechanisms could affect the transcriptional level and therefore also the translational level. In the last decade growing amount of hypothesis-free "omics" studies have shed light on the potential MS mechanisms and raised potential biomarker targets. To understand MS pathophysiology and discover a subset of biomarkers, it is becoming essential to take a step forward and integrate the findings of the different fields of "omics" into a systems biology network. In this review we will discuss the recent findings of the genomic, transcriptomic and proteomics fields for MS and aim to make an unifying model.

Introduction

Multiple sclerosis (MS) is a chronic demyelinating neurological disease which affects young adults [1,2]. The age of onset is 20-40 years and it affected females twice as often as men [2,3]. The total amount of people diagnosed with MS worldwide is approximately 2.5 million, and the incidence and prevalence rates differ based on the geographical locations [4]. For example, the prevalence of MS in Asia is approximately 5 per 100.000 people, whereas a prevalence of 100-200 per 100.000 people is observed for United Sates, Canada and Northern and Western Europe [4].

The course of MS pathology varies between individuals and has been divided in three different subtypes; a) the relapsing remitting (RR) subtype, which is characterized by periods of exacerbations and remissions. Initially about 85% of the MS patients are affected with this subtype; b) the primary progressive (PP) subtype, which is characterized by steady neurological and clinical decline from disease onset and affects approximately 10-15% of the MS patients; c) within 15 years from onset about 60% of the RRMS patients converse into a secondary progressive (SP) subtype, in which steady neurological decline with no relapses is observed [2,5,6].

The underlying disease mechanisms of MS remain unknown. Generally, auto-immune mediated demyelination followed by axonal degeneration is considered as the primary mechanism, however recent studies suggest oligodendrocyte and axonal loss as the initiating event [7,8]. The debate on whether the inflammatory or neurodegenerative mechanisms are the primary cause is on-going. However, it is evident that the combination of genetic susceptibility for MS and environmental factors can cause MS [4,9,10]. The epigenetic field can be observed as a potential "translator" between the environmental factors and gene expression. Therefore, epigenetic regulatory mechanisms on gene expression are becoming more important in understanding MS pathology [9,11]. As cellular functional units, proteins are interesting as potential biological markers (biomarkers) and therapeutic targets.

Despite the improvements in the MRI field and diagnostic criteria, the diagnosis of MS and MS subtypes remains challenging [12]. Furthermore the current therapies have low efficacies to attenuate MS and are primarily effective in RRMS [13,14]. Improvements in diagnosis of clinical definite MS and disease progression are required to be able to start therapies in early

stages and to target specific aspects of the pathological mechanisms. Therefore intensive efforts on discovering biomarkers for MS are on-going [15-18]. A biomarker is defined as "a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" [19]. Additionally, the discovery of disease specific MS biomarker(s) can clarify and offer more insight on the pathophysiology of MS. Based on heterogeneity and complexity of MS it is unlikely that a single molecule derived from a single biological level can represent MS pathophysiology and serve as a potential biomarker. Within the recent years different "omics" disciplines have reported multiple molecules that are associated with MS, and it is now time to take a step forward and use all the available hypothesis free "omics" data to gain more insight about MS pathophysiology.

Therefore, from a systems biology perspective, we evaluated recent "omics" data in order to identify unifying pathways associated with MS which are reflected into alterations in body fluids *in vivo*. Systems biological is the approach where the complete biological systems and the interactions with environment is studied [20]. We will incorporate "omics" data published from January 2010 till May 2013. Furthermore updates concerning independent replications of potential biomarkers published prior 2010 will be incorporated as well. We will avoid repetition of markers already published in previous reviews and aim for an interdisciplinary system biology approach [15,17,18,21-24].

Genome wide association studies for multiple sclerosis

As indicated above, the underlying pathophysiology of MS is considered to be caused by both genomic susceptibility and environmental triggers [25,26]. The major risk allele for MS is the HLA-DRB1*1501 haplotype tagged by SNP rs3135388 [25,27,28]. Heterozygosity for this SNP increases the risk for MS by approximately a 3 fold [27,28]. However the HLA-DRB1*1501 haplotype is thought to be responsible for approximately 50% of the genetic risk for MS, thus understanding the additional risk factors for MS at a genetic level could have an additional value for MS diagnosis and understanding the pathophysiology [28].

From 2005 till 2012 approximately 1350 genome-wide association (GWA) reports have been published, and approximately 30 GWAS addressing MS [29]. The online available database National Human Genome Research Institute (NHGRI) reports more than hundred associations of single nucleotide polymorphisms (SNPs) with over 80 diseases or traits [29,30]. Because most of the high throughput genotyping platforms are not efficient in genotyping copy number variations, insertions, inversions and deletions not much is known about these structural variations in association with MS as yet [31]. Therefore we will focus primarily on available SNP data in MS.

SNPs could cause synonymous or non-synonymous mutations. Synonymous polymorphisms do not change the amino acid sequence, whereas the non-synonymous polymorphisms change the amino acid sequence and are divided into missense or nonsense mutations [32,33]. Missense mutations alter the codon which results in a different aminoacid sequence, whereas nonsense mutations result in a stop codon and truncated proteins. Missense and non-sense mutations can occur in exons, introns and or promoter regions and can affect the protein phenotype, gene expression and alternative splicing [32-35].

Depending on the location and the type of mutations different consequences are observed, e.g. polymorphisms in promoter regions could affect the transcription factors binding sites which could result in altered levels of transcription of genes [32,33]. Polymorphisms between annotated genes are called intergenic regions and are the most prevalent, but their association with disease traits remain unknown. However, it is possible that these intergenic SNPs are located in the vicinity of miRNA regions and thus have indirect effects on gene regulations [36-38]. A single SNP in a heterogeneous disease like MS is unlikely to represent the disease pathogenicity or serve as a

potential genetic marker. It is therefore more likely that combinations of SNPs in MS associated genes could assist in MS diagnosis and related subtypes. Moreover, the genetic diversity between different ethnicities and the common SNP frequencies in the general public could make it challenging to include certain SNPs in a potential genetic profile for MS. It is more likely that rare variants of SNPs with a low frequency in general public contribute to the genetic makeup of MS [39,40]. Therefore, several requirements for SNPs that potentially could assist for creating genetic risk profiles for MS are; a) haplotypes or SNPs in different genes that preferably follow a Mendelian inheritance, this to have a stable genetic profile in subsequent descendants, b) contribute to increased risk for MS and preferably show phenotypic alterations in the subsequent gene products, c) the frequency of the risk SNPs in the general population should preferably be low [39,40].

A recent publication of a worldwide collaboration of different MS centres has led to validation and discovery of numerous loci associated with MS [41].This was performed by the International MS Genetic Consortium (IMSGC) with approximately 10.000 cases and 20.000 controls [41]. We will elaborate on the data published by IMSGC and only include SNPs that have been described or replicated by other GWAs as well. Moreover, we will make a selection of non-HLA genes, and exclude risk alleles of loci with high genotype frequencies in general public, which could result in decreased discriminative power and thus likely would be a less important addition to the possible genotype profile for MS (Table 1) [25,42,43]. To have an overview of the MS associated genes and their link to the two possible MS aetiologies we have divided the selected loci based on their functionality into a) immune system associated, b) neuro-glial associated.

Table 1. Overview MS associated loci reported by IMSG

Putative gene of interest	rsID	Chromosome	Risk Allele	OR (95% CI)
PTGER4	rs4613763	5	C	1.21 (1.15-1.28)
CD58	rs1335532	1	T	1.18 (1.12-1.24)
EVI5	rs11810217	1	T	1.15 (1.11-1.20)
CBLB	rs2028597	3	G	1.13 (1.06-1.21)
IRF8	rs13333054	16	T	1.12 (1.08-1.17)
IL2RA	rs3118470	10	C	1.12 (1.08-1.17)
CD6	rs650258	11	C	1.12 (1.08-1.16)
RGS1	rs1323292	1	T	1.12 (1.07-1.18)
IL7R	rs6897932	5	C	1.11 (1.06-1.16)
CD40	rs2425752	20	T	1.10 (1.06-1.14)

Immune system associated loci

Discovery and subsequent replications in different studies of SNPs in IL-7RA (rs6897932, odds ratio (OR):1.11) and IL-2RA (rs3118470, OR: 1.12) show associations with MS [41,44-46]. The cytokine IL-7 is primarily expressed within the thymus and bone marrow and promotes the lymphocyte homeostasis and affects the maturation and survival of immature and mature T and B-lymphocytes [47,48]. Binding of the cytokine IL-2 with the IL-2 receptor is necessary for the proliferation of T-cells [49,50]. The receptor for IL-7 consist of two subunits, the IL-7Rα and the common-γ chain receptor [51]. The IL-2 receptor consists of the IL-2Rα, IL-2Rβ and the same -γ chain receptor as for IL-7 [49,50]. The IL-7R rs6897932 SNP is located in exon 6 and the common form is a C whereas the mutant is a T. This polymorphism causes a missense mutation and changes the Threonine into an Isoleucine amino acid, and possibly affects the ratio of soluble and membrane-bound isoforms [45]. Surprisingly, the common C allele has a high frequency in approximately 64% of the people with North and West-European ancestry [45]. This C allele seems to be the risk factor whereas the T polymorphism indicates a possible protective mechanism [44,45]. The IL-2RA rs3118470 is located at the 5 end of intron 1, and the homozygous frequency of the ancestral allele T is 48%, whereas the homozygosity of the C/C is approximately 8.7% in the European population descents. For the rs3118470 IL-2RA SNP the C allele seems to be the risk factor for MS and it has shown to reduce expression levels of IL-2RA [46,52]. The IL-7R rs6897932 SNP risk allele has a high frequency in general public, whereas the IL2R rs3118470 risk allele is less present in the general European population. Therefore it is more likely that the IL-2R polymorphism is a valuable candidate to add to the genetic profile for MS.

Different loci within the Prostaglandin E Receptor 4 (PTGER4) gene are associated with Crohn's disease and multiple sclerosis [41,53-55]. The PTGER4 encodes for a Prostaglandin E receptor (EP_4) which is a member of the G-protein coupled receptors. This receptor family binds prostaglandin E2 (PGE2) [56]. This binding can result in inhibition of T-cell proliferation, inhibition of TNF-α and IL-6 synthesis by neutrophils and down regulation of MHC class II proteins in dendritic cells [56]. The proposed SNP rs4613763 (OR: 1.21) is within the vicinity of the PTGER4 gene and might

affect the transcription of PTGER4 [41,53,54]. The rs4613763 major homozygous T allele has a genotype frequency of 70.5% for the North – West European population, whereas the risk allele C has a 4.4% homozygous frequency. Based on the low homozygous frequency of the risk allele and the regulatory functions on T-cell proliferation, the SNP in PTGER4 could be a valuable addition to the genetic profile for MS.

The SNPs in the immunoglobulin genes CD6, CD40 and CD58 have been replicated in different populations of European descent in multiple studies [41,55,57,58]. The receptors encoded by CD6 and CD58 genes are both involved in T-cell co-stimulation and differentiation [59]. CD6 is primarily expressed on thymocytes and mature T- cells. Additionally CD6 and CD58 interact with ALCAM and CD2 which are expressed on antigen-presenting cells (APC), allowing APC-T-cell binding which primes the thymocytes and activates T-cells. The specific CD6 SNP rs650258 (OR: 1.12) reported by the IMSGC has not been reported by others [41,55]. This SNP is an intergenic SNP between the genes CD6 and CD5 and functional experiments are required to show if and how rs650258 affects CD6. Others have reported the CD6 intronic rs17824933 SNP with a risk allele C as risk for MS [55]. According to HapMap data a population of European descents does not possess the minor homozygosity for this allele; whereas a heterozygous allele frequency of 4.2% is observed. The low minor allele frequency for this SNP makes it a viable addition to the disease profiling SNP subset. The CD58 intronic rs1335532 (OR: 1.18) has been replicated in European descent and others have reported different SNPs for CD58 as well [27,41,55,57]. The risk allele is the ancestral T allele with a homozygote genotype frequency of approximately 70% in the European descent, while the C minor allele has a frequency of 2.7% suggesting a possible protective mechanism. The risk allele T has a high frequency in the general population of European descent and together with a low odds ratio the rs1335532 SNP is not a valuable addition to the subset.

The gene CD40 encodes for a trans-membrane receptor of the tumor necrosis factor gene superfamily [60,61]. This receptor is expressed on monocytes, B cells and other APC cells [60,61]. The binding of CD40 with the CD40 ligand expressed on mature T-cells is a co-stimulatory signal necessary for activation of T-cells and expression of cyto- and chemokines by these T-cells [60,61]. The SNP rs2425752 (OR: 1.10) was initially reported by IMSGC as a CD4 SNP, however according to HapMap and

other reports this polymorphism is located at the NCOA5 gene, thus it is unlikely that this SNP contributes to the observed CD40 MS associations observed by others [55]. Others report that the CD40 rs1883832 SNP (OR: 1.12) is associated with MS, Crohn's disease and Horton's disease [55,58,62]. This SNP is located at the 5' UTR region and might disrupt the Kozak sequence [58]. The Kozak sequence on mRNAs is recognized by ribosomes and is required for the initiation of translation; therefore rs1883832 possibly inhibits translation of the CD40 mRNA [55,58,62]. The minor allele T is also the risk allele with a homozygous frequency of 3.5% in the European descent. Both the decreased gene translation and low homozygous risk genotypes make this SNP a viable addition to the MS profile subset.

The Interferon Regulatory Factor 8 (IRF8) encodes for the IRF8 protein which binds to IFN-Stimulated Response Element (ISRE) and regulates the expression of the IFN-α and IFN-β [63,64]. Furthermore IRF8 is involved in activation of resting microglia, transformation of B-cells by Epsteinbar virus (EBV) and genetic variation in the IRF8 gene is associated with different circulating monocyte count in inflammatory diseases [63-65]. Several polymorphisms have been reported for IRF8; rs13333054 (OR: 1.12) and rs17445836 (OR: 0.84) [41,55,66]. The rs17445836 polymorphism has been shown to alter the expression of the downstream targets in the interferon response pathway [55]. However, this risk allele for rs17445836 is the ancestral allele G, with a homozygous genotype frequency of 53.4% in European descent population. The risk allele T for rs13333054 is the minor allele with a homozygous minor frequency of approximately 3%, making this SNP a more suitable polymorphism for genotype profiling models.

Genes associated with neuro-glial cell homeostasis

The IMSGC and others have reported several MS associated genes which are involved with cellular homeostasis, which are EVI5, CBLB and RGS1 [41,57,67-69]. The EVI5 gene encodes for the Ecotropic Viral Integration site 5 protein homolog (EVI5). This protein has a Rab GTPase activating protein (GAP) activity and localizes in the nucleus [70]. During anaphase EVI5 is considered to stabilize the centrosome and assist with the dynamics of the spindle apparatus [70,71]. The SNP rs11810217 (OR: 1.15) in the EVI5 intron has an ancestral C allele with a homozygous genotype frequency of 42%, with a homozygous minor risk allele frequency of 11.6%. The CBLB gene encodes for the Casitas B-lineage Lymphoma proto-

oncogene B (CBLB) and is an ubiquitin-transferring enzyme [72,73]. These ubiquitin transferring enzymes are important in regulation of the proteasome and self-tolerance [72-74]. For example, the activation of B- and T-cells are inhibited by CBLB, a possible "coast guard" mechanism to allow self-tolerance and inhibiting auto-inflammatory reactions [72-74]. The intronic SNP rs2028597 (OR: 1.13) in CBLB with the ancestral allele G, has homozygous frequency of 82% in different populations of European descent [41,57]. Polymorphisms in the gene encoding for the Regulator of G-protein signalling 1(RGS1) have been shown to be genome wide associated or showing a statistical trend of association with MS in two different populations of European descent and African Americans [41,55,75]. The RGS1 is expressed neurons and especially in B-cells and monocytes [76,77]. The RGS1 is considered to desensitize activation of B-cells and monocytes by conversion of bound GTP to GDP and subsequent alterations in cytokine receptors e.g. CXCL13 [76,77]. The polymorphism rs1323292 (OR: 1.12) is located near the RGS1 gene with the ancestral allele T [41]. The ancestral allele T is also the risk allele associated with MS with a homozygous genotype frequency of 65% and a minor allele frequency of 5.3% in European descent [41]. Overall the above discussed polymorphisms in EVI5, CBLB and RGS1 have risk alleles that are too prevalent in the general healthy population of North- and West-European descents, which makes these SNPs not valuable additions to a possible SNP profile for MS.

The list of genes associated with MS is growing and contains approximately more than 50 non HLA- genes [41,78]. More than half of these polymorphisms in MS associated genes have risk alleles with a high frequency in common European population, which makes these SNPs impractical in a biomarker sense for MS diagnosis/disease profiling. These genes have shed light on possible underlying mechanisms for MS. A big overlap of associated genes is observed between different autoimmune diseases indicating possible common disease mechanisms [78,79]. Further knowledge about the different genotype profiles, by discovery of novel polymorphisms associated with MS and validations of these polymorphisms by functional experiments is required. This could potentially lead to genotype profiles which are able to discriminate between MS and other neurological pathologies.

Epigenetics in MS

Different definitions of the term "epigenetics" have been used in the last decades [80]. In 2008, a consensus about the operational definition was made; "an epigenetic trait is a stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence" [81]. The epigenetic mechanisms that regulate gene expression are DNA methylation, histone modification and regulatory non-coding RNAs [81,82]. Growing evidence based on genomics and epidemiological data indicate that unknown environmental factors trigger MS in genetic susceptible individuals [2,9]. Considering that complex diseases such as MS are difficult to explain by genetic susceptibility only, interest in the influence of environmental factors on genetics, with epigenetics as a potential "translator" is growing in MS [11,83]. A good example of an environmental factor associated with MS is vitamin D [11,83-85]. Both geographical and biological data suggest that low vitamin D is associated with high risk of developing MS [84,85]. The mechanisms behind these associations are considered to be to the transcription factor activity of the Vitamin D- Receptor (VDR) complex recognizing vitamin D response elements (VDRE) on the genomic regions [85]. This binding is involved with regulation of expression of 229 different genes, e.g. IRF8, RGS1 and CD40 are regulated by VDR transcription factor [84-86]. Increasing evidence is showing the importance of VDR to be able to regulate different miRNAs and histone modifying enzymes [87-90].

Focus on the epigenetic link between genes and environment could assist in understanding what other environmental factors are able to induce MS in genomic susceptible individuals. A recent publication on DNA methylation patterns between 3 pairs of twins with discordance for MS reported no different DNA methylation or mRNA patterns between these twins, suggesting other downstream epigenetic mechanisms could be altered in MS [91]. The limitation of this report was the limited amount of twins available. However, to see whether differences in epigenetic mechanisms, such as DNA methylation, could explain the MS discordancy between the monozygotic twins remains an interesting question.

Another mechanism of epigenetics control on gene expression is the regulation on mRNA by miRNAs. Even though miRNAs do not interact with DNA directly, they are able to regulate mRNA translation and considered to be inherited to the daughter cells [92-94]. From a biomarker point of view, miRNAs or a combination of miRNAs have the ability to

serve as potential MS biomarker based on the following characteristics; a) miRNAs are relatively stable in different tissues and body fluids, b) miRNAs can be associated with specific tissues or cells, c) the quantification of miRNAs is more sensitive than immune assays, d) depending on the tissue of interest miRNAs can be obtained by minimally invasive sampling, e.g. urine, serum and plasma, and serum-derived miRNAs has been shown to reflect brain pathology in some diseases [95,96]. Different reports before 2010 showed promising results for the use of miRNAs profiles as biomarkers for MS [15,97]. Since then different groups have analysed the association of miRNAs with MS, however different results is observed between these studies [98-105]. A summary of the miRNAs associated with MS or MS subtypes based on these studies is shown in table 2. The primary explanations for the observed differences could be that these studies used different tissues or cell types and analysed a relatively small number of patients. Furthermore, different pre- and post-analytic issues, such as sample handling, methods of RNA isolation, the types of arrays used and data normalization methods have been shown to contribute to different results [96,106,107]. Due to lack of consensus between the studies, we here discuss miRNAs that are directly linked to important pathological mechanisms in MS. However division of these miRNAs into immune or neuro-glial MS aetiologies is not possible because of imperfect biding of miRNA seeds regions and thus having potentially multiple mRNA targets.

Table 2. Overview of miRNAs associated with MS

Name miRNA	Cell type/tissue	expression in MS	Subtype	Reference
miR-17	Cell free blood	↓	total MS	[98],[102]
miR-20a	Cell free blood	↓	total MS	[98]
miR-181a	B cells	↓	RR vs HC	[104]
Let-7a	EDTA plasma	↑	RR vs SP	[100]
miR-30e	EDTA plasma	↑	RR vs HC	[100]
miR-223	PBMC	↓	MS vs HC	[118]
miR-146a	PBMC	↑	RR vs HC	[105]
miR-142-3p	PBMC	↑	RR vs HC	[105]
miR-181c	CSF	↑	MS vs OND	[101]

MS associated miRNAs

The miR-17 and miR-20a were significantly decreased in different MS subtypes compared to healthy controls [98] . Interestingly, *in silico* experiments have shown that these miRNAs are associated with immune response pathways and T cell activation genes [98]. Moreover the miR-17-3p a member of the miR-17 family has been show to also bind Oligodendrocyte Lineage Transcription Factor 2 (OLIG2) mRNA and inhibits its expression [108]. Olig2 is required for oligodendrocyte and motor neuron specification in the spinal cord during prenatal development [108,109]. It has been reported that Olig2-positive progenitors cells (OPCs) are increased around MS lesions [109]. The authors concluded that a potential differentiation block of OPCs around chronic MS lesion is a major determinant in remyelination failure [109]. To gain more insight about this possible differentiation block, it would be interesting to see whether inhibition of miR-17 is the cause of increased Olig2 expression in lesions in chronic EAE models.

In B-cells of RRMS patients a twofold decreased expression was observed for miR-181a compared to healthy controls [104]. Moreover, in EDTA plasma of RRMS compared to healthy controls an elevated expression was detected for miR-30e (OR: 3.98) [100]. The miRNAs 181a and miR-30e are located on chromosomes 9 and 1 respectively. Both of these miRNAs are associated with Natural Killer (NK) cells [110,111]. Mir-181a down regulates the inhibitor of Notch pathway, allowing proliferation of NK cells [110]. MiR-30e targets the serine protease perforin expression, which is a granule peptide with cytolytic activity [111]. The inhibitory effects of miR-30e are required to decrease the cytotoxicity of activated NK cells [111]. In MS NK cells are considered to be involved in the regulation and dampening of the auto-inflammatory cells [112]. It is possible that the lower expression of miR-181a reduces the NK cells proliferation and could affect subsequent immune regulatory functions in MS [113]. Interestingly, an age-dependent decrease of miR-181a is considered to be associated with impaired naïve T-cell responsiveness, and reconstitution of miR-181a *in vitro* improved CD4+ T cell responsiveness [114]. The expression of miR-181a in T-cells derived from MS patients needs to be assessed, to see whether this decrease is also observed in MS patients.

The miRNA let-7a has been reported to be increased in RRMS compared to SPMS in EDTA plasma [100]. The association of miR-let-7a with different

subsets of T cell and IL-6, IL-10 and IL-13 production has been shown by *in silico* and *in vitro* studies [100,115-117]. Let-7a is associated with the anti-inflammatory cytokine IL-10 in CD4+ T cells [117]. The increased levels of let-7a in RRMS in comparison to SPMS might be explained by its inhibitory role on translation of the anti-inflammatory cytokines IL-10 and IL-13, which could lead to pro-inflammatory microenvironment and possibly exacerbations.

The miRNAs miR-223, miR-142-3p and miR-181c were shown to be differentially expressed in PBMCs and CSF of RRMS compared to healthy controls or other neurological diseases [101,118]. These miRNAs are involved in the regulatory pathway of hematopoietic cells, controlling the cell proliferation [119,120]. Interestingly miR-142-3p has been shown to inhibit cAMP in CD4+ T cells and CD4+ CD25+ T regulatory (reg) cells [121]. It is believed that low levels of cAMP is necessary for the activation of CD4+ T cells, whereas the regulatory function of CD4+ CD25+ Treg cells could be by transferring cAMP to adjacent target cells by gap junctions [121,122]. Interestingly, it has been shown that tumor associated macrophages secrete exosomes containing miR-223, which promotes breast cancer invasiveness [123]. Additional function of miR-223 where shown in two recent publications, in which the NLR Family, Pyrin Domain Containing 3(NLRP3) inflammasome protein expression was inhibited by miR-223 [124,125]. Noteworthy the mRNA of NLRP3 3' biding site binds an EBV miRNA, preventing activation of inflammasome [124,125]. It might be possible that a possible competition between the endogenous NLPR3 regulator miR-223 and the EBV miRNA prevents the normal inflammasome inhibition and thereby promotes macrophage activation in MS.

The miR-146a has been shown to be increased in PBMCs of RRMS individuals compared to healthy controls [105]. Stimulation of monocytes by bacterial endotoxins increases the transcription factor NF-$\varkappa$B, which increases miR-146a expression [126]. The subsequent targets for miR-146a are two signal transducers of the NF-$\varkappa$B activation pathway; by doing this the inflammatory response is dampened [126]. The association of miR-146a with different autoimmune disease has been shown, e.g. systemic lupus and rheumatoid arthritis [126-128]. More interestingly, a recent publication observed similar negative feedback response of miR-146a in astrocyte-mediated inflammatory responses [129]. An increase in the expression of miR-146a was observed upon the addition of IL-1β *in vitro*, with the

inhibition of the IL-6 and COX-2 as a consequence of miR-146a [129]. A higher expression of miR-146a in PBMCs of MS patients was observed, which contradicts the anti-inflammatory effects of miR-146a. However, it does indicate an imbalance of this regulatory pathway in MS in the periphery. The miRNAs and non-coding RNA are promising prospects for shedding light on the underlying mechanisms of MS and potentially could serve as biomarkers for MS. However this relative new field of MS requires more independent validation and replication of the observed results. Furthermore, a consensus guideline regarding the pre and post-analytic challenges for the non-coding RNAs is required and could assist in independent validation and replication studies [96,106,107].

Proteins associated with MS

The genetic code is uniformly present throughout all the cells of a single individual. The expression of this genetic code is tightly regulated in different tissues, resulting in a tissue dependent synthesis of proteins which is required for cell homeostasis. Furthermore, proteins undergo post-translational modifications such as phosphorylation or glycosylation [130]. Therefore, the study of proteins as the functional units of the cells for heterogeneous diseases like MS is essential [130]. Hypothesis-free proteomics for MS primarily has primarily focused on CSF [131-134]. Most of the proteins considered to be associated with MS prior 2010 have been divided into: a) inflammatory, b) demyelination, c) oxidative stress, d) repair and e) neuroaxonal damage [16-18]. In the recent years different proteomics studies have discovered novel and replicated protein targets associated with MS or MS subtypes [131-135]. In this section will include the proteins that have been reported by at least two different proteomic reports or have subsequently been replicated in independent cohorts by immunoassays (Table 3). For detailed discussion of other candidate protein biomarkers, we refer to recent excellent reviews [16,18,136].

Our selection is again divided into; a) immune associated proteins, b) neuro-glial associated proteins.

Table 3. Overview MS associated proteins

Protein name	Body fluid	expression in MS	Subtype	Reference
C3(f)	Serum & CSF	↑	MS vs NINDs	[134],[137]
Clusterin	CSF	↑	CIS & MS vs NINDs	[132],[137]
CHI3L1	CSF	↑	RR, SP vs OND CIS-conv vs CIS-non conv	[131],[133]
Kallikrein-6	CSF	↓	CIS-conv vs CIS-non conv & NINDs SP vs Con	[132],[133]
ACT	CSF	↑	MS vs NINDs	[132],[133], [137]
A2M	CSF	↑	MS vs NINDs	[132],[133], [137]
AGN	CSF	↑	cdMS vsCIS SP vs NINDs	[132],[133], [137]
Contactin-1	CSF	⇕	RR&PP vs CIS SP vs NINDs	[132],[133], [137]

Immune system associated proteins

The complement component 3 (C3) has been shown to be differentially expressed in MS [135,137,138,138]. Elevated levels of C3 in CSF and C3f in serum in MS patients compared to non-inflammatory neurological diseases (NINDs) have been observed [137,138]. Recent reports indicate that C3 together with other complement associated enzymes could serve as potential biomarker for MS prognosis [139-143]. Nevertheless, the underlying mechanisms of C3 and other complement enzymes in regards with MS pathology are not known. C3 is an important factor in the classical, alternative and leptin pathways for complement activation [142,143]. After pathogen recognition by the innate immune system, the mature C3 protein is cleaved into different fragments, e.g. C3a, C3b or the small (2 kDa) C3f, which results in activation of the downstream targets of these fragments and subsequently in inflammatory activation and opsonisation of the pathogens [142,143]. Earlier studies have shown that oligodendrocytes express low quantities of the complement C inhibitors, which makes them vulnerable to complement lysis [143,144]. Furthermore, *in vitro* and immune-staining experiments have shown that oligodendrocytes are a major source of C3 and other complement factors within the brain [145]. The inflammatory micro-environment in MS lesions might affect the expression of the complement factors in oligodendrocytes and thus possibly stimulate inflammation and impair remyelination.

Clusterin is elevated in CSF of clinically isolated syndrome (CIS) and MS patients compared to non-inflammatory neurological diseases (NINDs) [132,137]. Another recent study was not able to validate clusterin in CSF or in plasma of MS patients, however limited sample size was used [141]. Clusterin is an enigmatic protein with different biological functions such as chaperone binding to itself, lipids or components of the complement membrane attack complex [146]. The ability of clusterin to bind complement factors and thereby allowing remyelination was shown in *in vivo* models [147]. The elevated levels of clusterin in CSF of MS patients might be an indication of complement activation in the CNS and the binding of clusterin to complement factors possibly is an attempt to dampen the inflammatory responses in MS.

Different levels of chitinase-3-like protein (CHI3L1/YKL-40) were detected in CSF of CIS no-converters compared to CIS-converters, RRMS compared

to OND and SPMS compared to OND [131,133]. In plasma, different results were obtained in a study comparing patients in relapses with patients in remission and PPMS patients; however, they did observe a difference between PPMS compared to RRMS or healthy controls [148]. CHI3L1 is expressed in variety of cells, such as macrophages, neutrophils and fibroblasts [149]. Monocytes do not express CHI3L1; however the expression of CHI3L1 is strongly induced in macrophage differentiation and macrophage activation, and could be considered an activation marker for a subset of macrophages, the alternatively activated macrophages [149,150]. The biological function of CHI3L1 is considered to be tissue remodelling and inflammatory regulation [149,150]. *In vitro* studies on different cell lines have shown that IL-6, IL-13, IL-17, IFN-γ and TNF-α increase the mRNA expression of CHI3L1 [149,150]. The cellular responses to IL-1β and TNF-α is inhibited by CHI3L1 [149,150]. This suggests a possible negative feedback of CHI3L1 on the pro-inflammatory responses. Recent *in vitro* experiments showed that CHI3L1 expression enhanced the expression of the chemokines; IL-8, CCL5 and monocyte chemotactic protein-1 (MCP-1) resulting in enhanced migratory ability of T-cells and macrophages [151]. Overall, an increase in CHI3L1 was observed in CIS-converters and other MS subtypes, suggesting CHI3L1 is an early marker for activated macrophages in CIS patients who might convert to clinical definite MS via chemo-attraction of other immune cells and possibly worsening lesion formation. Another possible mechanism is that the increased expression of CHI3L1 in astrocytes due to gliosis could be the early initiator and attract macrophages and T-cells [152].

Neuro-glial cell homeostasis associated proteins

Kallikrein-6 is a member kallikrein serine protease family, of which lower levels in CSF of CIS converters and non-converters compared to OND was observed [132]. Others report differential levels of kallikrein-6 in SPMS compared to controls [133]. The kallikrein family is expressed by 15 different genes with homology at genetic and protein levels for all the members [153]. The members of the kallikrein family are involved in regulation and activation of inactive proteins in blood clothing pathway, blood pressure, proliferation and inflammation [153]. Different members are differentially expressed in different tissues [153].The expression of kallikrein-6 is high in the CNS, especially in the brain stem and CNS injury-activated

astrocytes express kallikrein-6 [153,154]. Kallikrein-6 is able to degrade extracellular matrix proteins, myelin basic protein and myelin glycoprotein [153-155]. Furthermore, recent studies have shown that kallikrein-6 increases protease-activated receptors (PAR) in CNS [156]. These receptors are involved in numerous functions in the CNS, e.g. proliferation, morphology, physiology and neuro-inflammation [156,157]. Activated PARs lead to pro-inflammatory cascade in CNS, therefore the differential levels observed for of kallikrein-6 via PAR activation could lead to exacerbations in MS which makes this protein an interesting therapeutic target in MS [158].

Interestingly, the protease inhibitors α-1-Antichymotrypsin (ACT) and α-2 Macroglobulin (A2M) can both bind kallikrein family members, and specific binding of ACT with kallikrein-6 has been shown [159]. Elevated CSF levels of ACT and A2M were observed in all subtypes of MS compared to NINDCs [132,133,137]. Additionally, ACT and A2M are able to bind growth factors, cytokines and hormones and are considered to dampen the effects of these molecules [160,161]. Furthermore, astrocytes express ACT in a pro-inflammatory environment as a potential inhibitory response to the inflammatory environment [162,163]. The elevated levels of ACT and A2M in tissue of all the MS subtypes may reflect attempts of astrocytes to attenuate the underlying pathology throughout the MS disease course. It is possible that ACT and A2M might target other harmful proteins than cytokines in MS pathology.

Interestingly, ACT is able to inhibit angiotensin-converting enzymes that are able to convert the inactive angiotensin I into active angiotensinogen II [164,165]. In different proteomics studies the precursor angiotensinogen has also been shown to be increased in CSF of SPMS compared to NINDCs and clinical definite MS compared to CIS [133,137]. The angiotensinogen (AGN) protein is cleaved by renin, which results in angiotensin I (AG1), which subsequently is converted in an octapeptide by angiotensinogen-converting enzymes into angiotensin II (AG2) [164,165]. Circulation of AG2 stimulates vasoconstriction and secretion of aldosterone, which results in Na+ reabsorption and water retention [164,165]. The primary source of AGT is the liver, however within the brain astrocytes are the main site of AGT expression [164,165]. AGT in CNS is considered to regulate autonomic nervous system, hypothalamus-pituitary adrenal (HPA) axis functioning, secretion of vasopressin and stimulation of salt en thirst appetite [164,165]. Whether the observed elevated CSF AGT levels in MS patients is linked to

the observed hyperactivity HPA axis needs to be studied in the future. Thus, A2M, ACT and AGN are related to each other and are all elevated in CSF of MS patients. Therefore, they can reflect a common mechanism or process, such as astrocytosis.

Differential levels were observed for the protein contactin-1, lower levels in RRMS and PPMS compared to CIS were observed, whereas for SPMS compared to OND elevated contactin-1 levels was observed [132,133,137]. Interestingly, validation of contactin-1 with an immunoassay showed highest levels of contactin-1 in RRMS during relapse [133]. Contactin-1 together with Contactin-associated protein (Caspr) are involved in the formation of axoglial junctions during neurogenesis and are primary expressed on oligodendrocyte precursor cells (OPC) [166,167]. The interaction between axons and oligodendrocytes during neurogenesis is accommodated by trans-membrane receptors between the cells and the extracellular matrix and contacting-1/Caspr complex is essential for the maintenance of axoglial interaction at the paranodes [166-168]. Furthermore the expression of contactin-1 on the cell surface of OPC and the subsequent binding with the receptor protein tyrosine phosphatase inhibits proliferation and promotes oligodendrocyte maturation [167]. Although different results were observed, elevated levels of contactin-1 in RRMS during relapse might be an indication of remyelination within the lesions. The decreased levels of contactin-1 in CSF of RRMS and PPMS compared to OND may have depended on which other neurological diseases were used as controls. The importance of defining controls and choosing the right controls possibly affects the outcome of the results [169]. Further validations of contactin-1 and comparing better defined control groups might offer more insight.

The promising field of proteomics has offered interesting targets that might result in biomarkers for diagnosis and prognosis MS, although more *in vitro* and *in vivo* experiments are needed to understand their possible association with MS pathophysiology. Most of the proteomic studies in comparison to transcriptomics reports have looked in CSF of MS patients. The CSF is a probably a more suitable body fluid for discovery phase of MS associated molecules, because of it close proximity to the CNS and is less likely to be influenced by systemic factors, therefore having possibly a better representation of molecules associated with MS pathology. Recent consensus guidelines regarding CSF biobanking and definition of control groups for MS biomarker studies have been proposed, which could lead to more

independent replications and validations of proteins targets in the near future [138,169].

Expert commentary & five year view

The last decades have seen advances in the biotechnological field, which has led to the "omics" era. These advances have led to obtaining a vast amount of data regarding different pathologies and biological processes at the genomic, transcriptomic and proteomic level. Despite the vast amount of available data sets no applicable biomarker(s) for MS has been discovered or validated. The traditional reductionist focus has led to successful discoveries on biological mechanisms. However, in the field of biomarkers for multifactorial diseases such as MS this reductionist view has been somewhat unsatisfying. A holistic approach is becoming more crucial for understanding MS pathology and subsequent biomarker discovery. Studies that apply the holistic approach are systems biology, in which the complete biological systems and the interactions with environment is studied [20]. The benefit of systems biology approach is to comprehend the complete underlying mechanisms of MS associated genes, RNAs and proteins. Applying this knowledge possibly provides a complex set of biomarker which represents MS pathology better than a single biomarker at a single biological level.

Criteria for a complete systems biology approach have been proposed and consist of; 1) quantification of biological information (e.g. genetic polymorphisms, proteins and miRNAs), 2) integration of information at different levels (e.g genetic polymorphisms that result in higher transcription of miRNAs and possibly increasing target inhibition, or polymorphisms that induce non-synonymous mutations resulting in less clearance at the proteomic level), 3) the dynamic changes of the biological system in response to environment and 4) testing and if necessary adaption of the model (e.g. prediction of MS progression in different environments or therapy response in different sub-groups) [20]. Once these criteria are met the assessment of this complete model could be used for diagnosis, subtyping and progression of MS.

The whole array of biological MS associated gene polymorphisms, miRNAs and proteins possibly allows dividing MS patients into sub-categories. These divisions can be made on the presence of different genetic susceptibility polymorphism for MS, and additionally it might distinguish patients who will respond to therapy and patients who will not. A hypothetical model based on systems biology could be applied to unravel the cause of MS and develop tailor-made diagnostic tools (figure 1). The numbers in the model represent

possible mechanisms; a) inflammatory, b) neurodegenerative and c) unknown causes (e.g. viral).

An example of an environmental factor influencing genetic expression is the transcription factor activity of vitamin D ligand and Vitamin D receptor (VDR) complex by binding to vitamin D response element (VDRE) on specific genomic regions, as shortly mentioned under "epigenetics" [85]. In a recent study the VDRE sites were shown to be enriched near genes associated with MS polymorphisms, such as IRF8, RGS1, CD40, CD58, IL7R and PTGER4. A hypothetical model representing the in this review described "omics" data as network in response to VDRE regulation is shown in figure 2.

The association of a VDRE binding site with IRF8 rs17445836 could potentially alter the expression of this gene. IRF8 encodes a the transcription factor that is involved in the transcription of IFN-α and IFN-β, and the IRF8 SNP rs17445836 has been shown to potentially increase the mRNA of interferon-response pathway genes in MS patients [55,63,64]. Therefore, the regulatory transcriptional functions of IRF8 on interferon-response pathway could be diminished by possible diminished binding capacity of VDR complex with the VDRE region, which potentially leads to pro-inflammatory downstream reactions via secretion of INF-α and IFN-β. Another example of MS associated genes is the PTGER4 (encodes for EP_4) rs4613763 which was reported by IMSGC and others and is located at a VDRE site [41,53,55,85]. Whether this SNP or other SNPs in linkage disequilibrium could alter gene expression and lead to altered EP_4 receptor levels is not known. A recent study showed elevated levels of the ligand PGE_2 in CSF of MS patients compared to healthy controls [170]. PGE_2 expression dampens acute inflammatory response and has a positive feedback on its own expression, which leads to enhanced expression of PGE_2 [171]. The enhanced expression of PGE_2 and possible altered expression of EP_4 receptor could lead to PGE2 predominance in chronic immune stages such as MS pathology [171].

The proteins encoded by CD40 and CD58 genes are both involved in T-cell co-stimulation [59]. A possible altered binding of the VDR complex at rs1335532 CD58 could alter gene expression and lead to imbalanced T-cell signalling and subsequent activation pathways. The specific role of CD40 and CD58 in response to vitamin D needs to be validated in functional analyses. A VDRE site associated with IL-7R rs6897932 was also shown [85].

Furthermore, this SNP is considered to alter the balance between soluble and membrane-bound isoforms of the encoded IL-7R proteins [45]. It is possible that impaired vitamin D homeostasis and subsequent transcriptional activity of VDR complex enhances the imbalance between soluble and membrane bound IL-7R isoforms, which could affect the IL-7 role in B and T-cell development and homeostasis [47,48].

Last example in this model, is the regulatory role of RGS1 on desensitization of B-cells by conversion of GTP to GDP in cytokine receptors (e.g. CXCR5) [77]. The ligand for CXCR5 is the CXCL13 chemokine, which is considered to be upregulated in CSF of MS compared to CIS and controls [172,173]. So hypothetically MS associated RGS1 rs1323292 near the VDRE site might affect the expression of RGS1, which could influence the inhibitory function of RGS1 on B-cells. This could result in activation of B-cells and subsequent inflammatory reactions, which can be measured as body fluid biomarkers. As shown by the question marks in figure 2, information regarding the consequences of the polymorphisms on VDRE sites by possible alterations in binding capacity of VDR complex, and the regulatory relation with environment by vitamin D on histone modification is still lacking for MS pathology [90]. The network nodes associated in the complex MS pathophysiology are becoming visible, however the interchangeable connections between these nodes require further functional experiments, e.g. potential differential gene expression of MS associated genes by SNPs in promoter or and VDRE regions, the role of vitamin D dietary intake on histone modification in MS pathology and so on.

For many of the discussed genes, miRNAs and proteins this model is incomplete and lacks system biology criteria 2 till 4 as we defined above. For example, expecting that small set of relatively weak susceptibility genes could be applied for MS profiling is unlikely, and furthermore *in vitro* and *in vivo* experiments are required to understand the possible role of this polymorphism in the associated MS genes [174]. However, the genomic level does provide the opportunity to classify the MS groups into different genetic sub-groups, which could lead to clear endophenotypes to be used to define patient subsets to target biomarker discovery. Based on the epidemiological and twin studies the environmental role in MS has become evident, and the interaction of epigenetic mechanism with the environment is a promising part of the system biology in MS. Since proteins are considered to be the

functional units within the cells, their association with MS pathology requires more knowledge of their dynamics in MS systems biology network.
The first step towards complete system biology for MS has been made, and we expect within the next five years independent replication and validation of MS associated genes, miRNAs and proteins and subsequent functional analysis of their consequences in MS pathology. This may lead to unraveling of the complete underlying MS mechanism, which can provide molecular signatures that allow the diagnosis and prognosis for MS in sub-groups or individuals.

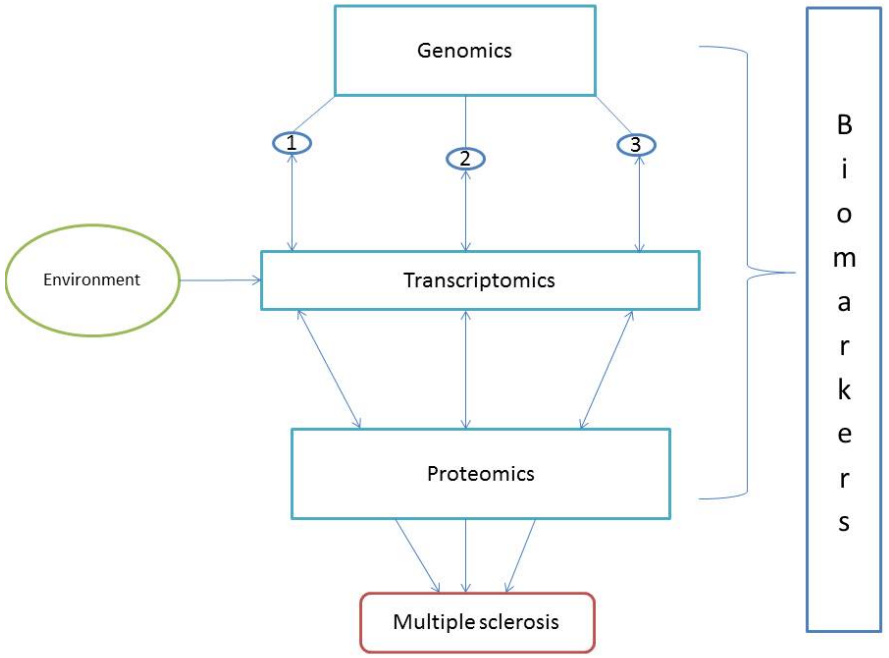

Figure 1. A hypothetical model representing system biology in MS. Systems biology provides the opportunity to use of biomarkers as a complete molecular fingerprinting through different biological fields.

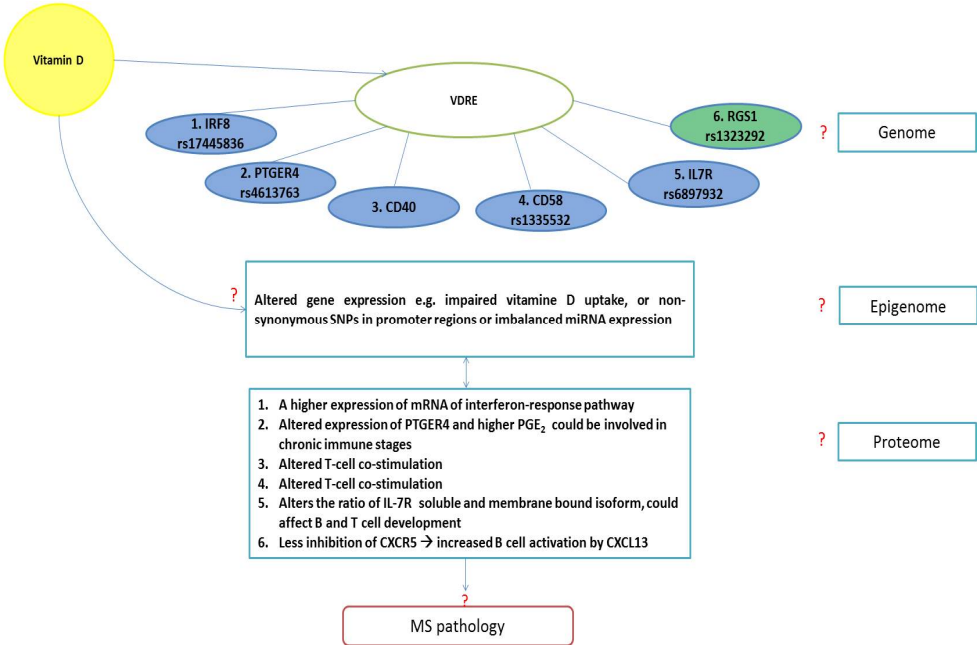

Figure 2. A hypothetical systems biology model based on the "omics" data for MS and vitamin D response element binding sites in MS associated loci. In blue the immune associated loci with possible associations with vitamin D are shown, whereas the neuro-glial association is shown in green. Question marks on different biological levels indicate the lack of information of the obtained "omics" data and their consequences in MS.

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