Transcriptional profiling of multiple sclerosis: towards improved diagnosis and treatment

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The development of high-throughput techniques, for example cDNA and oligonucleotide microarrays, for simultaneous analysis of the transcriptional expression of thousands of genes, even the entire genome, has provided new possibilities to get better insights into the pathogenesis of various diseases. This technology has also been applied to define biomarkers and, most importantly, possible new candidate targets for novel treatments. In multiple sclerosis, microarray studies have been performed on brain autopsy and biopsy specimens and peripheral blood. The effects of current treatments for multiple sclerosis, especially interferon-β and glatiramer acetate, on transcriptional profiles, have also been investigated. We review the main findings revealed from these studies. The emerging potential of microarray technology to define gene signatures, diagnostic and prognostic markers for disease course, and treatment response in multiple sclerosis will be discussed.

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Multiple sclerosis (MS) is a complex disease, in which many pathophysiological processes (e.g., inflammation, demyelination, axonal damage and repair mechanisms) are involved (FIGURE 1) [1,2]. There is a clinically variable phenotypic expression of the disease and the individual response to therapies. Current clinical and paraclinical (magnetic resonance imaging [MRI], CSF (cerebrospinal fluid) pleocytosis and presence of oligoclonal bands, evoked potentials [EPs]) diagnostic tools allow a reliable diagnosis of MS [3,4]. Initially, more than 80% of patients express a relapsing–remitting form of MS (RRMS), characterized by exacerbations of partially or completely reversible neurological deficits. The majority of RRMS patients progress to a secondary progressive phase (SPMS), which is characterized by steadily increasing irreversible deficits and neurodegeneration with or without superimposed relapses. In primary progressive MS (PPMS), continuous progression without distinguishable relapses occurs (FIGURE 2) [5,6].

Immune mechanisms are believed to play an important role in the disease process. Focal demyelinated plaques (the hallmark of MS) are infiltrated by heterogeneous populations of immune cells and soluble immune mediators, including T cells, B cells, macrophages and microglia, as well as cytokines, chemokines, complement and other toxic agents. Demyelinated axons are exposed to the inflammatory mediators leading to axonal damage and neuronal loss in the pathoanatomical substrate of irreversible functional impairment and disability [7]. Normal appearing white and gray matter are also different in MS compared with healthy controls.

Interferon (IFN)-β and glatiramer acetate (Cop-1) are the first drugs with proven beneficial effect on RRMS; they decrease the formation of plaques and the number of relapses by a third, compared with untreated patients [8]. However, the individual response is unpredictable and ranges from excellent to at best ineffective. The costs of these therapies are high, but we are currently unable to identify prospectively patients who will fail to respond to one or another of these drugs. Moreover, it seems that these drugs have less or virtually no impact on the relapse of unrelated, more diffuse tissue damage and resulting atrophy, and hence they are of limited value in the prevention of long-term disability [9,10]. Conversely, there is an accumulating body of evidence that the earlier therapeutic intervention is

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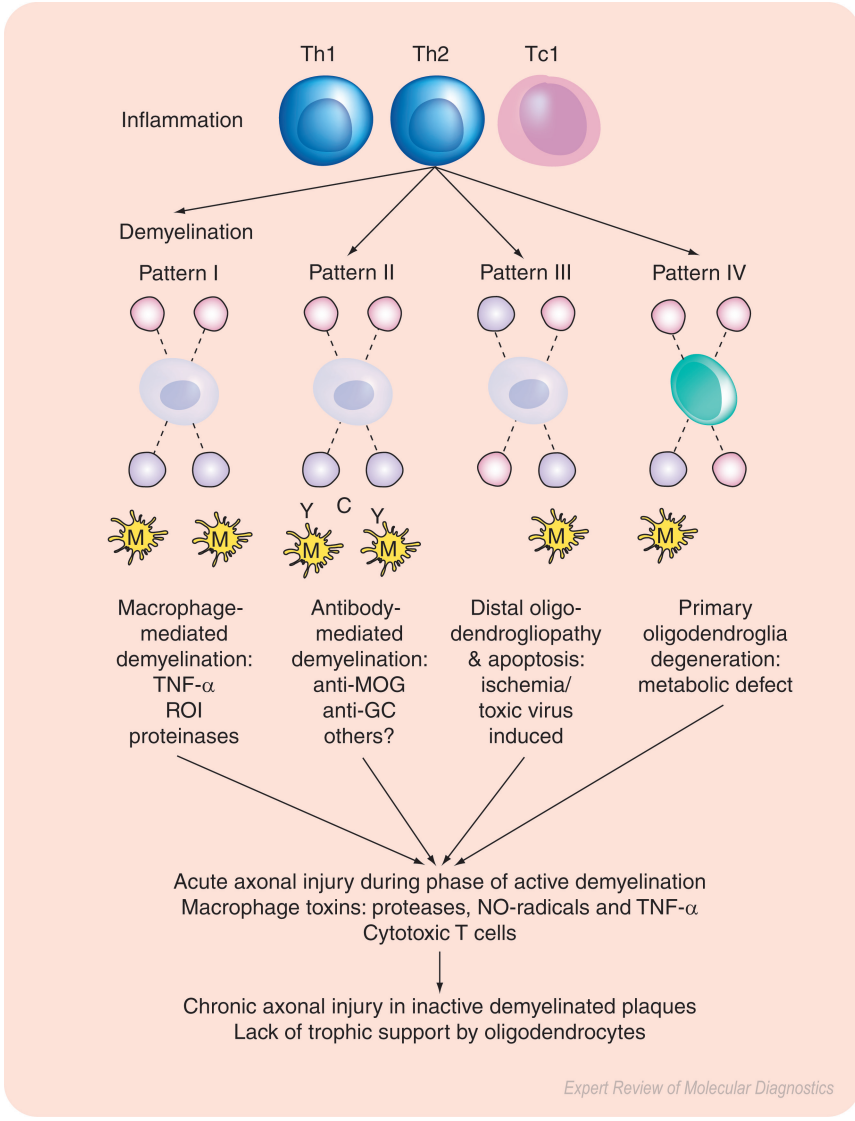

Figure 1. Pathogenetic mechanisms involved in the formation of multiple sclerosis lesions.
Demyelination may be induced by macrophages (M) and/or their toxic products (resulting in pattern I), by specific demyelinating antibodies and complement (C), resulting in pattern II), by degenerative changes in distal processes, in particular those of periaxonal oligodendrocytes (distal oligodendrogliopathy), followed by apoptosis (resulting in pattern III) or by a primary degeneration of oligodendrocytes followed by myelin destruction (resulting in pattern IV). GC: Galactocerebroside; Th: T helper; TNF: Tumor necrosis factor; MOG: Myelin oligodendrocyte glycoprotein; NO: Nitric oxide; ROI: Reactive oxygen intermediate.
Adapted with permission from [2].

applied in clinically isolated syndrome (CIS) and RRMS, the more favourable the outcome [11–13]. However, we are lacking diagnostic and prognostic markers for the early course of MS. During the last 7 years, high-throughput microarray technologies have been applied in order to identify such markers. The use of these tools has also revealed novel aspects of the pathogenesis of the disease and revealed new therapeutic targets [14].

Expression profiling of multiple sclerosis brain tissue

Published large-scale transcriptional profiling studies on brain autopsy and biopsy tissue in MS with microarray technology are summarized in TABLE 1 [15–24]. Real time RT-PCR technology has also been used for expression studies on MS brain tissue, but the number of targets analyzed is limited, from a few targets [25] to 56 genes [26]. The heterogeneity of tissues investigated (acute, active, silent lesion, normal appearing white matter [NAWM] and motor cortex), platform used (cDNA or oligonucleotide array [FIGURE 3] [27]), type of MS disease course (RRMS, SPMS or PPMS), and different statistical approaches make the comparison between various studies difficult. However, these studies have revealed a complex pattern of mostly known genes involved in inflammation, immune response, transcriptional control and neural homeostasis.

In the first large-scale transcriptional analysis by Whitney and colleagues [15], an acute active lesion of a PPMS patient was compared with donor-matched NAWM. Two different custom-made cDNA arrays with 1400 and 5000 genes were used. A total of 62 differentially expressed genes, including upregulation of the tumor necrosis factor (TNF)-α-receptor 2, interferon regulatory factor-2 and chemokine receptor were found, which suggests altered inflammatory processes. The study was extended 2 years later to 16 lesions with various activities of one PPMS patient and two lesions of RRMS patient [16]. The control white matter samples were obtained from normal controls. The main finding in the lesions was a strong up-regulation of 5-lipoxygenase (5-LO), a key enzyme in the biosynthetic pathway of leukotrienes, which are important inflammatory mediators. The presence of 5-LO mainly in macrophages was confirmed by immunohistochemistry.

Through a broad screening approach, it has been possible to identify 'new players' such as *osteopontin* (*OPN*) and *αβ-crystallin* [17], whose expression in lesions and NAWM has been confirmed with immunohistochemistry at the protein level [28]. Up-regulation of *OPN* was also shown in experimental autoimmune encephalomyelitis (EAE), an animal model of MS. Furthermore, OPN-deficient mice were resistant to EAE and produced more interleukin (IL)-10 and less interferon (IFN)-γ than their wild-type littermates [17]. However, controversial results in terms of a response to EAE in OPN-deficient mice with different strains have been reported [29]. Lock and colleagues demonstrated (in their microarray studies on MS brain autopsies) an increased transcription of inflammatory cytokines (e.g., *IL-6* and *-17*) and other immune-related molecules, such

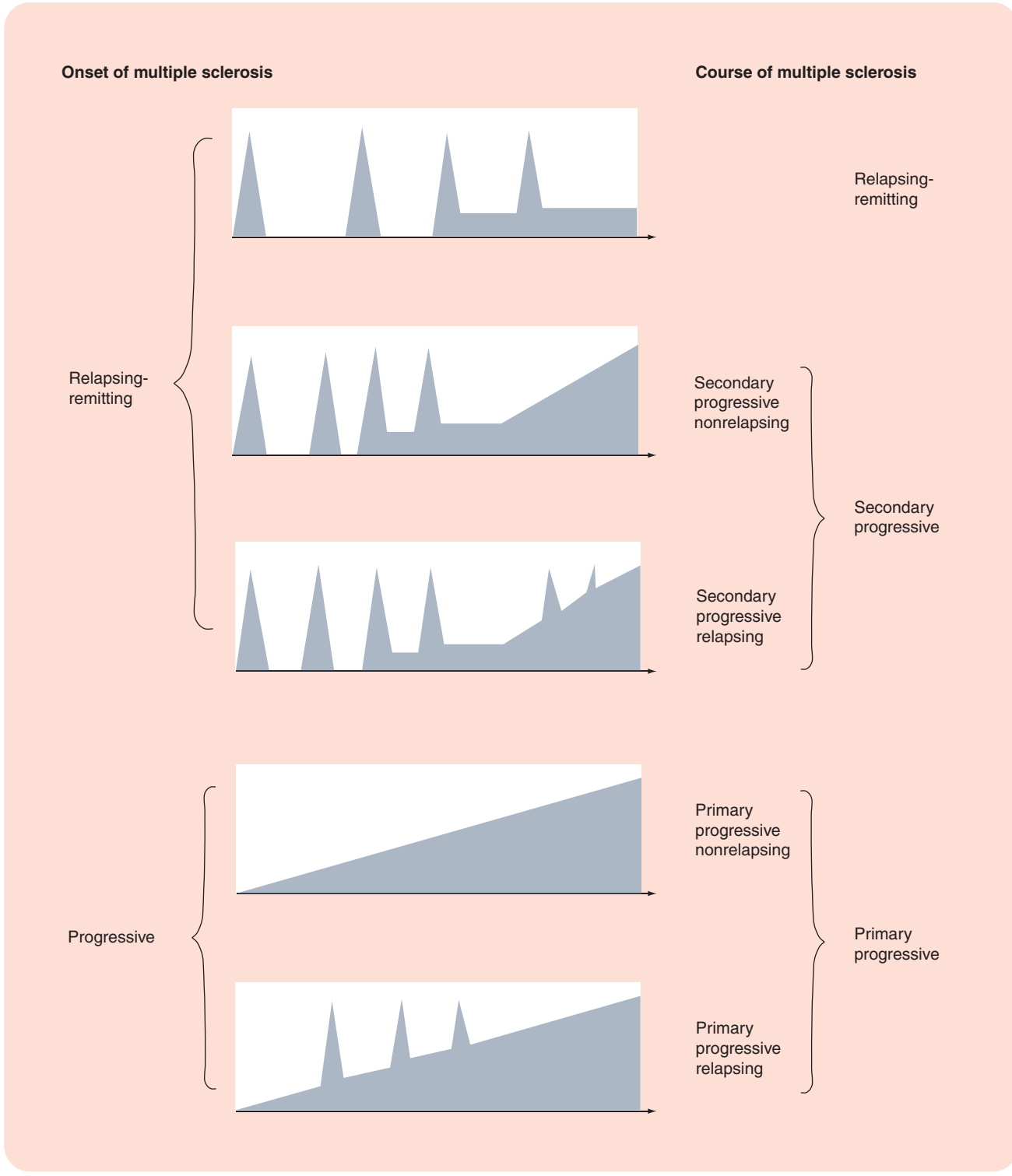

Figure 2. Proposed classification of the onset and course of multiple sclerosis. Adapted with permission from [6].

as major histocompatibility complex (*MHC*) class II and complement genes [18]. In the same study, the role of the immunoglobulin (Ig) Fc receptor common γ chain (*FcγRI*) and the granulocyte colony-stimulating factor (*G-CSF*) in MS, revealed from microarray studies, was investigated in EAE [18]. It was demonstrated that in FcγRi-knockout mice, disease was absent in the chronic and recovery stage of the disease, thus being concordant with the expression pattern in chronic lesions in MS. Conversely, the upregulation of G-CSF was in the acute stage of the disease in EAE.

Table 1. Gene expression studies on brain tissue of MS patients.

Type of MS	Type of lesion	n	Analytical platform	No. of targets	Differentially expressed genes	Main findings	Statistics	Ref.
PPMS	Acute vs NAWM	1	cDNA array, 'self-printed' glass slide	1400/5000	62 ⇑ ⇓	*Chemokine receptor*, *TNF-α R2*, *IRF2* ⇑ ⇒ inflammatory processes	NA	[15]
PPMS and RRMS	Acute, chronic active, chronic inactive	2	cDNA array, 'self-printed' glass slide	2798	62 ⇑ ⇓	*5-lipoxygenase* ⇑ ⇒ biosynthesis of the proinflammatory leukotrienes	NA	[16]
NA	Active, acute, inactive	3	cDNA library, 'self-constructed'	11,000 clones	54 ⇑ ⇓	*Osteopontin* (confirmed with EAE) and *αβ-crystallin*	Fisher's exact test	[17]
SPMS	Chronic active, chronic inactive	6	Oligonucleotide array, HuGeneFL7026 Affymetrix	7000	39 ⇑ 49 ⇓	*IgFcR* ⇑ in inactive ⇒ EAE, γ-KO ⇒ EAE ameliorated *G-CSF* ⇑ in active ⇒ treatment in EAE in acute phase	Permutation test, error model	[18]
SPMS	Chronic active, chronic inactive	4	cDNA array, nylon membrane (Atlas™, Clontech, CA, USA)	588	87/65 ⇑ and 69/22 ⇑ in margin/center in act and silent	DEGs correlates with lesion activity	NA	[19]
SPMS	Active, chronic active	5	cDNA array, glass slide Queensland Institute of Medical Research	5000	139 ⇑ ⇓	69 common genes expressed in all lesions (e.g. *αβ-crystallin*), 70 uniquely expressed according the activity of the lesion	T-test, Spearman's σ-analysis, Mann-Whitney	[20]
RRMS, SPMS, PPMS	NAWM	10	cDNA array, nylon membrane Atlas, Clontech	3528	NA	Ischemic preconditioning	Mann–Whitney	[21]
SPMS	Active vs NAWM	6	Oligonucleotide array, Human U95A Affymetrix	12,000	123 ⇑ ⇓ (lesion) 47 ⇑ ⇓ (NAWM)	MS is a generalized CNS disease, dysregulation of cellular immune response prevailing in NAWM, humoral immune response in lesions	T-test, Kruskal-Wallis, Mann–Whitney, ANOVA	[22]
SPMS	Chronic active, chronic inactive	4	cDNA array, nylon membrane Atlas, Clontech	588	50/15 ⇑ and 64/59 ⇑ in margin/center in act and silent	Active ⇒ inflammation Inactive ⇒ apoptosis	NA	[23]
SPMS (9), PPMS (1)	Motor cortex	10	Oligonucleotide array, Human U133A/ U133B Affymetrix	33,000	67 ⇑ 488 ⇓	Mitochondrial dysfunction ⇒ dysbalance in ion homeostasis ⇒ axonal degeneration in motor neurons ⇒ progressive disability	Two-tailed group-wise t-test, permutation test + FDR	[24]

ANOVA: ANalysis Of VAriance; DEG: Differentially expressed gene; EAE: Experimental autoimmune encephalomyelitis; FDR: False discovery rate; G-CSF: Granulocyte colony-stimulation factor; IgFcR: Receptor for Fc domain of immunoglobulin; IRF: Interferon regulatory factor; KO: Knockout; MS: Multiple sclerosis; NA: Not available; NAWM: Normal appearing white matter; PP: Primary progressive ; RR: Relapsing-remitting; SP: Secondary progressive; TNF: Tumor necrosis factor.

Tajouri and colleagues investigated expression profiles in acute and chronic active MS lesions with microarrays and compared those with patient-matched white matter [20]. A total of 139 differentially expressed genes were identified, in which 69 of those showed common patterns in both lesion types, in contrast with 70 genes, which were expressed uniquely in either of the lesions (acute or chronic) studied. Interestingly, expression differences were significantly higher in acute plaques compared with chronic lesions, suggesting that quantitative rather than gross qualitative differences in the gene expression pattern may define the progression from an acute to a chronic active lesion. In this study, upregulation of *αβ-crystallin* was found and confirmed with quantitative real-time RT-PCR, being consistent with the findings published by others [17].

In two separate reports, Mycko and colleagues studied gene expression between margins and centers of chronic active and chronic inactive lesions from autopsy samples of four SPMS patients [19,23]. Significant differences in the transcriptional profiles of these two lesion types, in both marginal and central areas, were found. The genes related to inflammation (e.g., *TNF* and *IL-6* were present in both the margins and centers of active plaques, whereas they were under-represented in inactive lesions). In contrast, many apoptosis and death-related genes, such as *bcl-x*, *growth factor receptor-bound* gene, heat shock proteins *(HSP)90A* and *HSP70*, were present in inactive lesions. The overexpression of HSPs in MS lesions at the protein level has been documented in several studies [30–32]. Graumann and colleagues recently reported transcriptional upregulation of *HSP70* in NAWM in MS [21]. They also found the upregulation of *hypoxia inducing factor* (*HIF*)-1α and, consequently, genes such as *platelet-derived growth factor B* (*PDGF-B*), transferrin receptor and insulin growth factor-binding protein *(IGFBP)1* were induced. The key finding of their study was the upregulation of gene expression related to oxidative stress and ischemic preconditioning, suggesting autoprotective mechanisms in the NAWM.

A comparative microarray analysis of NAWM and donor-matched lesions in six SPMS patients was recently reported [22]. From four patients, matched lesion and NAWM tissues were studied. From one patient, only active lesion, and from one patient only NAWM tissue was available. The gene expression patterns in diseased specimen were compared with those of control subjects, who died from non-neurological diseases. The study revealed 123 and 47 differentially expressed genes in lesions and NAWM, respectively. In active lesions, the largest number of regulated genes was involved in neural homeostasis. Functional genes (i.e., dynamin and synaptosome-associated protein), which are essential for cell trafficking and exocytosis in nerve terminals, were upregulated. The lesions distinguished themselves from NAWM by a higher expression of genes related to immunoglobulin synthesis and neuroglial differentiation, while cellular immune response elements were equally dysregulated in both tissue compartments. These results provide molecular evidence of a continuum of dysfunctional homeostasis and inflammatory changes between lesions and NAWM, and support the concept of MS pathogenesis being a generalized process that involves the entire CNS.

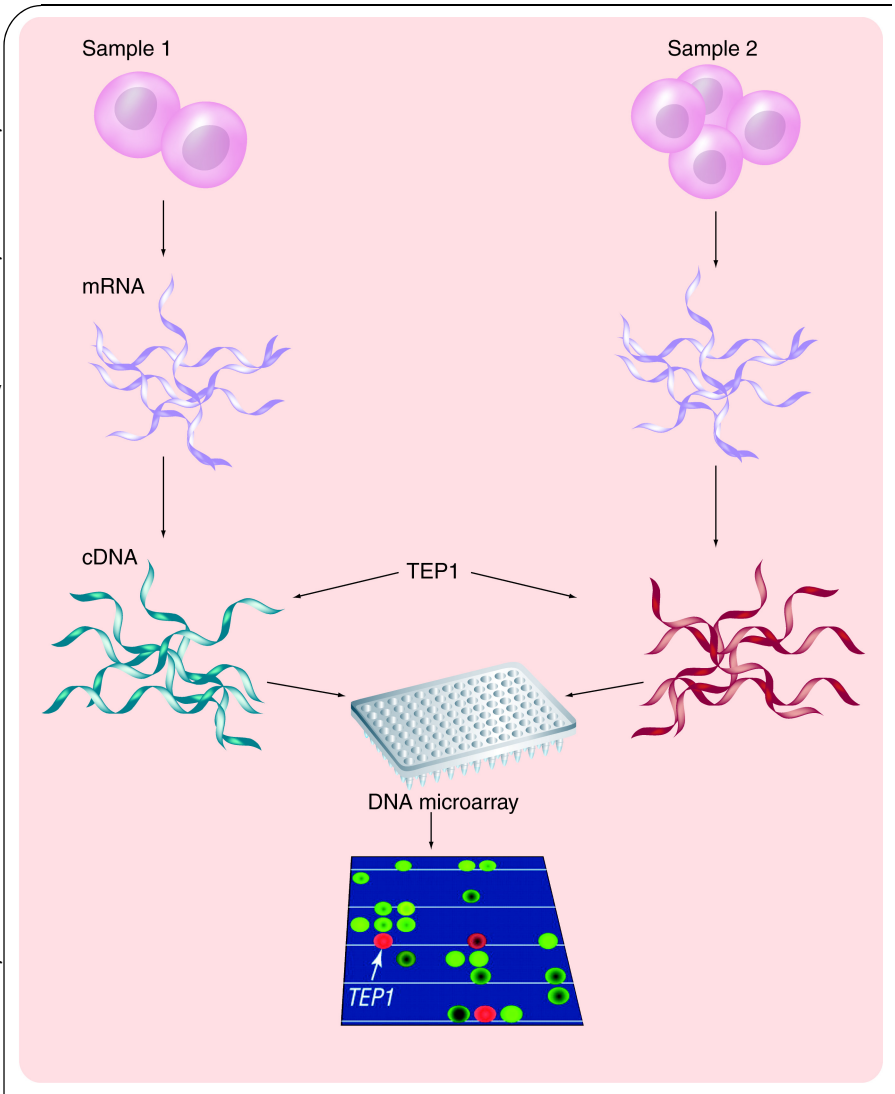

Figure 3a. Principles of cDNA arrays. cDNA microarrays contain double-stranded cDNA sequences of interest that have been synthesized by PCR and then 'spotted', 'immobilized', on the glass slide or on the nylon membrane. Thousands of genes can be spotted on one array. Dye-labeled RNA populations are mixed and hybridized on the array. The RNA from each sample hybridizes to each spot in quantitative manner and therefore relative expression levels in various samples can be determined.
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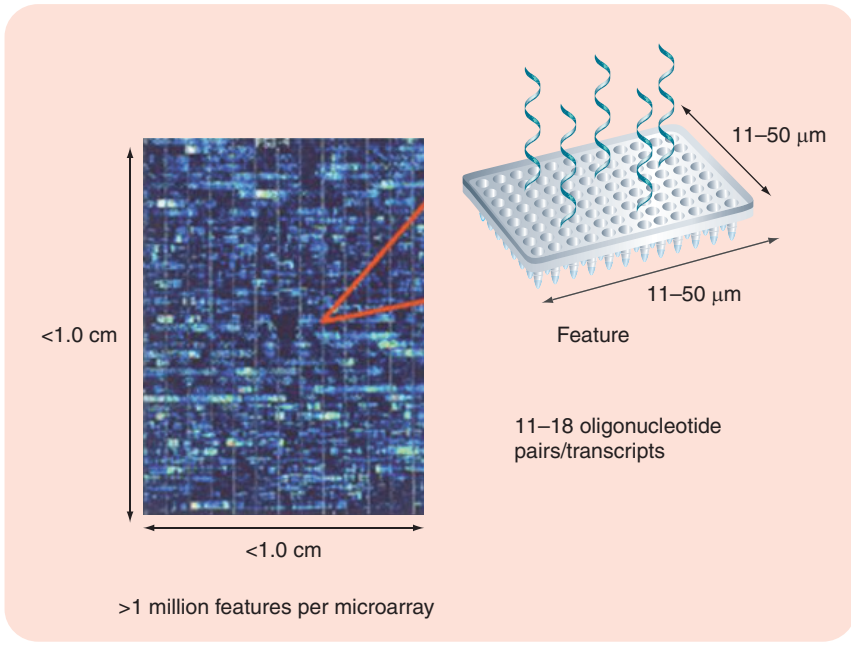

Figure 3b. Principles of cDNA arrays. Oligonucleotide microarrays are prepared using specific oligonucleotides synthesized directly onto a quartz or silicon wafer using combinatorial chemistry and photolithography. One microarray may contain more than 1 million different oligonucleotides, which act as probes in individual 'features' on the microarray surface. Fluorescent-labeled cRNAs derived from a single test sample are hybridized on the microarray. Expression levels of even the entire human genome can be measured in the test sample with one microarray. Adapted and modified with permission from [27].

Most of the gene expression studies on brain tissue of MS patients have been performed on lesions with various disease activities or normal appearing white matter. Dutta and colleagues recently reported the first large-scale gene expression study on cortical neurons in MS patients [24]. Nonlesioned motor cortex from six SPMS patients and six controls were analyzed. A total of 555 significantly differentially expressed genes included 488 down- and 67 upregulated genes. Transcripts were classified into gene ontology-based biological processes according to their significance in the following processes: oxidative phosphorylation, synaptic transmission, cellular transport, MHC related, antigen presentation, antigen processing and translational initiation. The expression of 26 nuclear-encoded mitochondrial genes was decreased. Functional assays confirmed that the activity of mitochondrial respiratory chain complexes I and III was also consequently reduced. Another interesting finding in this study was a decreased expression of several genes related to the inhibitory neurotransmitter γ-aminobutyric acid (GABA) system. The *GABA A α1* and *β3* receptor subunits and GABA A receptor associated protein (*GABRAP*) were downregulated in the motor cortex of MS patients. Other presynaptic inhibitory related genes, such as *GAD67*, parvalbumin, cholecystokinin and tachykinin were also decreased in MS samples. The authors proposed that reduced ATP production in demyelinated segments of upper motor neuron axons impacts on ion homeostasis, induces Ca^{2+}-mediated axonal degeneration and contributes to progressive neurological disability in MS patients. Understanding the mechanisms that regulate nuclear-encoded mitochondrial genes in upper motor neurons may lead to therapeutics that increase ATP production.

Expression profiling of peripheral blood cells of multiple sclerosis patients

Transcriptional profiling studies on peripheral blood cells in MS are summarized in TABLE 2 [33–47]. Also in these reports, the heterogeneity of subjects included, target cells studied, platform and analytical/statistical approaches applied makes the comparison between various studies difficult. Intraindividual and interindividual variations have also been demonstrated to play an important role in gene expression in peripheral blood [48,49]. In principle, there are three different categories of findings: first, gene signatures of several hundreds of genes [35,40–42,47]; second, groups of a few genes (pairs, triplets up to 34 genes) [35,39,44]; and third, single genes [33,34,36,37,43,46], have been explored. The effect of IFN-β [36–41,44,47] and glatiramer acetate [39] on gene expression in MS patients has also been investigated.

In the very first large-scale expression study on peripheral blood mononuclear cells (PBMCs), Ramanathan and colleagues found 34 differentially expressed genes in 15 RRMS samples compared with 15 matched healthy volunteers, among 4000 genes studied on the array [33]. The majority (13 genes) had inflammatory and immune functions, such as *IL-7 receptor* and *LCK*, a Src family kinase that is important in T-cell development, activation and proliferation. In general, the number of altered genes allowing RRMS to be distinguished from controls was limited. Bomprezzi and colleagues used an advanced computational approach on gene expression data in PBMCs obtained from RRMS and SPMS patients and healthy volunteers, aiming to identify a panel of molecular markers indicative of disease status [35]. They could define more than a thousand pairs of genes that could distinguish MS samples from controls. The strongly dominating genes included *HSP70* and *CDC28* protein kinase *(CKS)2*, which combined with the H1 histone family member (*HIF*)2 and platelet-activating factor acetylhydrolase, isoform 1b, α subunit (*PAFAH1B1*), respectively, discriminated well between MS and controls. These pairs also had 80% 'predictor' value to classify an independent sample into the correct class. Of interest, when they used strong feature sets based on gene triplets (rather than pairs), the misclassification error did not improve; therefore, pairs were used for further analysis. The most discriminating gene pairs were also related to MS relevant biological pathways and thus would also be indicative of disease pathophysiology. Such molecules, which were highly expressed in MS, were *CD27*, the *TNF* receptor,

Table 2. Gene expression studies on peripheral blood mononuclear cells of MS patients .

Subjects	Treatment	Target tissue	Analytical platform	No of genes	Statistical methods	Main findings	Ref.
15 RRMS (11 F/4 M) 15 controls	No	PBMC *ex vivo*	cDNA array GeneFilters® GF211, Research Genetics (Invitrogen)	4000	Pathways 2.0 Excel + SPSS Paired t-test	34 genes Up: *P protein*, *LCK*, *cAMP-rem*, *IL-7R MMP19*, *M130 antigen* Down: *STRL22* (C-C chemokine receptor)	[33]
6 RRMS Controls (n = 2)	Treatment longitudinally (IFN-β)	PBMC-NK cells and Th1 cells *in vitro* and PBMC *ex vivo*	cDNA array 'custom-printed' glass slide	3035 known genes + 3397 ESTs	Sigma-Stat t-test Wilcoxon's signed-rank test ANOVA + Dunett	*IL-12Rβ2* chain and *CCR5* upregulated *in vitro* and *ex vivo*	[34]
24 MS (18 RR + 6 SP, 15F + 9M) 19 controls (5F + 14M)	No	PBMC *ex vivo*	cDNA array glass slides ResGen (Invitrogen)	Set 1: 6500 clones Set 2: 7500 clones	'Classifiers' defined based on published computer-publications	Expression profiles can distinguish MS patients from controls. Identification of disease relevant pathway 'Expression signature'	[35]
10 RRMS (F), 6 responders 2 non-responders, 2 INR (initially no response)	0, 2, 4, 6 months (IFN-β)	PBMC *in vitro*, PBMC *ex vivo*	cDNA array Mini-Lymphochip	3035 known genes + 3397 ESTs (see [34]) or double amount of genes	Two-sided t-test	*Ex vivo*: 25 and *in vitro*: 87 IFN-regulated genes Novel finding: downregulation of *IL-8* in responders. Apoptotic genes regulated (down: *IEX-1L*, *TSC-22R*, up: *BNIP3*, *TRAIL*)	[36]
13 RRMS (9F + 4M), 3 controls (pooled)	0, 3 and 6 months (IFN-β)	$CD3^+$ vs CD3- T-cells + monocytes + B c ells + NK cells *ex vivo*	cDNA array 'custom-printed' glass slide	1263	Cyber-T, t-test + Bayesian inference of variance	21 genes after treatment: 9 IFN-responsive promoter elements, no changes in Th1 or Th2 marker genes, *TSG-6* ⇒ decreased protease activity	[37]
8 RRMS (6F + 2M) no controls	0, 1, 2, 4, 8, 24, 48, 120, 168 h, 3 and 6 months	PBMC-monocytes *ex vivo*	cDNA array, GeneFilter GF211, Research Genetics	>4000	Pathway 4.0 Excel + SPSS 6.1 software, SAS	Antiviral response, Jak-Stat pathway, immune activation markers	[38]
30 patients (RR and SPMS, mixed F/M ?) 9 controls (6F + 3M)	2–8 years (IFN-β or GA)	PBMC *in vitro* and *ex vivo*	cDNA array, nylon membrane, 'custom spotted'	34 selected genes + β-actin + neg control	Scananalyze 2.5, Shapiro-Wilk test, t-test, Mann-Whitney test	Effect of Nabs of IFNβ, Different effect of IFNβ and GA	[39]

ANOVA: ANalysis Of VAriance; CIS: Clinically isolated syndrome; DEG: Differentially expressed gene; EAE: Experimental autoimmune encephalomyelitis; EST: Expressed sequence tag; F: Female; GA: Glatiramer acetate; HC: Healthy controls ; Jak: Janus kinase; IBIS: Integrated Bayesian inference system; IFN: Interferon; IL: Interleukin; INR: International normalized ratio; LOOCV: Leave-One-Out-Cross-Validation; M: Male; MMP: Matrix metalloproteinase; MS: Multiple sclerosis; NA: Not available; NK: Natural killer; RR: Relapsing–remitting; PBMC: Peripheral blood mononuclear cells; RT: Reverse transcription; SAS: Statistical Analysis Software; SLE: Systemic lupus erythematosus; SP: Secondary progressive; SPSS: Statistical product and service solution; STAT: Signal transducers and activators of transcription; Th: T helper; TNoM: Threshold number of misclassification.

Table 2. Gene expression studies on peripheral blood mononuclear cells of MS patients (Cont.).

Subjects	Treatment	Target tissue	Analytical platform	No of genes	Statistical methods	Main findings	Ref.
17 patients 14 RR, 2SP, 1CIS (12F + 5M) 7 controls	8 no treatment + 9 IFN-β	PBMC *ex vivo*	Oligonucleotide array, HuGeneFL Affymetrix	6800	Affy Software Own developed statistical tools	553 differentially expressed genes Gene signature of enhanced immune cell activation E2F pathway ⇒ EAE model	[40]
12 in relapse 14 in remission	5 treated, 7 nontreated 8 treated, 6 nontreated, respectively, (IFN-β)	PBMC *ex vivo*	Oligonucleotide array, U95Av2 Affymetrix	12000	ScoreGenes, t-test, nonparametric tests, Bayesian classifier LOOCV	1109 genes signature, irrespective of disease activation or treatment 721 gene signature for disease activation	[41]
13 RRMS 9F + 4M 5 SLE 4F + 1M 18 controls 16F + 2M	No	PBMC *ex vivo*	Oligonucleotide array, U95Av2 Affymetrix	12000	ScoreGenes, t-test, nonparametric tests, TNoM Bayesian classifier LOOCV	541 gene signature for MS/SLE vs HC 1031 gene signature for MS 1146 gene signature for SLE	[42]
21 RRMS 11 bowel disease 19 healthy controls	No	$CD4^+$ and $CD8^+$ *ex vivo*	NIA immunoarray	NA	ANOVA and t-test Tukey-Kramer Gene Cluster Genespring	*CYFIP2* is increased in $CD4^+$ cells in MS and is involved in T-cell adhesion	[43]
33 responders 19 poor responders	IFN-β	PBMC *ex vivo*	RT-PCR	70	Quadratic discriminant analysis-based IBIS	9 sets of gene triplets have predictive value for response to IFN-β	[44]
65 RRMS 7 SPMS 22 healthy controls	No	T cells and non-T cells: *ex vivo* and *in vitro*	cDNA array 'custom-printed' glass slide	1258	Cyber-T IBIS	173 DEGs in T cells 50 DEGs in non-T cells Apoptosis related genes regulated	[45]
10 RRMS 8 PPMS 12 healthy controls	No	PBMC *ex vivo*	Oligonucleotide array, U95Av2 Affymetrix	12000	Mann-Whitney U-test	16 DEGs in RRMS vs HC 1 DEG in PPMS vs HC CX_3CR1 downregulated NK cells	[46]
65 RRMS 7 SPMS 22 healthy controls	IFN-β	T cells *ex vivo*	cDNA array 'custom-printed' glass slide	1258	Pierre of the "R"-statistical package	286 DEGs in T cells (untreated MS vs HC) 4 MS-subclusters, 5 geneclusters IFN-β responders and nonresponders have different gene expression patterns	[47]

ANOVA: ANalysis Of VAriance; CIS: Clinically isolated syndrome; DEG: Differentially expressed gene; EAE: Experimental autoimmune encephalomyelitis; EST: Expressed sequence tag; F: Female; GA: Glatiramer acetate; HC: Healthy controls ; Jak: Janus kinase; IBIS: Integrated Bayesian inference system; IFN: Interferon; IL: Interleukin; INR: International normalized ratio; LOOCV: Leave-One-Out-Cross-Validation; M: Male; MMP: Matrix metalloproteinase; MS: Multiple sclerosis; NA: Not available; NK: Natural killer; RR: Relapsing–remitting; PBMC: Peripheral blood mononuclear cells; RT: Reverse transcription; SAS: Statistical Analysis Software; SLE: Systemic lupus erythematosus; SP: Secondary progressive; SPSS: Statistical product and service solution; STAT: Signal transducers and activators of transcription; Th: T helper; TNoM: Threshold number of misclassification.

which functions as a costimulatory molecule during T-cell activation, the T cell receptor α locus and its ζ-chain associated protein kinase (*ZAP70*), and the zinc finger protein *(ZNF)148*, which is known to be involved in the activation of transcription of TCR genes. Interestingly, the IL-7 receptor (*IL-7R*), which is required for B- and T-cell development, was also strongly upregulated. Similar findings were reported in another microarray study on PBMCs of MS patients [33]. Downregulated genes in MS included *HSP70* and *CKS2*, which are both implicated in the regulation of apoptosis. HSP70 has been previously suggested to be an autoantigen in MS [50], but it may also be involved in the mRNA degradation in the ubiquitin–proteasome pathway [51]. Activation of extracellular matrix-remodeling processes was evident from upregulation of matrix metalloproteinase (*MMP*)*-19* and downregulation of a tisue inhibitor of metalloproteinase (*TIMP1*) 1 [35].

Gene signatures for MS and disease pathophysiology have been defined in several studies [40–42]. Iglesias and colleagues identified a set of 553 differentially expressed genes in RRMS compared with healthy controls, 87 of which were highly discriminated [40]. Among the differentially expressed genes (DEGs), a signature of enhanced immune-cell activation and costimulation in MS could be defined. These included several interferon-responsive genes, such as the Th1 cytokine *IL-12*, *CD40*, cytotoxic T-lymphocyte antigen 4 (*CTLA4*), chemokines, T-cell receptors, immunoglobulins, *IL-6 receptor*, *IL-8 receptor*, and adhesion molecule genes and integrins, such as *VLA4* and *VLA6*. Interestingly, the activation of the E2F pathway was evident from upregulation of several pathway-related genes, (i.e., *E2F2*, *E2F3*, *CDC25A*, *CDK2*), thymopoietin (*TMPO*), B-cell leukemia/ lymphoma (*BCL*) and DNA primase (*PRIM1*). The importance of the E2F pathway in MS was validated in EAE. E2F1-deficient mice manifested only a mild disease course of EAE. A study by Iglesias and colleagues supports the role of the microarray approach as a tool to define gene signatures for MS and altered biological pathways, which might also lead to better understanding of pathophysiology of the disease and thus new treatment approaches.

Gene signatures for MS disease activity have also been described. Achiron and colleagues identified a signature of 1109 genes in PBMCs from 26 MS patients compared to healthy volunteers, irrespective of disease activation state [41]. The signature was validated with the 'leave-one-out-cross validation' (LOOCV) method [52], which yielded only two classification errors, proving that the patterns observed represent a true biological phenomenon. These included genes involved in T-cell expansion and activation, inflammatory stimuli (cytokines and integrins), epitope spreading, and apoptosis. Comparison of expression profiles in PBMCs from MS patients in relapse and remission revealed a signature of 721 genes. Lysosomal cystein protease L, cathepsin L (*CTSL*), which has a regulatory function on epitope spreading, and monocyte-specific chemoattractant proteins *MCP1* and *MCP2*, were up-regulated during relapse. The expression of several mitogen-activated protein kinases (MAPKs), which are involved in several immune responses, was also increased. By contrast, several apoptosis-related genes (e.g., cyclin G1 and caspases [*CASP*] 2, 8 and 10) were downregulated.

A specific gene signature for 'autoimmune disease', including MS and systemic lupus erythematosus (SLE), has been reported [42]. Expression profiles of PBMCs from 13 RRMS, five SLE patients and 18 age- and gender-matched healthy volunteers were compared. A signature of 541 genes was identified for both diseases (MS/SLE) compared with controls. The autoimmune signature included genes that are related to the apoptosis pathway, such as TNF receptor-associated factor 5 (*TRAF5*), *CASP8*, *BCL2*, immediate early response (*IER*)3 and IL-1β (*IL1B*), and genes that are involved in stimulation of inflammation, proliferation and immune response (e.g., C-terminal binding protein [*CTBP*]1, IL-11 receptor α (*IL-11RA*), vascular endothelial growth factor (*VEGF*), B-cell-translocation gene 1 and 2 (*BTG1/2*), amphiregulin (*AREG*) and *CD19*. Interestingly, the most prominent cluster in this 'autoimmunity signature' contained several genes associated with the MMP pathway, (e.g., *TIMP*), being consistent with the report by Bomprezzi and colleagues [35].

The same cohorts were used to identify MS- and SLE-specific signatures of 1031 and 1146 genes, respectively. The main characteristics of the MS signature was downregulation of cell death-related genes, (e.g. nuclear factor-κ B1 [*NFKB1*], baculoviral IAP repeat- containing 2 and 3 [*BIRC2/3*], *HSPA1A*, *HSPA5* and *HSPA1B*), and signal transduction-related genes (e.g., *IL-8*, *GRO3* [cytokine] and guanine nucleotide binding protein α 15 [*GNA15*]). Conversely, inflammation genes, such as *CD24*, *IL15*, *defensin a3* (*DEFA3*), *nuclear factor of activated T cell* (*NFATC*)*3* and *PTGS2*, and adhesion molecules, such as integrins and LY75, were upregulated. The SLE expression pattern included mainly upregulated genes associated with inflammation, such as *IFI16*, *BAT1* and DNA damage/repair-inducible molecules (e.g., *POLS*, *MBD4*, *ERCC2* and *MSH3*. In addition, genes related to negative regulation of proliferation (e.g., *DDX17*) and apoptosis (e.g., *TIAL1*) were induced. *NXP2*, antinuclear matrix protein antigen, *TOPBP1*, DNA topoisomerase I antigen and *IFI16*, interferon-inducible antigen were upregulated, which are targets for development of autoantibodies, and are connected to SLE pathogenesis.

Gene expression profiling of PBMCs and specific cell subpopulations has been used to study the effect of IFN-β in MS patients (TABLE 2), and has provided evidence that the biological mechanism of IFN-β is more complex than the postulated shift of proinflammatory T helper (Th)1 cells to anti-inflammatory Th2 phenotypes. Wandinger and colleagues reported the first microarray study regarding the effect of IFN on the expression profile of PBMC from one MS patient and two healthy controls [34]. Although the number of subjects was small, this study revealed some interesting findings. As expected, several IFN-inducible genes (e.g., *1–8U*, *1–8D*, oligo A synthetase [*OAS*] and myxovirus resistance 1 [*MxA*]) were upregulated. Gene expression of Th1-markers, *IL12Rβ2* and *CCR5*, was significantly upregulated *in vitro*, which was

also confirmed *in vivo* in six patients treated with IFN-β for a period of up to 6 months. Interestingly, an anti-inflammatory molecule (*IL-10*) was also upregulated. Stürzebecher and colleagues studied the effect of IFN-β on gene expression in PBMCs from ten RRMS patients (six responders, two nonresponders and two initial nonresponders) [36]. In total, 25 and 87 IFN-regulated genes in *ex vivo* and *in vitro*, respectively, were found. The cytokines *IL-8* and fms-like tyrosine (flt) kinase-3, (costimulatory cytokine for hematopoietic progenitors) were significantly downregulated both *ex vivo* and *in vitro*, which correlated with responder/nonresponder status of the patients. Four pro-apoptosis related genes, namely *BCL2*/adenovirus *E1B* 19kDa interacting protein (*BNIP*)3, TNF-related apoptosis-inducing ligand (*TRAIL*), immediate early gene, apoptosis inhibitor (*IEX-IL*) and transforming growth factor β stimulated clone 22-related gene (*TSC22-R*), were regulated *ex vivo* in responders.

Weinstock-Guttman and colleagues studied the dynamics of the gene expression cascade induced by an IFN-β treatment in eight RRMS patients [38]. As expected, antiviral response genes (e.g., double-stranded RNA-dependent protein kinase, myxovirus resistance proteins 1 and 2, and guanylate-binding proteins 1 and 2) were rapidly induced within 1–4 h of intramuscular administration of IFN-β. These transcriptional changes are faster than changes in the protein markers for IFN-β response, such as neopterin, β_2-microglobulin and MxA protein, which have been used previously. Changes in gene expression in the Jak-Stat pathway, the main intracellular pathway transmitting actions of IFN-β, also occured early. IFN receptors 1 and 2, *Jak1* and *Tyk1* (kinases that phosphorylate IFN receptors and stat 1 and 2) and *p48*, which binds to receptor-heterodimer and is needed to constitute (IFN-stimulated transcription factor [*ISGF*]3) were all upregulated within 1.7–4.4 h.

The same expression data set has been used for evaluation of the various filtering approaches and statistical analysis [53]. Parametric, semi- and nonparametric filtering methods were compared. The analysis of variation with bootstrapping, class dispersion and Pareto with permutation methods was applied. Each method was differentially sensitive to specific variability in the gene expression data. This powerful statistical analysis revealed three clusters of genes, whose regulations were interdependent. The importance of the information for a better understanding of a therapeutic measure of IFN-β needs further evaluation.

Several studies have attempted to define gene signatures and sets of altered genes for IFN response, rather than identifying single genes as an indicator of treatment effect. Hong and colleagues characterized a novel gene array-based profiling tool to define biomarkers for monitoring treatment efficacy [39]. They selected 34 genes, which are known to be involved in inflammation and are important in the regulation of current MS treatments (IFN-β and glatiramer acetate). The array was evaluated with PBMC samples of 30 RRMS patients, who had been treated for 2–7 years either with IFN-β (n = 18) or glatiramer acetate (n = 12). A total of 15 untreated RRMS patients served as controls. IFN-β and glatiramer acetate had distinct effects on expression profiles of selected genes. In particular an opposite outcome was seen in the expression of *MMP9*, *Fas*, *IL-1b* and *TNF-α*, while a synergistic effect on *IP-10* and *CCR5*, was found. The importance of the tool for identification of neutralizing antibody-positive patients (NAb^+) was evaluated by expression profiles of known IFN-inducible genes. NAbs exhibited a blocking effect on some, but not all genes regulated by IFN-β. IFN-β activates complex signaling pathways; therefore, more detailed studies of NAb effects on various target genes are needed.

By applying advanced data-mining and predictive computational modeling tools, Baranzini and colleagues identified nine sets of gene triplets, whose expression, when tested before the initiation of the treatment, could predict the response to IFN-β in RRMS patients [44]. The data set was derived from expression studies of 70 genes in PBMC samples from 52 MS patients by a quantitative real-time RT-PCR technique. Sample classification was performed by analysing all 54,740 possible three-gene combinations of 70 genes. The most discriminant three-gene sets were '*caspase 2, caspase 10, FLIP*' and '*caspase 2*, *caspase 3*, *IRF4*' and '*IL4Ra*, *MAP3KI*, one apoptosis molecule'. With the same approach, gene triplets were defined for IFN-β response. Interestingly, the most discriminant genes for the poor responders included apoptosis-related genes. Unfortunately, NAb status was not determined in these studies. However, the combined large-scale expression analysis and advanced data mining may be able to identify a set of markers that can predict the treatment response of IFN-β.

Satoh and colleagues used microarray-analysis for specific subpopulations of blood cells, such as $CD3^+$ T cells [45]. Orphan nuclear receptor Nurr1 (*NR4A2*), receptor-interacting serine/threonine kinase (*RIPK*)2 and silencer of death domains (*SODD*) were upregulated, while *TRAIL*, *BCL2* and death-associated protein 6 (*DAXX*) were downregulated, which suggests a counterbalance between promoting and preventing apoptosis. The same cohorts were used to study the effect of IFN-β on gene expression [47]. Based on 286 (DEGs) in T cells, four patient subgroups and five gene clusters were identified. However, only a slight association between the patients exhibiting the most active disease course (measured by frequency of relapses, no of lesions on T2-weighted MRI and EDSS score before IFN-β treatment) with the gene cluster including chemokines, cytokines, and growth factors and their receptors was found. After 2 years of treatment with IFN-β, the responders were clustered in two of four-patient groups.

Satoh and colleagues recently studied the effect of IFN-β on gene expression profiles *in vitro* in PBMCs from two healthy volunteers and one RRMS patient [54]. Interestingly, IFN-β induced immediately, within 3–24 h, a set of genes, including expected conventional IFN-response markers, IFN-signaling genes, chemokines and cytokines. A surprising finding was the upregulation of several pro-inflammatory genes. The novel finding was the upregulation of expression of *CXCR3* and *CCR2* ligand chemokines. The importance of the early

response of pro-inflammatory chemokines and cytokines to IFN-β and their clinical relevance for early adverse effects in MS patients requires further investigation.

An individual gene (i.e., chemokine receptor [*CX_3CR1*]) revealed from large-scale expression analysis in subpopulations of T cells, has been identified and proposed as a marker for disease activity [46]. *CX_3CR1* was shown to be downregulated in RRMS and PPMS patients compared with healthy volunteers. The finding was confirmed by real-time RT-PCR and a flow cytometric analysis in independent cohorts. Natural killer (NK) cells were found to be responsible of the phenotype, while the expression of CX_3CR1 was not altered in cytotoxic $CD8^+$ cells in MS patients compared with controls. Another example of single gene findings in microarray analysis is the description of upregulation of the cytoplasmic binding protein of fragile X protein (*CYFIP2*) in $CD4^+$ cells from RR MS patients [43]. Although the exact mechanism of action of CYFIP2 is still not established, adenoviral-mediated overexpression and down-regulation with an antisense oligonucleotide approach in Jurkat cells, suggest that CYFIP2 facilitates T-cell adhesion. Therefore, inhibition of *CYFPI2* gene expression may provide a new treatment target.

Expert commentary

The application of novel and 'state-of-the-art' technologies (i.e., large-scale expression profiling microarrays) on brain autopsy specimen and PBMC from MS patients has provided some novel insights into the molecular mechanisms involved in the pathogenesis and pathophysiology of MS. The same technologies are also starting to reveal better understanding of the mode of action of current therapies.

The limitations of the published studies are the small number of individuals, and the heterogeneity of subjects included, target cells studied, platform used and analytical/statistical approaches applied. Therefore, the comparison between various studies is also very difficult. Suboptimal standardization of sampling procedures (e.g., diurnal variation, caloric intake, and hormonal status of the subjects and sample processing) has a significant impact for the noise of the data generated and thus resulting false-positive findings. The major problem in gene expression profiling studies on peripheral blood is the choice of a target cell population. The RNA from whole blood, including all types of cells, and specific cell subpopulations, has been used for expression studies. The advantage of the whole blood approach is that the expression profiles reflect the actual time point of blood drawing due to the added stabilization compound in the blood collection tube, which prevents the degradation of RNA and stops the transcription. However, the impact of specific cell types on transcriptional changes cannot be determined. Conversely, during the separation of cell subpopulations *ex vivo*, RNA is prone to degradation and transcriptional changes, which makes the interpretation of the results difficult. Transcriptional profiling yields hundreds of thousands data points; therefore, sophisticated data analysis tools and bioinformatics are needed to fully explore the information. Bioinformatics uses techniques developed in computer science and statistics to facilitate the understanding of how the expression profiles generated are related to the biological systems being studied.

Transcriptional changes do not always reflect alterations at protein and small molecule metabolite levels; therefore, functional assays are needed to validate biological consequences of dysregulated gene expression profiles. A 'multiplex approach' combining transcriptomics with protein expression and metabolite profiles will provide more comprehensive views of altered biological processes and increase our understanding of pathophysiology of MS and, thus, provide a basis for the development of novel therapeutic strategies.

The elucidation of important gene-expression patterns during disease allows for identification of genetic susceptibility markers, biomarkers of disease progression and new therapeutic targets. Microarray studies of MS have provided candidate genes as markers for disease course and treatment response in MS. Not only single genes, but a set of 'tens' of genes, have been proposed as diagnostic or prognostic tools for MS. However, none of the suggested tools have been validated with large independent cohorts [55]. Confirmation of findings in large numbers of subjects with MS and other neurological, noninflammatory and inflammatory diseases is a requisite of establishment of diagnostic and prognostic tools.

Five-year view

The development of genomic microarrays has allowed the rapid accumulation of new information on gene expression in many diseases, including MS. High-density microarrays have a great

Key issues

- Multiple sclerosis (MS) is an immune-mediated demyelinating and neurodegenerative disease of the CNS.
- Since immune dysregulation is a key event in the disease course, it is obvious that current immunomodulatory therapies (e.g., Interferon [IFN]-β, glatiramer acetate and natalizumab) are effective in decreasing relapse rates. However, they are less effective in preventing disease progression.
- Large-scale expression studies on brain tissue and peripheral blood mononuclear cells of MS patients have provided novel insights into the pathogenesis and pathophysiological processes in MS. Recent findings favor the neuroprotection and repair-promoting approaches as promising new treatment strategies.
- Reliable diagnostic, predictive and prognostic markers for MS and its course are needed. Indicators to identify responders/nonresponders to current treatments are necessary for better management of the disease.

potential for a better understanding of disease pathogenesis and identification of biomarkers for diagnosis and prognosis of disease course. However, their application as clinical 'bedside' diagnostic tools is difficult owing to the high costs and the requirement for special instrumentations. On the contrary, PCR-based 'low-density arrays', analyzing a limited number of genes in one assay, have prospects to be established as a rapid test for prognosis and disease course evaluation of MS and for a treatment response. A small amount of RNA required, short analysis time (1–2 h) and low costs makes the technology ideal for routine clinical applications, which can be performed in any analytical laboratory. The PCR-based low-density arrays have been used in cancer diagnostics and prediction of treatment response [56]. This area of research and technology development will increase dramatically during the next few years. Patients are likely to benefit from this research activity as it may lead to more rapid and definitive testing of clinical specimen and, eventually, improved disease management by personalized treatments.

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