FOCUS ON BIOMARKERS

BIOMARKERS FOR MULTIPLE SCLEROSIS

Promising biomarkers such as HLA or myelin antibodies have not fulfilled expectations, but many other biomarkers are in the pipeline to fill this niche.

by Pablo Villoslada

BIOMARKERS FOR THE MANAGEMENT OF COMPLEX DISEASES

Complex diseases are characterized by a multifactorial pathogenesis that leads to wide clinical variability (1). For this reason, the course of the disease and the response to therapy can vary largely between patients, even if groups can be established of similar clinical phenotypes. The unpredictability of complex diseases represents a significant challenge for healthcare systems and is an important priority from a patient's perspective. For this reason biomarkers offer a promising approach to identify disease stages or subgroups based on the ongoing pathogenic events, in order to improve our predictive abilities. In addition, the development of effective biomarkers is one of the more basic steps in the path of developing personalized medicine (2).

The definition of a biomarker has been clarified recently for regulatory purposes. Thus, biomarker is a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacological responses to a therapeutic intervention (3). A surrogate endpoint is a biomarker that is intended to substitute for a clinical endpoint and is expected to predict the effect of a therapeutic intervention. A clinical endpoint is a characteristic or variable that measures how a patient feels, functions or survives (3). Prentice's criteria define two main properties of a biomarker: 1) the biomarker strongly and significantly correlates with the clinical endpoint; 2) the biomarker must fully capture the net effect of the treatment on the true clinical endpoint (4). Although the first principle is included in the definition of biomarkers, the second is often not considered and in general, it is very difficult to fulfill. Because the majority of chronic diseases have a complex pathogenesis, an individual biomarker is likely to reflect only one of the many ongoing pathogenic processes. This specificity of a biomarker for an individual process is one reason why it does not serve as a surrogate for the clinical outcome of a complex disease. However, it also implies that the clinical outcome, which reflects all the pathophysiological processes that contribute to the disease, is too insensitive to capture the full effect of any therapeutic application specific to an individual process.

False-positive and -negative biomarkers are an important limitation to the discovery of true biomarkers (5). False-positive biomarkers may arise if the changes in the biomarker reflect the effect of treatment, although these effects are either irrelevant to the pathophysiology of the disease or they are clinically unimportant. False-negative biomarkers reflect clinically relevant changes in the pathophysiology but they do not capture the mechanistic effects of the treatment applied (e.g., T2 lesion load in magnetic resonance imaging [MRI] for neuroprotection trials). In addition, they may arise if the biomarker is more sensitive than the clinical marker or if the clinical marker is irrelevant to a subset of the patients, to a novel mode of action of the drug or to its new indication (5).

SUMMARY

The pursuit of personalized medicine requires the development of biomarkers to predict disease course, monitor disease evolution, stratify patient subgroups by disease activity and to predict and monitor response to therapies. Multiple sclerosis (MS) is a common neurological disease in young adults with an unpredictable course that may be associated with significant disability, diminishing the patient's quality of life. Currently, disease prognosis is based on clinical information (relapse rate and disability scales) and diagnostic tests (brain MRI or the presence of oligoclonal bands in the cerebrospinal fluid). However, the ability of neurologists to make an accurate prognosis is very limited based on such information, a situation perceived by patients as one of their biggest concerns. Although many recent studies have identified different molecules and imaging techniques associated with the course of MS, in most cases the diagnostic accuracy of such technologies has not been properly assessed. This shortcoming is partly due to the failure to validate such biomarkers, which impedes their application in clinical practice. However, the recent validation of anti-aquaporin-4 antibodies for Devic's disease and the development of optic coherent tomography for MS, are examples of the benefits that the development of MS biomarkers can offer. Indeed, it may currently be necessary to redress the bias in research towards clinical validation rather than discovery in order to promote translational research and improve patient's quality of life.

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Biomarkers can be divided into different categories: 1) prognostic markers, 2) predictive markers and 3) pharmacodynamic biomarkers (Fig. 1A) (6). Prognostic biomarkers seek to predict the natural course of the disease, distinguishing between good and bad outcomes. Predictive biomarkers or response biomarkers aim to establish the probability a given patient will respond to a specific therapy. Finally, pharmacodynamic biomarkers measure the near-term treatment effects of a drug on the disease, in order to guide the doses required. Thus, at the treatment level, the prognostic biomarker addresses the decision to "treat or not treat", the predictive biomarker "which drug", and the pharmacodynamic biomarker the "dose" (6). In addition to this diagnosis-therapeutic approach, an alternative approach is to search for pathway-based biomarkers in order to stratify patients into subgroups based on the main pathogenic processes responsible for the disease that will drive their response to therapy. This pathway-based approach is the main focus of the systems biology drug and biomarker discovery approach (7).

The Food & Drug Administration (FDA) categorizes biomarkers as 1) exploratory biomarkers, 2) possible valid biomarkers, 3) known valid biomarkers and 4) regulatory biomarkers (Box 1) (8). The regulatory process at the FDA is guided by the *Clinical Laboratory Improvement Amendments* (CLIA) and by the *CE mark* of the European Medicines Agency (EMA). Discovering biomarkers is a multistep process involving biomarker identification, assay development, biomarker validation, regulatory approval and translation to clinical practice (Fig. 1B). In order to validate biomarkers, it is necessary to perform well-controlled observational studies using the Standards for Reporting of Diagnostic Accuracy (STARD) criteria (www.consort-statement.org\stardstatement.htm) (9). The STARD criteria are equivalent to the CONSORT (Consolidated Standards of Reporting Trials) criteria for reporting clinical trials but they are aimed at reporting the diagnostic accuracy of a test. Calculating the diagnostic accuracy requires establishing the number of patients that must reach the clinical endpoint and calculating the sensitivity, specificity, positive and negative predictive value, accuracy and the area under the receiver operating characteristic (ROC) curve (AUC). Although there are many articles describing new exploratory biomarkers every year, in most cases these discoveries are not followed-up by systematic studies to validate the marker. Moreover, new studies often fail to reproduce the original observation due to methodological differences, poor study design, the use of nonstandardized assays or misleading statistical analysis based on small sample sizes. This problem has been particularly evident in the field of cancer, where many biomarkers have been proposed but few have been validated, a situation which has led to the release of a set of standards (REMARK, REporting recommendations for tumor MARKer prognostic studies) to report biomarker discovery (10).

THE NEED FOR BIOMARKERS IN MULTIPLE SCLEROSIS

Biomarkers in multiple sclerosis (MS) can play several different roles. They can be used to improve our diagnostic capacity when screening individuals at risk of suffering the disease, allowing early diagnosis and preventive therapy. Although the sensitivity and specificity of diagnosing MS based on current criteria is high (11, 12), identification of new biomarkers of the disease may improve our capacity to diagnose more complex cases or those where confounding diseases imply the need for a differential diagnosis. Even if the number of individuals requiring such differential diagnosis is small compared with the overall population of patients, augmenting the accurate diagnosis of the disease will improve how the disease is managed as a whole.

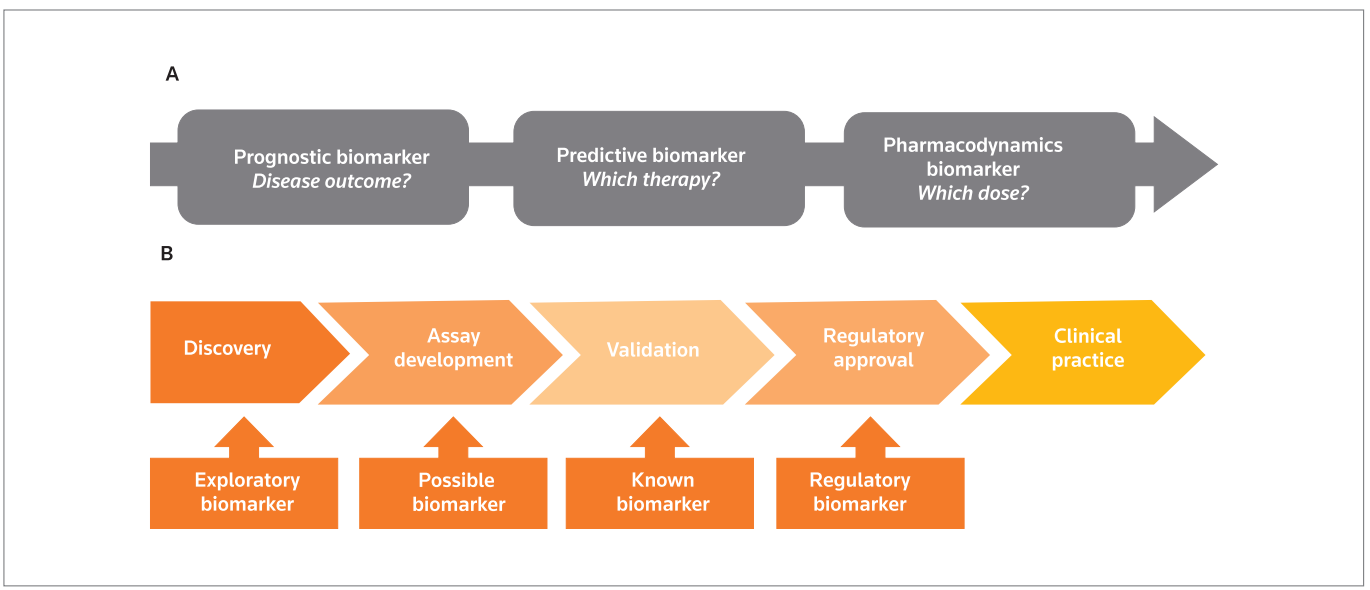

Figure 1. Role of biomarkers in patient management and biomarker development. A) Biomarkers are useful tools to address specific questions regarding patient management and therapy. B) Development of a biomarker follows a process to some degree similar to drug development, until reaching clinical application.

Box 1. *Biomarker definition (FDA)*

- Valid (regulatory) biomarker: A biomarker that is measured in an analytical test system with well-established performance characteristics and for which there is an established scientific framework or body of evidence that elucidates the physiologic, toxicologic, pharmacologic or clinical significance of the test results.
- Known valid biomarker: A biomarker that is measured in an analytical test system with well-established performance characteristics and for which there is widespread agreement in the medical or scientific community about the physiologic, toxicologic, pharmacologic or clinical significance of the results.
- Probable valid biomarker: A biomarker that is measured in an analytical test system with well-established performance characteristics and for which there is a scientific framework or body of evidence that appears to elucidate the physiologic, toxicologic, pharmacologic or clinical significance of the test results.

Source: U.S. FDA. *Guidance for Industry: Pharmacogenomic Data Submissions*.

The most important unmet need in MS is the identification of biomarkers for the prognosis of the disease (prognostic biomarkers). MS is a very variable and unpredictable disease, this situation being one of the most disturbing aspects referred to by patients (13). The uncertainty about the short- and long-term disease course poses significant difficulties to reach the appropriate balance between risk and benefits for therapies, as well as when making decisions about personal life. In addition, obtaining predictive biomarkers—those providing information about response to therapy—is also a priority since decisions about drug prescription are currently based on population information (efficacy of the therapy in cohorts of patients with a similar phenotype). By identifying which patients are more likely to respond well to a given therapy, or which are at risk of developing serious side effects (e.g., progressive multifocal leukoencephalopathy [PML]), we can maximize the patient's benefits for a given therapy, significantly improving the patient's quality of life and healthcare management. Moreover, development of pharmacodynamic biomarkers will permit the most convenient dosage to be selected and adjusted over time.

Finally, biomarkers can be specific and informative of the pathogenic processes active in a given patient. As explained above, in complex diseases like MS there may be many pathogenic processes ongoing at different levels and in different individuals. Hence, more knowledge of the active pathways would allow physicians to make a more precise diagnosis at the physiopathological level, which will drive disease management. Indeed, different biomarkers have been proposed for the inflammatory process (both innate and immune system response), infections, demyelination, axonal damage and neurodegeneration, although these markers still remain to be validated (14, 15).

PROPOSED BIOMARKERS FOR MS

In a PubMed search using the key words "multiple sclerosis" and "biomarker", we obtained 2,980 entries reporting the identification of new molecular, cellular, imaging or clinical variables associated with the course of MS or its response to therapy. However, a search using the same terms plus the key words suggested by the STARD criteria ("diagnostic accuracy", "sensitivity", specificity" or "AUC") produced only 308 hits. This indicates the bias in research (and publishing) towards the screening for new biomarkers rather than validating previously reported markers. This could reflect the fact that exploratory studies can be done with a small cohort of patients, whereas validation requires prospective multicenter studies, which are significantly more complex and expensive.

The *Biomarkers Module* of *Thomson Reuters Integrity*℠ (http://thomsonreutersintegrity.com) is a specialized, manually curated text-mining database that contains biomarker information published in scientific papers and patents. A search of this database using the condition "Multiple Sclerosis" identified 319 entries, and it was striking that the majority of these were in the exploratory stage (including emerging, experimental, early and late studies in humans), and none had reached the known or regulatory level. Moreover, the majority of the biomarkers were related with new high throughput techniques (genomics and proteomics; Fig. 2A) (16). In terms of their medical application, they were mainly focused on the diagnosis of MS, including differential diagnosis (Fig. 2B), and only a few addressed the unmet need in MS for prognostic and predictive biomarkers.

A list of proposed biomarkers (molecular and imaging) for MS is shown in Tables I and II, which may not be complete if any molecules or imaging techniques exist that have not been described as biomarkers for MS. The best known biomarkers for MS at the molecular level is the presence of oligoclonal bands (OCB) in cerebrospinal fluid (CSF) or an increase in the IgG index (11). OCB have a known biomarker status, although they may never reach the regulatory status due to the lack of current commercial interest. OCB have a high sensitivity and specificity for the diagnosis of MS and in addition, the presence of OCB is a prognostic biomarker for the conversion from clinically isolated syndrome to relapsing-remitting MS (17, 18). Also, the presence of IgM OCB seems to be associated with a more aggressive disease (19, 20).

The field of genomics, including genetic studies (genetic association studies and linkage studies) and gene expression studies (DNA arrays, real time PCR), has been particularly fruitful in providing new candidate markers, although outside of the human leukocyte antigen (HLA) class II it has been quite poor in validating them (21, 22). However, recent genome-wide association studies have started to provide validations for some such markers (Table I), although the low odds ratio of such genes might prevent their usefulness as biomarkers of MS. The case of HLA is a particular example of the difficulties in biomarker discovery since even if the association between *HLA-DRB1**1501 exists and it is associated with a significant risk of suffering the disease (23), its utility is significantly impaired by the fact that it is also a common allele in

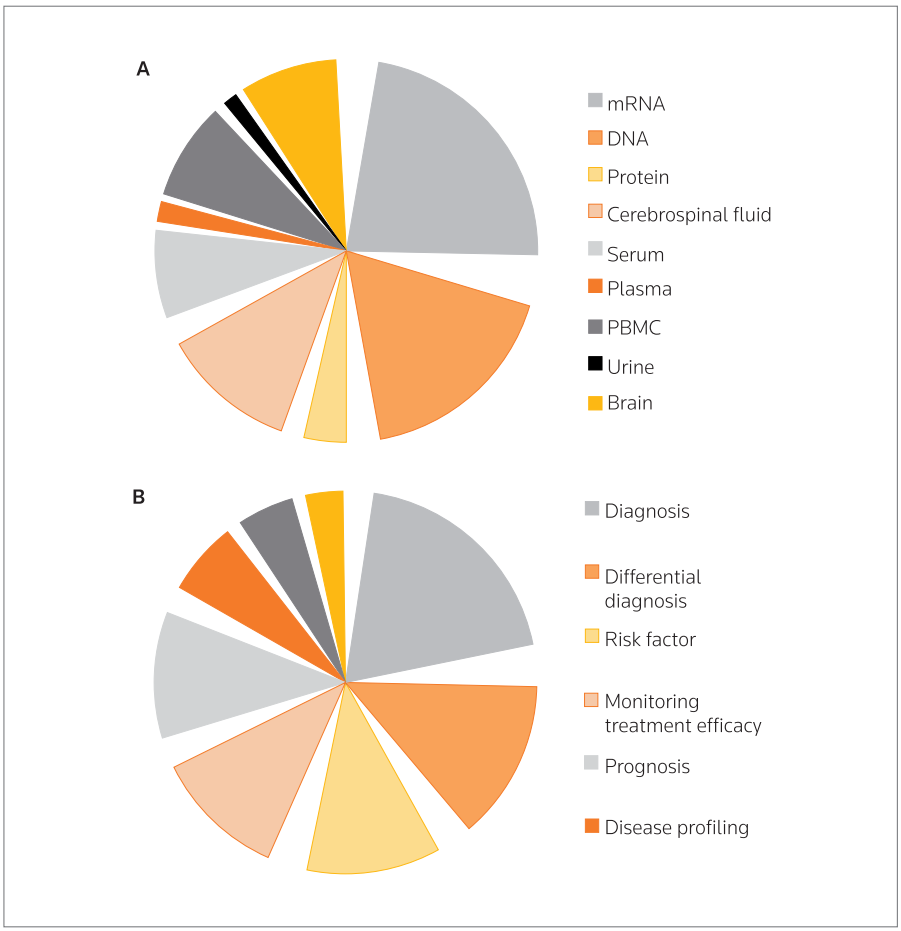

Figure 2. Current biomarkers in multiple sclerosis. A) Distribution of proposed multiple sclerosis biomarkers (excluding imaging markers) available in the *Biomarker Module* of *Thomson Reuters Integrity*SM database based on the technology and tissue and B) their role in clinical practice.

the general healthy population (30–60% of the Caucasian population).

A new field in the search of biomarkers for MS is pharmacogenomics and in a wider perspective, biomarkers of the response to therapy (predictive and pharmacodynamics biomarkers) (24, 25). The first studies conducted in this field have provided a list of candidate biomarkers related with the response to the most common therapies for MS, such as interferon β or glatiramer acetate (Table I) (26-29). Again, the complexity of the disease and the mode of action of these drugs make identifying markers difficult, as does the lack of a good definition of the response to therapy, the pleiotropic activity of the drug and the heterogeneity of the disease.

Another hot topic in MS is the discovery of self-antibodies in the blood. Antibodies are ideal candidates as biomarkers because they are very stable, specific for the antigen and related to the pathogenesis of the disease. In MS, the search for antibodies has been extensive, reporting many candidate auto-antibodies (15, 30). However, none of these have been validated to date, even despite some extensive work as in the case of anti-myelin-oligodendrocyte glycoprotein antibodies (including the standardization of many assays and well-conducted prospective studies) (31, 32). However, in the case of neuromyelitis optica (NMO), the discovery of the anti-aquaporin-4 antibody (anti-AQP4) was further validated as having good sensitivity and high specificity for the diagnosis of the disease (33).

At the protein level, many of the candidate markers reported are related to the study of the pathogenesis of the disease, mainly at the immunological level. They include molecular mediators of the immune system such as cytokines, chemokines, activation markers or adhesion molecules (Table I) (34, 35). However, even after decades of research and the confirmation that a few of these molecules are involved in the pathogenesis of the disease, none have been confirmed as valid biomarkers (known or regulatory biomarker). The field of proteomics is now starting to generate a long list of candidates that will ultimately require further validation (36-40).

Among the other biomarkers explored are metabolites, lipids, self-antigens, cell phenotypes or viruses. Metabolomics is a growing area of interest due to the recent technological advances enabling thousands of metabolites to be screened. Due to the critical role of intermediate metabolism in the immune response and the response of brain tissue to damage, such molecules are actively screened in other fields like cancer and a few of them have been reported in MS (Table I) (41). More recently, the role of vitamin D in the susceptibility of MS has been strengthened and it is actively pursued as a marker of the disease (42). Although lipids have been a topic of interest in MS research given their prominent role in demyelination-remyelination, technical limitations in the study of lipids have hampered significant advances in this field. However, new technologies may open the door to the efficient analysis of lipids and to the new field of glycomics (43-45). Antigens represent a specific set of molecules with a distinct chemical nature (and for this reason with different technological needs) that are recognized by the adaptive immune response. Identifying the antigen targeted by the immune response will allow the immune response and the pathogenetic process to be monitored. Since the explosion of molecular immunology in the 1980s and the interest in autoimmune diseases, a prominent search for antigens involved in the pathogenesis of MS has been carried out (34). However, we have failed to definitely probe the main antigen in MS, despite significant advances in NMO with the discovery of AQP4. New technologies to screen hundreds of antigens in a chip open new avenues to promote such discovery (46).

Cell phenotyping has been another area of intensive research due to the development of cellular immunology. Although numerous

Table I. *Candidate molecular biomarkers for multiple sclerosis.*

Type	Examples	Status[1]
DNA		
HLA	*HLA-DRB1* (1501, 1503, 0801, 0301, 0401, 1401), *DRB5*, *HLA-DQA*, *HLA-DQB* (0603), *HLA-C*	Possible
Genes[2]	*IL7R*, *IL2RA*, *CLEC16A*, *CD68*, *CD226*, *RPL5*, *DBC1*, *ALK*, *FAM69A*, *TYK2*, *CD6*, *IRF8*, *TNFRSF1A*, *SCIN*, *IL12A*, *MPHOSPH9*, *RGS1*, *KIF21B*, *TMEM39A*	Possible
Genes[3]	*ADAMTS14*, *AGER*, *ALS2*, *ALOX5*, *BANK*, *CD226*, *CCDC97*, *CYP2S1*, *CTLA4*, *FAM5A*, LECAM2, GCCR, *GSK3B*, *GPC5*, *AFGF*, *E1BAP5*, *ITGA4*, *ICAM1*, *IRF1*, *IFNGR1*, *IFNGR2*, *IL10*, IL12, *IL13*, *IL2RA*, *IL23R*, *IL3*, *IL4*, *IL4R*, *IL5*, *IL6*, *IL7*, *IL7R*, *IL9*, *CMT2A*, *GLOD1*, *PTPRC*, *FDC*, *LFA3*, *MMP7*, *MMP9*, *TIMP3*, *MICB*, *MAPT*, *SLC25A8*, *MBP*, *MAG*, *MPO*, *CMT1F*, *NPAS3*, *NPTXR*, *NT3*, *CARD15*, *OPN*, *CMT1A*, *PAI1*, *PECAM1*, *PLA2G7*, *PRR2*, *POU2AF1*, *GGF2*, *NKNA*, *JAG1*, *PKCA*, HIP, *PON1*, *STAT1*, FLJ22950, *LAP18*, *MMP3*, *SOD1*, *SYN3*, *PLAT*, *TCF7*, *TGFB1*, *TGFB2*, *TNFA*, *NGFR*, *GITR*, *TNFR2*, *TNFRSF5*, *4-1BB*, *AXL*, *VEGF*, *VAMP*	Exploratory
Genes[4]	*CASP3*, *TRAIL*, *FLIP*, *COL25*, *GPC5*, *HAPLN1*, *CAST*, *STAT1*, *IFNAR1*, *IFNAR2*, *MX1*, *IFNG*, *IL10*, *GRIA3*, *CIT*, *ADAR*, *ZFAT*, *STARD13*, *ZFHX4*, *FADS1*, *MARCKS*, *IRF2*, *IRF4*, *IL4R*, *CASP10*, *CASP7*, *IL8*, *IFIT3*, *RASGEF1B*, *IFIT1*, *OASL*, *IFI44*, *IFIT2*, *HLA-DRB1**1501, *TCRB*, *CTSS*	Exploratory
mRNA	*PDGFRA*, *BAX*, *BCL2*, *APAF1*, *API1*, *CASP1*, *CASP2*, *CASP6*, *CASP8*, *CASP10*, *P53*, *COL3A1*, *DOCK10*, *ADAM17*, *EGR2*, *EPHX2*, *EAAT1*, *G3PD*, *C11*, *HBB*, *HAVCR*, *IFI6*, *IFITM1*, *IFITM3*, *IFNAR1*, *IFNAR2*, *ISG15*, *MX1*, *G10P1*, *G10P2*, *IL1B*, *IL1A*, *IL10*, *IL12*, *IL4*, *IL5*, *CLEC5B*, *LY6E*, *LT*, *LAPTM5*, *MIF*, *MBP*, *MYD88*, *SIR2L1*, *NOTCH2*, *FLJ00340*, *EBP-1*, *RIP15*, *PRDX5*, *PLSCR1*, *PSEN2*, *PDCD2*, *PDCD4*, *PARK7*, *JAG1*, *PKB*, *RSAD2*, *EB9*, *HIP*, *NOGO*, *STK17A*, *TLR4*, *TLR6*, *NFKB3*, *TGFB1*, *TRIB1*, *TNFA*, *TRAIL*, *TNFSF12*, *APRIL*, *FASL*, *TNFRSF12A*, *UBE4B*, *XIAPAF1*, *RASGEF1B*, *OASL*, *MARKS*	Exploratory
Proteins		
Oligoclonal bands (OCB)	IgG index, IgG OCB, IgM OCB, light chains	Known
Antibodies	Anti-MBP, anti-MOG, anti-GalC, anti-PLP, anti-OSP, anti-CNPase, anti-transaldolase, anti-proteasome, anti-β-arrestin, anti-Gangliosides, anti-CRYAB, anti-HSP60, anti-HSP70, anti-HSP90, anti-ATP2C1, anti-KIAA1279, anti-PACSIN2, anti-SPAG16, anti-hnRP B1, anti-Alu repeats, anti-NG2, anti-phosphatidyl-choline, anti-NF, anti-NogoA, anti-tubulin, anti-enolase, anti-glycan, anti-triosephosphate isomerase (TPI), anti-GAPDH Anti-AQP4[5] Neutralizing antibodies of interferon β or natalizumab	Exploratory Known Possible
Cytokines	IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-15, IL-17A, IL-18, IL-23, TNF-α, TGF-β, interferon β, interferon γ	Exploratory
Chemokines	CCR2, CCR5, CCR7, CCL1, CCL2, CCL3, CCL4, CCL5, CCL8, CCL17, CCL21, CCL22, CXCR3, CXCR4, CXCL5, CXCL10, CXCL12, CXCL13	Exploratory
Complement	C3, C3d, C4, C7 neoC9	Exploratory
Adhesion molecules	ICAM-1, VCAM-I, E-selectin, L-selectin, LFA-1, VLA-4	Exploratory
Activation markers	CD25, CD40, CD80, CD86, CD26, CD30, OX40, Fas, TRAIL, OPN, CD127, CD45, CD47, CD16, CD279, CD163, T-bet, CD1d, CD266, GITR, TNFR2	Exploratory
Other[6]	αβ-Crystallin, neurofilaments (light-chain), tau, actin, tubulin, 14-3-3, neuronal enolase Nogo-A, Lingo, ALDH, α1B glycoprotein, α2-HS-glycoprotein, α-synuclein, Aβ, ANX1-5, ApoA (I, IV, B, D), API1, βADRBK1, Arrestin, beta 1, beta-End, NGF, BDNF, CNTF, BRCA1, CRP, CB2, CD276, CD44, chitotriosidase-1, chromogranin A, clusterin, contactin1, cystatin C, CD26, Mac-2 BP, gelsolin, GFAP, haptoglobin, iNOS, IGFBP-3, interferon α, interferon γ, MxA, IL-1ra, kallikrein-1, kallikrein-6, Manan-binding lectin serine protease-1, MMP-9, TIMP-3, MICB, MBP, MAG, NT3, OLIG2, P2X7R, PDGFB, PD-L1, PD-L2, IGFBP3R, COX-2, DJ-1, PACSIN2, protein C inhibitor, S100A, S100B, RBP4, secretogranin I, transferrin, serum paraoxonase/arylesterase 1, Stat-1, SCN2A, Sox-9, Sox-10, SPAG16, MMP-3, SOD1, tetranectin, tPA, transferrin receptor, TGF-β, peripheral benzodiazepine receptor, transthyretin, TNFSF12, tissue factor, Fas, vitamin D-binding protein, VDAC1, AZGP1	Possible Exploratory
Metabolites	Folic acid, homocysteine, prostaglandin E_2, vitamin D, vitamin B_{12}, vitamin B_6, hydroxyindoleacetic acid, iron, malonaldehyde, *N*-acetylaspartate, neopterin, nitrates, orosomucoid, sorbitol, thiobarbituric acid reactive species, cholesterol, 24*S*-hydroxycholesterol	Exploratory
Lipids	Galactocerebroside, gangliosides, sphingolipids, phosphatidyl-serine, oxidized cholesterol derivatives	Exploratory

Continued

Table I. Cont. Candidate molecular biomarkers for multiple sclerosis.

Type	Examples	Status[1]
Antigens	MOG, MBP, PLP, β-arrestin, contactin 2, AQP4[5]	Exploratory Known
Cell phenotypes	Treg (Foxp3+, Tr1, CD8reg) Breg, NK cells, CD4, CD8, B cells (CD5+), macrophages, DC (myeloid and plasmacytoid)	Possible Exploratory
Viruses	EBV, HHV-6, MSRV, VZV	Exploratory

[1]Status: Exploratory biomarker (including emerging, experimental, early and late studies in humans according to the *Biomarkers Module* of *Thomson Reuters Integrity*SM); possible biomarker, known biomarker, regulatory biomarker (as per FDA definitions; see text).
[2]Genes discovered in genome-wide association studies and validated in subsequent studies.
[3]Genes reported as biomarkers in the *Biomarkers Module* of *Thomson Reuters Integrity*SM.
[4]Genes identified in pharmacogenomics studies for interferon-β therapy.
[5]Biomarker for neuromyelitis optica.
[6]Proteins identified by molecular biology methods (ELISA, Western blot), histology (immunohistochemistry or immunofluorescence) or proteomic methods in different tissues (CSF, serum, brain, etc.).

cell surface markers and cell populations have been proposed to be associated with MS (47), difficulties in the standardization of the techniques and the dynamic nature of the process have hampered the validation of a given cell phenotype as a biomarker of the disease. Nevertheless, the identification of regulatory T cells that are impaired in MS and that are modified by disease-modifying drugs is currently a very active area of research (48-50).

Finally, measuring pathogen levels is also another area in which there is an intense search for biomarkers, particularly focusing on viruses. The search for a pathogen associated with MS has been constant and although a role for environmental agents has been proposed, along with associations with different viruses and bacteria, to date no such agent has been fully validated as a biomarker (51, 52). This could be due to the complex interaction between pathogens and the immune system in the pathogenesis of MS, which might prevent a single pathogen from being detected in samples from patients with MS. Nevertheless, in recent years an association between Epstein-Barr virus and MS has been clearly established, although currently quantifying viral load and Epstein-Barr virus antibody titers is not sufficiently sensitive for it to become a biomarker for the disease (53, 54). Similarly, the association between human herpes virus 6 or the MS-associated retrovirus is promising, even though they have not yet achieved the status as biomarkers for the disease (55, 56).

BIOMARKERS BASED ON SAMPLE TYPE OR PATHOGENIC PROCESS

Identification of biomarkers is most often closely related with the samples available for study. By contrast to oncology, where the main focus is the target tissue, in MS the study of the brain is extremely unusual (only a few cases are study by biopsy), excluding post-mortem studies. For this reason, the main focus is the analysis of blood and CSF, and to a lesser extent urine. Biomarkers determined in blood will be the most convenient ones due to its availability and because it is a tissue that can reflect the activity of the immune system. Indeed, its relationship with the target tissue, it seems an ideal place for looking for pathogenic markers of the disease. For this reason, most of the studies performed to date have been performed on serum, plasma or blood cells, screening for DNA, messenger RNA, proteins, metabolites, antibodies, cytokines, viruses, etc. (Fig. 2B). Indeed, the CSF is currently the only tissue that has provided a known biomarker, namely the OCB (if we exclude brain MRI). Moreover, several biomarkers of axonal damage and neurodegeneration have been proposed, including light-chain neurofilaments, tau, β-amyloid, protein S100-B, 14-3-3 protein, glial fibrillary acidic protein (GFAP) or neuronal enolase (57-59). Recently, a new standard in the collection and biobanking of CSF has been proposed which is going to ultimately improve the ability of detecting biomarkers in this fluid (60). Urine has also been studied, which has the advantage of collecting molecules produced by metabolism but the disadvantage of being quite far from the two systems of interest, the brain and the immune system. Several biomarkers in urine have been proposed, including neopterin, nitrate, γ-globulins, myelin basic protein, interleukins, prostaglandin or β-microglobulin, but to date none of these have been validated (61-64).

Another important area of research is the discovery of biomarkers related to pathological processes, and several markers of inflammation have been proposed, including cytokines, chemokines, antibodies, complement, adhesion molecules, antigen presentation, cell cycle/apoptosis, etc. The proposed markers of demyelination are myelin basic protein-derived peptides or myelin antibodies, while the markers of axonal damage include neurofilaments, tau, β-amyloid, protein S100-B, GFAP or neuronal enolase (14, 59).

IMAGING AS A BIOMARKER FOR MS

Magnetic resonance imaging

Imaging is one of the most rapidly growing areas of interest in the development of biomarkers for complex diseases (65). In the case of MS, MRI has completely changed the diagnosis of the disease due its sensitivity to identify brain lesions and given that the MS MRI criteria (Barkoff-Tintore criteria) are also very specific (11, 12, 66). Several imaging markers have been described as prognostic biomarkers for MS, including the presence of black hole, as well as the num-

Table II. Imaging markers for multiple sclerosis.

Technology	Marker	Status[1]
MRI		
MS MRI criteria (Barkoff–Tintore)	3–4 criteria: periventricular (3), juxtacortical, gad+, infratentorial, spinal cord lesions	Known
T1	Number and volume of T1 lesions, "black-holes"	Known
T2	Number and volume of T2 lesions	Known
Gadolinium (gad+)	Number and volume of gad+ lesions	Known
Ion particles contrast	Number and volume of iron+ lesions	Exploratory
Double inversion recovery	Cortical lesions	Exploratory
Brain volume	Brain parenchymal fraction, grey matter volume, white matter volume, spinal cord (cervical) volume, regional volumes	Known
Magnetization transfer MRI	Magnetization transfer ratio	Possible
Spectroscopy	NAA, glutamate, glutamine, GABA, choline, creatinine, myoinositol glutathione, ascorbic acid	Possible Exploratory
Diffusion MRI	Mean diffusivity, diffusion tensor (tractography and voxelwise analysis)	Possible
Fractal dimension (FD)	White matter FD, grey matter FD	Exploratory
Texture analysis	Gradient matrix, run-length matrix, grey level co-occurrence matrix, autoregressive model, wavelet analysis	Exploratory
fMRI	Regional activation (BOLD)	Known
Optic coherent tomography	Thickness retinal nerve fiber layer and quadrants Macular volume	Possible
Positron emission tomography	Peripheral benzodiazepine receptor ligand (PK-11195, [^{11}C]-vinpocetine)	Exploratory
Echography	Hypoechogenicity/hyperechogenicity Deep nuclei, third ventricle	Exploratory

[1]See explanation in Table I. MS, multiple sclerosis; MRI, magnetic resonance imaging.

ber and volume of T1, T2 and gadolinium-enhancing lesions (Table II) (11, 67). These phenomena have been validated in previous studies, becoming considered as known biomarkers, and they are used as surrogate endpoints in phase II clinical trials. However, they have still not reached the status of regulatory biomarkers approved by the FDA or EMA as valid surrogate endpoints in phase III clinical trials.

At the pathogenic level, MRI markers are partially informative of the disease process. Indeed, there is a close relationship between the breakdown of the brain–blood barrier and presence of gadolinium enhancement, although we know that not all brain–blood barrier damage is captured by this marker. T1 lesions are more closely associated with axonal loss and tissue damage, although such an association is far from perfect. However, the pathogenic substrate of T2 lesions is more complex, including the presence of edema, demyelination, inflammation, astrogliosis or axonal loss. Hence, T2 lesions would appear to lack specificity as a biomarker of pathogenic processes (67, 68).

Brain atrophy is the imaging counterpart of tissue destruction and tissue loss is currently captured with brain volume measurements using voxel-based morphometry. Several markers have been described along such lines, including the brain parenchymal fraction, the volume of grey and white matter, cervical cord or regional volumes (e.g., thalamus volume) (68). Such measurements are associated with disease phenotype and disability, although they remain to be validated as prognostic markers of the disease.

Other MRI techniques that offer more information about the disease and that may serve as biomarkers of MS are magnetic transfer imaging to calculate the magnetic transfer ratio, MR spectroscopy, diffusion weighted imaging, functional MRI, fractal dimension analysis or texture analysis (69). Magnetic transfer ratio provides a quantitative measurement that is related to the extent of demyelination, although difficulties in standardizing the technique have prevented it from entering multicenter studies (70). MR spectroscopy provides quantitative information about several brain metabolites (Table II) and although it is currently used in the differential diagnosis of several brain diseases (71), it has not been validated as a marker for MS. Diffusion weighted imaging is a rapidly advancing technology that has provided several markers of brain damage related to the restricted movement of molecules, yet validation studies are still necessary before it can produce biomarkers for MS (72). Functional MRI provides information about brain activation by measuring changes in blood flow. While it is extensively used in cognitive sciences and it has revealed how brain damage impairs cognition in MS, it is still not a validated marker for MS (68). Our group developed fractal dimension analysis of the grey and white matter as a marker for MS (73, 74).

Fractal dimension captures the topological complexity of an object like the brain, and it is sensitive to the brain atrophy and tissue changes related to MS, even at the early stages of the disease. Indeed, fractal dimension by MRI is currently in the process of being validated as a biomarker for MS.

Optic coherence tomography

Optic coherence tomography (OCT) provides high-resolution measurements of the thickness of the retinal nerve fiber layer (RNFL) or the macula (75). The visual system is frequently affected by the disease, leading to axonal loss, which is captured by the RNFL measurement at the head of the optic nerve. We performed a study to validate the RNFL thickness measured with OCT as a prognostic biomarker of MS, detecting high specificity and intermediate sensitivity for predicting future relapses and increases in the Expanded Disability Status Scale 2 years later (76). OCT holds promise as a biomarker of the neurodegenerative processes in MS and to become a surrogate marker for neuroprotective therapies (68). The technological advance represented by spectral-domain OCT, with a very high spatio- temporal resolution will significantly help make OCT a useful biomarker for MS (77).

COMBINING BIOMARKERS WITH THE CLINICAL MARKERS OF MS

Decades of research in the natural history of MS have identified several clinical variables as markers of MS (Table III). By contrast to the biomarker field, the search for clinical markers of MS has led to the validation of many known markers, such as those for the time to the second relapse, relapse rate, progressive subtype or reaching several disability steps (78). Recently, low contrast visual acuity was accepted as another known marker of the disease, which is also used as an endpoint in clinical trials (79). Neurophysiological studies, including the measurement of evoked potentials, can identify subclinical damage of brain pathways and they are useful in the diagnosis of the disease. Visual evoked potentials and motor evoked potentials have been explored as prognostic markers of the disease, although more validation studies will be necessary for them to become useful markers (80).

The challenge in biomarker and clinical research for MS is now how to combine all the information and measurements these techniques have made available in order to better predict disease evolution and response to therapy. As explained before, the complexity and multifactorial nature of MS makes it difficult that a single biomarker will capture all the dimensions of the disease, proving accurate as a prognostic marker. For this reason, developing algorithms or scores that combine clinical, imaging and biological information is a promising approach to the problem. Accordingly, the use of computational classifiers developed in the field of medical informatics (neuronal networks, decision trees, Bayesian classifiers or regression classifiers) could be a good way to select the most useful information and maximize its predictive abilities (81-83).

DEFINING THE RESPONSE TO THERAPY

Predictive biomarkers are also of critical interest in MS due to the limited efficacy, cost and side effects associated with current therapies. Current efforts are focused on identifying biomarkers to identify responders and nonresponders to first-line therapies (interferon β and glatiramer acetate), or to predict and identify individuals at risk of developing severe side effects at early stages (e.g., PML in natalizumab-treated patients) (84, 85). Most of the main efforts along these lines have been made in pharmacogenomics and imaging for interferon β therapy. Several genes have been associated with the response to interferon β (Table I) and the persistence of gad^+ lesions, or the increase in the T1 or T2 lesion load, are being pursued as markers of failure to respond to the therapy (27, 85, 86). This is a growing

Table III. *Clinical markers for multiple sclerosis.*

Clinical variable	Marker	Status[1]
Disease subtype	CIS, RR, SP, PP, PR	Known
Demographics	Sex (male), age at onset (> 40 years) Month of birth, familiar MS	Known Exploratory
Topography first relapse	Motor, cerebellar, polyregional: bad outcome; Sensitive or visual: good outcome	Known
Disability after first relapse	EDSS > 0: bad outcome	Known
Time to second relapse	< 1 year: bad outcome	Known
Relapse frequency	Number of relapses previous 2 years	Known
Disability steps	Time to EDSS 4.0 Time to EDSS 6.0 Time to EDSS 7.0	Known
Low contrast visual acuity	2.5%, 1.25% contrast visual acuity	Possible
Visual evoked potentials	Latency P100	Possible
Motor evoked potentials	Latency, amplitude	Possible

[1]See explanation in Table I. CIS, clinically isolated syndrome; RR, relapsing-remitting; SP, secondary-progressive; PP, primary-progressive; PR, progressive-relapsing; EDSS, Expanded Disability Status Scale.

area of interest that physicians, patients and pharmaceutical companies are now addressing under the paradigm of personalized or stratified medicine, and it will clearly pay dividends in the near future (2).

CONCLUSIONS

Research into biomarkers for MS is a very active area that should move from the discovery phase to the validation stage in order to transfer the advances of biomedical research to clinical practice, and to provide benefits to patients and society. This process is going to require an effort by clinicians, researchers, funding agencies and journal editors, placing emphasis on the validation steps in order to provide more effective markers. Validation requires conducting prospective multicenter studies on large, statistically relevant cohorts, the standardization of the techniques, the evaluation of intercenter or intermachine variability, reporting the diagnostic accuracy of the biomarkers using accepted criteria such as STARD, and applying for regulatory approval when appropriate. In the process of developing personalized medicine, we must move from population data (statistics) to individual predictions. Biomarkers must be easy to apply in clinical practice, robust to the center and platform differences, and cost effective. In the field of demyelinating diseases, we have seen dramatic changes in the management of NMO through the discovery of a biomarker such as anti-AQP4 antibody. However, in terms of MS, we have seen that promising biomarkers such as HLA or myelin antibodies have not fulfilled expectations, although many other biomarkers are in the pipeline to fill this niche.

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REFERENCES

1. Rees, J. *Complex disease and the new clinical sciences.* Science 2002, 296(5568): 698-700.
2. Trusheim, M.R., Berndt, E.R., Douglas, F.L. *Stratified medicine: strategic and economic implications of combining drugs and clinical biomarkers.* Nat Rev Drug Discov 2007, 6(4): 287-93.
3. *Biomarkers and surrogate endpoints: preferred definitions and conceptual framework.* Clin Pharmacol Ther 2001, 69(3): 89-95.
4. Prentice, R.L. *Surrogate endpoints in clinical trials: definition and operational criteria.* Stat Med 1989, 8(4): 431-40.
5. Bielekova, B., Martin, R. *Development of biomarkers in multiple sclerosis.* Brain 2004, 127(Pt 7): 1463-78.
6. Sawyers, C.L. *The cancer biomarker problem.* Nature 2008, 452(7187): 548-52.
7. Villoslada, P., Steinman, L., Baranzini, S.E. *Systems biology and its application to the understanding of neurological diseases.* Ann Neurol 2009, 65(2): 124-39.
8. FDA, *Guidance for Industry: Pharmacogenomic Data Submissions*. U.S. FDA, Washington, D.C., 2005.
9. Bossuyt, P.M., Reitsma, J.B., Bruns, D.E. et al. *The STARD statement for reporting studies of diagnostic accuracy: explanation and elaboration.* Clin Chem 2003, 49(1): 7-18.
10. McShane, L.M., Altman, D.G., Sauerbrei, W., Taube, S.E., Gion, M., Clark, G.M. *REporting recommendations for tumor MARKer prognostic studies (REMARK).* Nat Clin Pract Oncol 2005, 2(8): 416-22.
11. Polman, C.H., Reingold, S.C., Edan, G. et al. *Diagnostic criteria for multiple sclerosis: 2005 revisions to the "McDonald Criteria".* Ann Neurol 2005, 58(6): 840-6.
12. Montalban, X., Tintore, M., Swanton, J. et al. *MRI criteria for MS in patients with clinically isolated syndromes.* Neurology 2010, 74(5): 427-34.
13. Janssens, A.C., van Doorn, P.A., de Boer, J.B., van der Meche, F.G., Passchier, J., Hintzen, R.Q. *Perception of prognostic risk in patients with multiple sclerosis: the relationship with anxiety, depression, and disease-related distress.* J Clin Epidemiol 2004, 57(2): 180-6.
14. Tumani, H., Hartung, H.P., Hemmer, B., Teunissen, C., Deisenhammer, F., Giovannoni, G., Zettl, U.K. *Cerebrospinal fluid biomarkers in multiple sclerosis.* Neurobiol Dis 2009, 35(2): 117-27.
15. Reindl, M., Khalil, M., Berger, T. *Antibodies as biological markers for pathophysiological processes in MS.* J Neuroimmunol 2006, 180(1-2): 50-62.
16. Ibrahim, S.M., Gold, R. *Genomics, proteomics, metabolomics: what is in a word for multiple sclerosis?* Curr Opin Neurol 2005, 18(3): 231-5.
17. Villar, L.M., Garcia-Barragan, N., Sadaba, M.C. et al. *Accuracy of CSF and MRI criteria for dissemination in space in the diagnosis of multiple sclerosis.* J Neurol Sci 2008, 266(1-2): 34-7.
18. Tintore, M., Rovira, A., Rio, J. et al. *Do oligoclonal bands add information to MRI in first attacks of multiple sclerosis?* Neurology 2007, 70(13): 1079-83.
19. Villar, L.M., Sadaba, M.C., Roldan, E. et al. *Intrathecal synthesis of oligoclonal IgM against myelin lipids predicts an aggressive disease course in MS.* J Clin Invest 2005, 115(1): 187-94.
20. Thangarajh, M., Gomez-Rial, J., Hedstrom, A.K., Hillert, J., Alvarez-Cermeno, J.C., Masterman, T., Villar, L.M. *Lipid-specific immunoglobulin M in CSF predicts adverse long-term outcome in multiple sclerosis.* Mult Scler 2008, 14(9): 1208-13.
21. Oksenberg, J.R., Baranzini, S.E., Sawcer, S., Hauser, S.L. *The genetics of multiple sclerosis: SNPs to pathways to pathogenesis.* Nat Rev Genet 2008, 9(7): 516-26.
22. Fugger, L., Friese, M.A., Bell, J.I. *From genes to function: the next challenge to understanding multiple sclerosis.* Nat Rev Immunol 2009, 9(6): 408-17.
23. Barcellos, L.F., Sawcer, S., Ramsay, P.P. et al. *Heterogeneity at the HLA-DRB1 locus and risk for multiple sclerosis.* Hum Mol Genet 2006, 15(18): 2813-24.
24. Pappas, D.J., Oksenberg, J.R. *Multiple sclerosis pharmacogenomics: maximizing efficacy of therapy.* Neurology, 74 Suppl 1: S62-9.
25. Martinez-Forero, I., Pelaez, A., Villoslada, P. *Pharmacogenomics of multiple sclerosis: in search for a personalized therapy.* Expert Opin Pharmacother 2008, 9(17): 3053-67.
26. Baranzini, S.E., Mousavi, P., Rio, J. et al. *Transcription-based prediction of response to IFNbeta using supervised computational methods.* PLoSBiol 2005, 3(1): e2.
27. Byun, E., Caillier, S.J., Montalban, X. et al. *Genome-wide pharmacogenomic analysis of the response to interferon beta therapy in multiple sclerosis.* Arch Neurol 2008, 65(3): E1-E8.
28. O'Doherty, C., Favorov, A., Heggarty, S. et al. *Genetic polymorphisms, their allele combinations and IFN-beta treatment response in Irish multiple sclerosis patients.* Pharmacogenomics 2009, 10(7): 1177-86.
29. Grossman, I., Avidan, N., Singer, C. et al. *Pharmacogenetics of glatiramer acetate therapy for multiple sclerosis reveals drug-response markers.* Pharmacogenet Genomics 2007, 17(8): 657-66.
30. Berger, T., Reindl, M. *Biomarkers in multiple sclerosis: role of antibodies.* Dis Markers 2006, 22(4): 207-12.

31. Lalive, P.H., Menge, T., Delarasse, C. et al. *Antibodies to native myelin oligodendrocyte glycoprotein are serologic markers of early inflammation in multiple sclerosis.* Proc Natl Acad Sci U S A 2006, 103(7): 2280-5.
32. Polman, C.H., Killestein, J. *Anti-myelin antibodies in multiple sclerosis: clinically useful?* J Neurol Neurosurg Psychiatry 2006, 77(6): 712.
33. McKeon, A., Fryer, J.P., Apiwattanakul, M. et al. *Diagnosis of neuromyelitis spectrum disorders: comparative sensitivities and specificities of immunohistochemical and immunoprecipitation assays.* Arch Neurol 2009, 66(9): 1134-8.
34. Sospedra, M., Martin, R. *Immunology of multiple sclerosis.* Annu Rev Immunol 2005, 23: 683-747.
35. Hauser, S.L., Oksenberg, J.R. *The neurobiology of multiple sclerosis: genes, inflammation, and neurodegeneration.* Neuron 2006, 52(1): 61-76.
36. Han, M.H., Hwang, S., Roy, D.B. et al. *Proteomic analysis of active multiple sclerosis lesions reveals therapeutic targets in the coagulation cascade.* Nature 2007, 451: 1076-81.
37. Berven, F.S., Flikka, K., Berle, M., Vedeler, C., Ulvik, R.J. *Proteomic-based biomarker discovery with emphasis on cerebrospinal fluid and multiple sclerosis.* Curr Pharm Biotechnol 2006, 7(3): 147-58.
38. Ottervald, J., Franzen, B., Nilsson, K. et al. *Multiple sclerosis: Identification and clinical evaluation of novel CSF biomarkers.* J Proteomics 2010, 73(6): 1117-32.
39. Satoh, J.I., Tabunoki, H., Yamamura, T. *Molecular network of the comprehensive multiple sclerosis brain-lesion proteome.* Mult Scler 2009, 15(5): 531-41.
40. Stoop, M.P., Dekker, L.J., Titulaer, M.K. et al. *Quantitative matrix-assisted laser desorption ionization-fourier transform ion cyclotron resonance (MALDI-FT-ICR) peptide profiling and identification of multiple-sclerosis-related proteins.* J Proteome Res 2009, 8(3): 1404-14.
41. Sinclair, A.J., Viant, M.R., Ball, A.K. et al. *NMR-based metabolomic analysis of cerebrospinal fluid and serum in neurological diseases - a diagnostic tool?* NMR Biomed 2010, 18: 41.
42. Ascherio, A., Munger, K.L. *Environmental risk factors for multiple sclerosis. Part II: Noninfectious factors.* Ann Neurol 2007, 61(6): 504-13.
43. Kanter, J.L., Narayana, S., Ho, P.P. et al. *Lipid microarrays identify key mediators of autoimmune brain inflammation.* Nat Med 2006, 12(1): 138-43.
44. Podbielska, M., Hogan, E.L. *Molecular and immunogenic features of myelin lipids: incitants or modulators of multiple sclerosis?* Mult Scler 2009, 15(9): 1011-29.
45. An, H.J., Kronewitter, S.R., de Leoz, M.L., Lebrilla, C.B. *Glycomics and disease markers.* Curr Opin Chem Biol 2009, 13(5-6): 601-7.
46. Quintana, F.J., Farez, M.F., Viglietta, V. et al. *Antigen microarrays identify unique serum autoantibody signatures in clinical and pathologic subtypes of multiple sclerosis.* Proc Natl Acad Sci U S A 2008, 105(48): 18889-94.
47. Kasper, L.H., Shoemaker, J. *Multiple sclerosis immunology: The healthy immune system vs the MS immune system.* Neurology 74(Suppl. 1): S2-8.
48. Venken, K., Hellings, N., Liblau, R., Stinissen, P. *Disturbed regulatory T cell homeostasis in multiple sclerosis.* Trends Mol Med, 16(2): 58-68.
49. Martinez-Forero I, Garcia-Munoz R, Martinez-Pasamar A et al. *IL-10 suppressor activity and ex vivo Tr1 cell function are impaired in Multiple Sclerosis.* Eur J Immunol 2008, 38(2): 576-86.
50. Zozulya, A.L., Wiendl, H. *The role of regulatory T cells in multiple sclerosis.* Nat Clin Pract Neurol 2008, 4(7): 384-98.
51. Ascherio, A., Munger, K.L. *Environmental risk factors for multiple sclerosis. Part I: the role of infection.* Ann Neurol 2007, 61(4): 288-99.
52. Fotheringham, J., Jacobson, S. *Human herpesvirus 6 and multiple sclerosis: potential mechanisms for virus-induced disease.* Herpes 2005, 12(1): 4-9.
53. Villoslada, P., Juste, C., Tintore, M., Llorenc, V., Codina, G., Pozo-Rosich, P., Montalban, X. *The immune response against herpesvirus is more prominent in the early stages of MS.* Neurology 2003, 60(12): 1944-8.
54. Lunemann, J.D., Ascherio, A. *Immune responses to EBNA1: biomarkers in MS?* Neurology 2009, 73(1): 13-4.
55. Alvarez-Lafuente, R., Garcia-Montojo, M., De Las Heras, V., Dominguez-Mozo, M.I., Bartolome, M., Benito-Martin, M.S., Arroyo, R. *Herpesviruses and human endogenous retroviral sequences in the cerebrospinal fluid of multiple sclerosis patients.* Mult Scler 2008, 14(5): 595-601.
56. Alvarez-Lafuente, R., Garcia-Montojo, M., De las Heras, V., Bartolome, M., Arroyo, R. *Clinical parameters and HHV-6 active replication in relapsing-remitting multiple sclerosis patients.* J Clin Virol 2006, 37(Suppl. 1): S24-6.
57. Brettschneider, J., Petzold, A., Junker, A., Tumani, H. *Axonal damage markers in the cerebrospinal fluid of patients with clinically isolated syndrome improve predicting conversion to definite multiple sclerosis.* Mult Scler 2006, 12(2): 143-8.
58. Hein Nee Maier, K., Kohler, A., Diem, R. et al. *Biological markers for axonal degeneration in CSF and blood of patients with the first event indicative for multiple sclerosis.* Neurosci Lett 2008, 436(1): 72-6.
59. Teunissen, C., Dijkstra, C.D., Polman, C. *Biological markers in CSF and blood for axonal degeneration in multiple sclerosis.* Lancet Neurol 2005, 4(1): 32-41.
60. Teunissen, C.E., Petzold, A., Bennett, J.L. et al. *A consensus protocol for the standardization of cerebrospinal fluid collection and biobanking.* Neurology 2009, 73(22): 1914-22.
61. Giovannoni, G., Thompson, E.J. *Urinary markers of disease activity in multiple sclerosis.* Mult Scler 1998, 4(3): 247-53.
62. Malcus-Vocanson, C., Giraud, P., Broussolle, E., Perron, H., Mandrand, B., Chazot, G. *A urinary marker for multiple sclerosis.* Lancet 1998, 351(9112): 1330.
63. Dobson, R., Miller, R.F., Palmer, H.E. et al. *Increased urinary free immunoglobulin light chain excretion in patients with multiple sclerosis.* J Neuroimmunol 2010, 220(1-2): 99-103.
64. 't Hart, B.A., Vogels, J.T., Spijksma, G., Brok, H.P., Polman, C., van der Greef, J. *1H-NMR spectroscopy combined with pattern recognition analysis reveals characteristic chemical patterns in urines of MS patients and non-human primates with MS-like disease.* J Neurol Sci 2003, 212(1-2): 21-30.
65. Barkhof, F., Filippi, M. *MRI—the perfect surrogate marker for multiple sclerosis?* Nat Rev Neurol 2009, 5(4): 182-3.
66. Tintore, M., Rovira, A., Martinez, M.J. et al. *Isolated demyelinating syndromes: comparison of different MR imaging criteria to predict conversion to clinically definite multiple sclerosis.* AJNR 2000, 21(4): 702-6.
67. Miller, D.H. *Biomarkers and surrogate outcomes in neurodegenerative disease: lessons from multiple sclerosis.* NeuroRx 2004, 1(2): 284-94.
68. Barkhof, F., Calabresi, P.A., Miller, D.H., Reingold, S.C. *Imaging outcomes for neuroprotection and repair in multiple sclerosis trials.* Nat Rev Neurol 2009, 5(5): 256-66.
69. Zhang, Y., Zhu, H., Mitchell, J.R., Costello, F., Metz, L.M. *T2 MRI texture analysis is a sensitive measure of tissue injury and recovery resulting from acute inflammatory lesions in multiple sclerosis.* Neuroimage 2009, 47(1): 107-11.
70. Filippi, M., Agosta, F. *Magnetization transfer MRI in multiple sclerosis.* J Neuroimaging 2007, 17(Suppl. 1): 22S-6S.

71. De Stefano, N., Filippi, M. *MR spectroscopy in multiple sclerosis.* J Neuroimaging 2007, 17(Suppl. 1): 31S-5S.
72. Rovaris, M., Agosta, F., Pagani, E., Filippi, M. *Diffusion tensor MR imaging.* Neuroimaging Clin N Am 2009, 19(1): 37-43.
73. Esteban, F., Sepulcre, J., Ruiz de Miras, J. et al. *Fractal dimension analysis of grey matter in multiple sclerosis* J Neurol Sci 2009, 282(1-2): 67-71.
74. Esteban, F.J., Sepulcre, J., de Mendizabal, N.V. et al. *Fractal dimension and white matter changes in multiple sclerosis.* Neuroimage 2007, 36(3): 543-9.
75. Frohman, E.M., Fujimoto, J.G., Frohman, T.C., Calabresi, P.A., Cutter, G., Balcer, L.J. *Optical coherence tomography: a window into the mechanisms of multiple sclerosis.* Nat Clin Pract Neurol 2008, 4(12): 664-75.
76. Sepulcre, J., Murie-Fernandez, M., Salinas-Alaman, A., Garcia-Layana, A., Bejarano, B., Villoslada, P. *Diagnostic accuracy of retinal abnormalities in predicting disease activity in MS.* Neurology 2007, 68(18): 1488-94.
77. Menke, M.N., Dabov, S., Knecht, P., Sturm, V. *Reproducibility of retinal thickness measurements in healthy subjects using spectralis optical coherence tomography.* Am J Ophthalmol 2009, 147(3): 467-72.
78. Confavreux, C., Vukusic, S. *Natural history of multiple sclerosis: a unifying concept.* Brain 2006, 129(Pt 3): 606-16.
79. Baier, M.L., Cutter, G.R., Rudick, R.A. et al. *Low-contrast letter acuity testing captures visual dysfunction in patients with multiple sclerosis.* Neurology 2005, 64(6): 992-5.
80. Leocani, L., Comi, G. *Neurophysiological markers.* Neurol Sci 2008, 29(Suppl. 2): S218-21.
81. Villoslada, P., Oksenberg, J. *Neuroinformatics in clinical practice: are computers going to help neurological patients and their physicians?* Future Neurology 2006, 1(2): 1-12.
82. Bates, D.W., Gawande, A.A. *Improving safety with information technology.* N Engl J Med 2003, 348(25): 2526-34.
83. Tegner, J.N., Compte, A., Auffray, C. et al. *Computational disease modeling - fact or fiction?* BMC Syst Biol 2009, 3: 56.
84. Villoslada, P., Oksenberg, J.R., Rio, J., Montalban, X. *Clinical characteristics of responders to interferon therapy for relapsing MS.* Neurology 2004, 62(9): 1653.
85. Rio, J., Comabella, M., Montalban, X. *Predicting responders to therapies for multiple sclerosis.* Nat Rev Neurol 2009, 5(10): 553-60.
86. O'Doherty, C., Villoslada, P., Vandenbroeck, K. *Pharmacogenomics of Type I interferon therapy: a survey of response-modifying genes.* Cytokine Growth Factor Rev 2007, 18(3-4): 211-22.

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