

Multiple sclerosis cerebrospinal fluid biomarkers

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Abstract. Cerebrospinal fluid (CSF) is the body fluid closest to the pathology of multiple sclerosis (MS). For many candidate biomarkers CSF is the only fluid that can be investigated. Several factors need to be standardized when sampling CSF for biomarker research: time/volume of CSF collection, sample processing/storage, and the temporal relationship of sampling to clinical or MRI markers of disease activity. Assays used for biomarker detection must be validated so as to optimize the power of the studies. A formal method for establishing whether or not a particular biomarker can be used as a surrogate end-point needs to be adopted. This process is similar to that used in clinical trials, where the reporting of studies has to be done in a standardized way with sufficient detail to permit a critical review of the study and to enable others to reproduce the study design. A commitment must be made to report negative studies so as to prevent publication bias. Pre-defined consensus criteria need to be developed for MS-related prognostic biomarkers. Currently no candidate biomarker is suitable as a surrogate end-point. Bulk biomarkers of the neurodegenerative process such as glial fibrillary acidic protein (GFAP) and neurofilaments (NF) have advantages over intermittent inflammatory markers.

Keywords: Multiple sclerosis, interferon beta, glatiramer acetate neutralizing antibodies, immune tolerance

1. Introduction

Despite extensive literature on CSF biomarkers in multiple sclerosis (MS) only qualitative and quantitative methods for determining the intrathecal production of immunoglobulins are used as an aid to the clinical diagnosis of MS [1]. Positive studies of other potential MS biomarkers are seldom reproducible, mainly due to methodological problems. Unfortunately, the majority of MS CSF biomarker studies are not performed and reported in a standardized way and published results lack sufficient detail to allow a critical review of the study and reproduction of the study design. A similar problem in the field of oncology with the reporting of tumor markers has prompted the formulation of a set of guidelines to address this issue and which are directly applicable to the field of MS. The aim of this pa-

per is to review the principles underpinning MS-related CSF biomarker studies and to recommend some guidelines [2].

2. Definitions

A **biomarker** or biological marker is “a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention [3]”.

A **clinical end-point** is “a characteristic or variable that reflects how a patient feels, functions or survives [3]”.

A **surrogate end-point** is “a biomarker intended to substitute for a clinical end-point. A surrogate end-point is expected to predict clinical benefit (or harm, or lack of benefit or harm) based on epidemiologic, therapeutic, pathophysiologic or other scientific evidence” [3].

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3. Why study CSF biomarkers in MS?

MS is believed to be a complex disease with no definitive cause having been identified as yet [4]. By definition it is a pre-theoretical disease [5], i.e. it is polythetic (requires more than one criterion to be fulfilled). The latest version of these criteria [6], requires the synthesis of intrathecal oligoclonal IgG bands detected by established methods (preferably isoelectric focusing) [7] or a raised IgG index [8] to help support the pathological specificity of MRI changes in attack-onset MS or as the pivotal criterion in making a diagnosis of primary-progressive MS. Intrathecal oligoclonal bands have similar positive and negative predictive values with regard to the future risk of developing MS as does an abnormal baseline MRI in subjects presenting with a clinically isolated syndrome compatible with demyelination [9].

MS lesions are rarely biopsied. Therefore, CSF is the closest one can get to the pathology of MS and is therefore often referred to as being a “*liquid biopsy*”. Changes in the cellular and biochemical constituents of CSF are used to (1) test hypotheses concerning the pathogenesis of MS, (2) to study disease heterogeneity, (3) to search for potential aetiological agents, (4) to monitor disease processes with the aim of identifying potential prognostic factors and (5) to identify surrogate end-points to monitor the effects of therapeutic interventions.

4. MS Pathogenesis and key pathological processes

Current dogma states that MS is an organ-specific autoimmune disease mediated by antigen-specific autoreactive T-cells [10]. The evidence for this is indirect and is largely based on experiments in experimental allergic encephalomyelitis (EAE). Others believe that MS may be caused by an as yet unidentified infectious agent [11]. These polarized views are not mutually exclusive and it is conceivable that an infectious agent could trigger or drive an organ-specific autoimmune reaction. Despite uncertainties about the pathogenesis the underlying pathological processes are relatively well defined (Fig. 1) and are key targets for the evaluation of appropriate biomarkers.

5. Variables to considered when studying biomarkers in the CSF

5.1. Assay

Before interpreting the results of any biomarker study the performance of the assay has to be scrutinized. This variability of the assay is particularly important as it impacts directly on the power of the study to detect meaningful differences between comparator groups [12]. The greater the variability of the assay the larger the number of subjects required to detect significant differences. As a general rule any assays with inter- and intra-coefficients of variation of greater than 10% are unacceptable.

5.2. Bulk vs. intermittent markers

A bulk biomarker is released in proportion to the volume of tissue it represents, e.g. plasma creatinine is proportional to muscle mass. Intermittent biomarkers are only released as part of a defined physiological or pathological process. In MS most inflammatory markers and putative markers of tissue destruction and repair are released intermittently. To measure and quantify these specific processes over time the biomarkers have to be measured serially to allow an area under the curve analysis. However a bulk biomarker needs to be measured less frequently since it acts as a natural integrator. A simple analogy is random blood glucose vs. haemoglobin (HbA1c): the former is a intermittent marker of short-term (minutes) glucose homeostasis, whereas the latter is a bulk marker of glucose homeostasis over the preceding months [13]. Bulk markers are simpler and easier to use, particularly when repeated invasive procedures like a lumbar puncture are difficult to justify.

5.3. Anatomical constraints

When considering the significance of CSF biomarkers the anatomical and physiological relationships between the CNS and the systemic compartments have to be considered. The majority of total CSF (70%) is formed from the choroid plexus and the remainder from the interstitium and meninges [14]. The relative contribution from each anatomical structure to the CSF varies depending on the anatomical location from which CSF is sampled. For example, ventricular CSF is produced almost entirely by the choroid plexus, with only a small contribution from the interstitial fluid derived from the

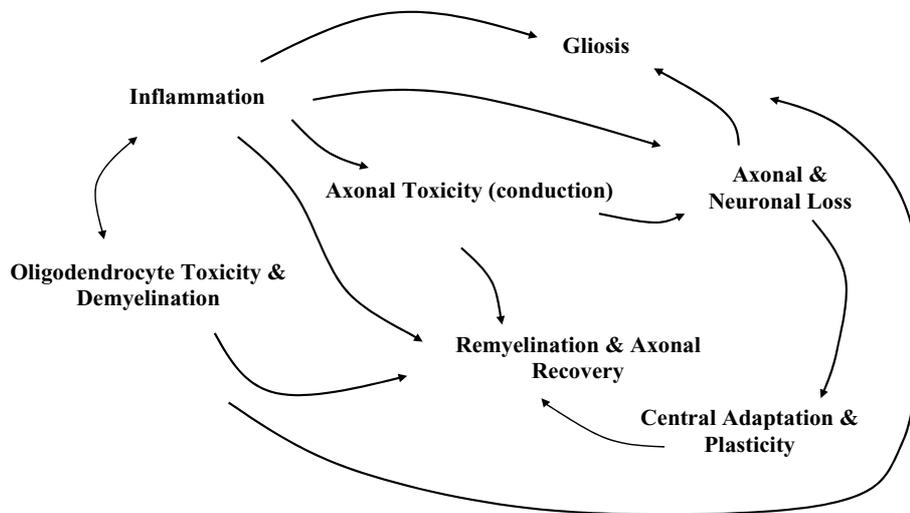


Fig. 1. Key pathological processes in multiple sclerosis.

adjacent paraventricular areas and none at all from the meninges. Conversely, a significant proportion of lumbar CSF is derived from the dorsal roots. The production rate of choroid plexus CSF is not constant, having a large diurnal variation. The maximum production rate of ~ 40 mL/hour occurs at ~ 0 h00 and falls to as low 5 mL/hour at ~ 12 h00 [15]. Levels of CNS-derived proteins in the CSF are likely to be higher during times of low CSF production and lower during periods of high CSF production. Anatomical factors in relation to the CSF flow pathways need to be considered when evaluating CSF analytes. The lumbar sac, the site most commonly sampled, is a *cul-de-sac*. Although mixing of lumbar with ventricular CSF occurs it is not constant and is affected by posture, levels of physical activity and changes in intrathoracic and intra-abdominal pressure. A CSF rostral-caudal gradient exists for most brain-derived proteins and metabolites [16]. This is particularly important in children and when interpreting CSF neurotransmitter levels [17]. Pathological processes occurring on the surface of the brain stem and brain, distal to the outflow pathway of the fourth ventricle, are less likely to be detected in lumbar CSF. For example, MBP levels in the lumbar CSF tend to be normal in acute optic neuritis, a demyelinating condition that one would expect to be associated with raised MBP levels. CSF tumor markers are less likely to be raised in deep parenchymal lesions compared to superficial lesions close to CSF [18]. The closer the lesions are to the CSF pathways the more significant the changes are likely to be; acute paraventricular and superficial spinal cord lesions are more likely to result in changes in lum-

bar CSF. In MS patients with single active lesions, soluble VCAM-1 levels in the CSF were inversely related to distance of the lesion from the ventricular surface [19].

5.4. Lesion clustering and temporal relationship of sampling to relapse

The majority of MS disease activity detected using frequent MRI is clinically asymptomatic. Whether a specific lesion causes symptoms mainly depends on the site involved. Lesions in eloquent sites such as the optic nerves, brainstem and spinal cord are more likely to be symptomatic, compared with lesions in the paraventricular and deep white matter. The size of the lesion, whether it involves a previously compromised pathway, and the qualitative nature of the inflammatory reaction are other, less well-defined, factors that may determine whether or not a lesion causes symptoms. Clinical attacks or relapses are associated with Gd-enhanced MRI activity and indicate at any one time that the MS disease process is more likely to be active. Therefore sampling CSF in close temporal proximity to a relapse increases the chances of detecting raised markers of inflammatory disease activity. This does not mean to say that when sampling CSF in subjects who have not had a relapse that inflammatory markers are unlikely to be raised; the chances are just lower.

5.5. Frequency of sampling

Trends over time and area under the curve analyses, which are often required to define the significance

of intermittent changes in specific biomarkers, require multiple measures. The more frequent the sampling the more robust the analysis. Sampling that is too infrequent will not capture all the information. In comparison, single or two time-points are all that is necessary when assessing bulk biomarkers (see above).

5.6. Duration of follow-up and selection of clinical comparator

When evaluating whether or not a biomarker can be used as a surrogate end-point or a substitute for a clinical end-point, the duration of follow-up and the nature of the “comparator” clinical end-point are vital. One could argue that the poor correlation between MS-related biomarkers and clinical end-points is due to a combination of a too short a period of follow-up and the poor performance of the expanded disability status scale (EDSS) the most commonly used clinical end-point. The EDSS is an imperfect tool and its failings have been highlighted [20]; at the lower end of the scale EDSS is an impairment scale and with disease progression the EDSS functions more as a disability scale or ambulation index. The EDSS is non-linear, poorly reactive, has ceiling and floor effects and is weakened by relatively large inter- and intra-rater variability. In my opinion as long as the MS biomarker field continues to use the EDSS as the clinical “gold-standard” we will not find an appropriate surrogate end-point to predict clinical course in the short- to mid-term. With regard to MRI biomarkers the correlation between early disease activity and the development of disability using the EDSS took 14 years to emerge [21]. To achieve correlations in which at least 40% of the variance (correlation coefficient or r -value of ≥ 0.63) of the independent variable (disability) can be explained by the dependent variable (biomarker), a long term follow-up of an impractical order of magnitude would be required.

6. Inflammatory markers

MS-related inflammatory biomarkers have been reviewed [22]; a detailed review of the literature is beyond the scope of this manuscript. The following are some general points that can be made in relation to MS-related inflammatory biomarkers:

1. Studies that have recruited subjects in relation to a clinical attack are more likely to be positive compared to studies recruiting subjects with no recent history of clinical activity [9].

2. There are both quantitative and qualitative differences between subjects with MS and controls subjects, and between the different clinical subtypes of MS.
3. When compared to controls and subjects with non-inflammatory diseases of the CNS, subjects with MS clearly have evidence of intermittent inflammatory activity within the CNS and periphery [23,24].
4. Inflammatory biomarkers correlate with MR markers of disease activity [9].
5. Some investigators have described differences between subjects with relapsing-remitting MS and secondary progressive MS [25] and between relapse-onset disease and primary progressive MS [26].

Despite these broad generalisations, it is difficult to draw any firm conclusions from the published literature on MS-related inflammatory biomarkers due to the reasons discussed above; most of the studies on which these conclusions are based are small, non-standardized and have not been reproduced. In summary, there are no current inflammatory CSF biomarkers that have been shown to predict disease progression reliably, nor have there been any studies with a sufficiently long period of follow-up.

7. Neurodegenerative and neuro-restorative CSF markers

CSF biomarkers of neurodegeneration and neuro-restoration make no assumptions about the pathogenesis of MS; they focus on intrinsic neurobiological processes that are not necessarily unique to MS. They offer one opportunity to monitor pathological processes linked to disease course.

7.1. Demyelination

Myelin basic protein (MBP) is a unique protein, found in the inner myelin layer. During demyelination, MBP and/or its fragments are released into the CSF and can be used as an index of active demyelination [27]. During clinical attacks CSF MBP levels are raised in $\sim 80\%$ of subjects. In comparison they are only raised in $\sim 40\%$ of subjects with non-relapsing progressive disease and in a minority of subjects with clinically-stable disease. CSF levels remain raised for a period of 5–6 weeks after the onset of a clinical attack. Raised CSF MBP levels are associated with MRI activity and

are reduced by corticosteroid therapy. Levels of CSF MBP correlate weakly with clinical disability and are associated with other markers of intrathecal inflammation [28]. MBP levels in the lumbar CSF are rarely raised in acute optic neuritis, presumably because the pathology is too distal to the CSF outflow path of the fourth ventricle [29].

7.2. Neuroaxonal loss

Although the cytosolic enzymes creatine kinase (CK) and neurone-specific enolase (NSE) are used as biomarkers in acute neuro-destructive disorders [30–32], levels are usually normal in subjects with MS [33–35].

Neurofilaments (NF) are structural neuron-specific proteins composed of three polypeptides: NF-light chain (NF-L), NF-medium chain (NF-M) and NF-heavy chain (NF-H). In a longitudinal 2-year study on 60 patients with relapsing-remitting MS, 78% of patients had raised concentrations of CSF NF-L [36], which correlated weakly with EDSS ($r = 0.27–0.34$) and the relapse rate over the study period ($r = 0.38$) and in the preceding 2 years ($r = 0.56$). CSF NF-L levels were particularly high soon after the onset of an attack and decreased with time [36]. CSF concentrations of actin, tubulin and NF-L are increased in MS, particularly in subjects with non-relapsing progressive MS compared to subjects with relapsing-remitting disease [37]. CSF concentrations of actin, tubulin and NF-L correlated with disability [37]. In another study a higher proportion of subjects with non-relapsing progressive MS (secondary and primary progressive MS) had an increase in CSF NF-H levels between baseline and 3-year follow-up compared with subjects with relapsing-remitting disease [38]. CSF NF-H levels were higher at baseline compared to subjects with non-relapsing progressive disease. Baseline CSF NF-H correlated with the EDSS, ambulation index (AI) and 9-hole peg test (9HPT) at the 3-year follow-up [38]. An increase in CSF markers of axonal damage in progressive MS and the correlation of these markers with clinical outcome suggests that cumulative axonal loss is responsible for sustained disability in MS, and that these axonal markers may be suitable biomarkers.

Anti-NF IgG and IgM antibodies are found in both the serum and CSF of subjects with MS [39]. An anti-NF-L index correlated with brain parenchymal fraction, T2 and T2 lesion loads, and MRI markers of tissue damage in a cohort of subjects with MS [40]. In another study serum anti-NF-L IgG antibodies were sig-

nificantly elevated in subjects with primary progressive MS [41]. This indicates that anti-NF-L antibodies may serve as a marker of tissue damage in MS.

Tau or microtubule-associated phosphoprotein is found predominantly within axons. Tau promotes the assembly and stabilization of microtubules [42,43]. A number of neurological diseases or tauopathies are associated with abnormal tau metabolism. A raised CSF tau level is non-specific and has been described in many neurological diseases. In a small study of 35 subjects with MS and 28 control subjects, tau levels were increased in MS compared to controls and were higher in subjects with progressive disease [44]. This has not been confirmed in other studies [45,46].

14-3-3 is a highly conserved protein that is not brain-specific and is present in most mammalian tissues. It exists mainly as a soluble cytoplasmic protein with small amounts bound to synaptic membranes. Different isoforms of 14-3-3 are associated with different neuron types and/or membrane compartments. 14-3-3 exists in different phosphorylated states and has several functions [47,48]. Raised CSF 14-3-3 levels are useful diagnostically in Creutzfeldt-Jacob Disease. In one study, 5 out of 38 (13%) subjects with clinically isolated syndrome had detectable 14-3-3 protein in the CSF; the presence of 14-3-3 in the CSF was an independent predictor for a shorter time to conversion to definite MS (RR = 4.1; 95% CI 1.1 to 15) and to reach an EDSS ≥ 2 at the end of follow-up (OR 14.8; 95% CI 2.86 to 76.8) [49]. This was not confirmed by another group [50]. A separate group detected 14-3-3 in the CSF in 38% subjects with either clinically isolated syndrome or MS; in this study the presence of CSF 14-3-3 correlated with disease severity [48]. The latter has been confirmed in a larger cohort of subjects [51]. 14-3-3 deserves to be investigated more thoroughly, preferably using a quantitative assay [52].

7.3. Markers of astrocyte activation and gliosis

S-100b is an acidic calcium binding protein located in the cytoplasm of astrocytes and Schwann cells. Raised CSF S-100b is found in all conditions associated with astrocytosis or gliosis. CSF and serum levels of S-100b are raised in a proportion of subjects with MS, particularly during clinical relapse [34,53, 54]. Raised CSF levels are found from day 5 after the onset of the attack and reach a maximum after a period of 2–3 weeks [54]. CSF S-100b levels are raised in a greater proportion of chronic progressive than relapsing-remitting patients [54]. Levels of CSF

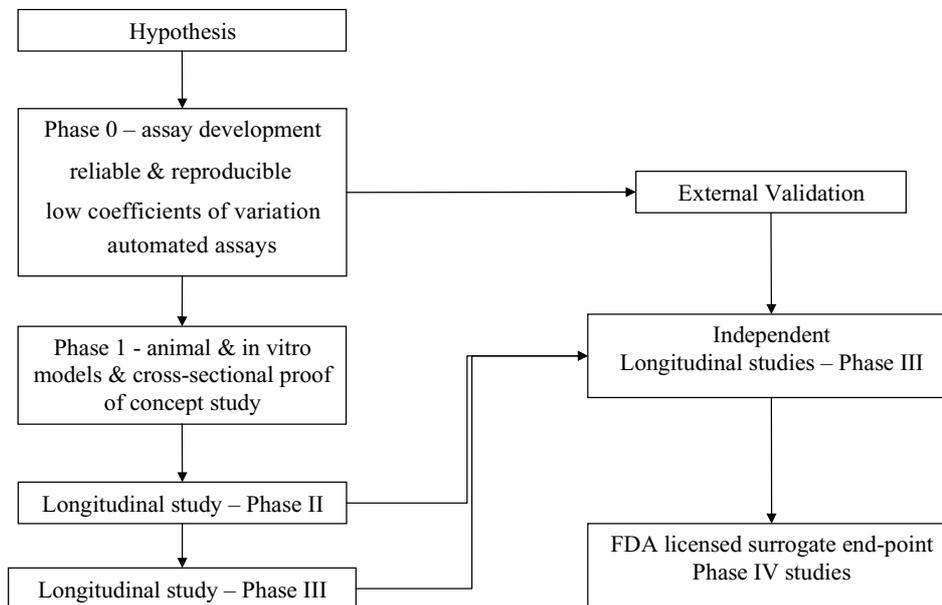


Fig. 2. Proposed methodological process for investigation of prognostic biomarkers in multiple sclerosis.

Table 1
Reasons for studying CSF Biomarkers in MS

I.	Diagnostic testing
	– Positive & negative predictive testing
II.	Pathogenesis
	– Immunology
	– Aetiology
	– Disease progression & recovery
	– Disease heterogeneity
III.	Monitor disease processes (surrogate end-points)
	– Prognosis (high vs. low risk patients)
	– Monitoring effect of therapeutic interventions

S100B are higher in subjects with primary progressive compared to subjects with secondary progressive or relapsing-remitting disease [55].

Glial fibrillary acidic protein (GFAP) is the major structural protein of the glial intermediate filament of astrocytes and its level in CSF increases in association with astrocytosis. GFAP was first isolated from chronic MS plaques where there is a high concentration of fibrous astrocytes [56]. CSF concentrations of GFAP are increased in a varying proportion (9–39%) of patients with MS [57]. A longitudinal study which measured CSF GFAP concentrations in 13 patients with relapsing-remitting MS found that CSF levels are raised compared to controls and that levels increased over the study period of 24 months from a baseline [58]. The latter increase correlated strongly with the increase in clinical deficit scores and was not associated with clinical relapse [58]. Subjects with MS with severe disability have significantly higher CSF GFAP levels than

less-disabled subjects, with a moderate correlation between CSF GFAP levels and ambulation in subjects with secondary progressive MS [55]. These findings imply that CSF GFAP levels may be used as a bulk marker of astrocytosis but this clearly needs to be confirmed.

7.4. Membrane markers of membrane or myelin turnover

24S-hydroxycholesterol is a CNS-specific cholesterol metabolite that transports cholesterol into the periphery [59]. CSF concentrations of 24S-hydroxycholesterol appear to be increased in MS patients with gadolinium-enhancing lesions [60]. As 24S-hydroxycholesterol is CNS-specific serum levels may be as suitable as CSF levels for monitoring CNS membrane turnover (see Teunissen et al. for review) [61].

7.5. Markers of remyelination and repair

CSF biomarkers of remyelination and repair have not been evaluated systematically. Potential candidates include the neural cell adhesion molecule (NCAM), which is involved in neuronal and glial adhesion. CSF NCAM is low in subjects with inactive MS and increases approximately a week after the onset of a clinical relapse and stays elevated for up to 5 weeks [62, 63]. Increased levels of CSF ciliary neurotrophic factor (CNTF), an oligodendrocyte survival factor, have been described in MS [64].

Table 2
Reporting recommendations for MS biomarker prognostic studies

<p>I. Introduction</p> <p>1. State the marker examined, the study objectives, and any pre-specified hypotheses.</p> <p>II. Materials and Methods</p> <p><i>Patients</i></p> <p>2. Describe the characteristics (e.g. disease stage) of the study patients, including their source and inclusion and exclusion criteria.</p> <p>3. Describe treatments received and how chosen (e.g. randomised, rule-based).</p> <p><i>Specimen characteristics</i></p> <p>4. Describe type of biological material used (including controls), and preservation and storage methods.</p> <p><i>Assay methods</i></p> <p>5. Specify the assay method used and provide (or reference) a detailed protocol, including specific reagents or kits used, quality control procedures, reproducibility assessments, quantitation methods, and scoring and reporting protocols. Specify whether and how assays were performed blinded to the study end point.</p> <p><i>Study design</i></p> <p>6. State the method of case selection, including whether prospective or retrospective and whether stratification or matching (e.g. by stage of disease, age) was employed. Specify the time period from which cases were taken, the end of the follow-up period, and the median follow-up time.</p> <p>7. Define precisely all clinical end points examined.</p> <p>8. List all candidate variables initially examined or considered for inclusion in models.</p> <p>9. Give rationale for sample size; if the study was designed to detect a specified effect size, give the target power and effect size.</p> <p><i>Statistical analysis methods</i></p> <p>10. Specify all statistical methods, including details of any variable selection procedures and other model-building issues, how model assumptions were verified, and how missing data were handled.</p> <p>11. Clarify how marker values were handled in the analyses; if relevant, describe methods used for cut-point determination.</p> <p>III. Results</p> <p><i>Data</i></p> <p>12. Describe the flow of patients through the study, including the number of patients included in each stage of the analysis (a diagram may be helpful) and reasons for dropout. Specifically, both overall and for each subgroup examine and report the numbers of patients and the number of events.</p> <p>13. Report distributions of basic demographic characteristics (at least age and sex), standard prognostic variables and the biomarker, including numbers of missing values.</p> <p><i>Analysis and presentation</i></p> <p>14. Show the relation of the marker to prognostic variables.</p> <p>15. Present univariate analyses showing the relation between the marker and outcome, with the estimated effect (e.g. hazard ratio). Preferably provide similar analyses for all other variables being analysed. For the effect of a biomarker on a time-to-event outcome, a Kaplan – Meier plot is recommended.</p> <p>16. For key multivariate analyses, report estimated effects (e.g. hazard ratio) with confidence intervals for the marker and, at least for the final model, all other variables in the model.</p> <p>17. Among reported results, provide estimated effects with confidence intervals from an analysis in which the marker and standard prognostic variables are included, regardless of their significance.</p> <p>18. If done, report results of further investigations, such as checking assumptions, sensitivity analyses, internal validation, etc.</p> <p>IV. Discussion</p> <p>19. Interpret the results in the context of the pre-specified hypotheses and other relevant studies.</p> <p>20. Discuss implications for future research and clinical value.</p>	<hr/> <p>8. The future</p> <p>The application of discovery based technologies or the so-called “omics” are likely to identify new candidate biomarkers in MS, for example the recently-identified inflammatory molecule, osteopontin [65]. Once identified, candidate biomarkers then need to be rigorously investigated. This requires a hypothesis-driven approach to identify the role of the candidate in the pathogenesis of MS and to ensure that it fulfils a set of pre-specified criteria for use as a surrogate end-point in clinical studies. A consensus on the latter has yet to be reached.</p>	<p>9. Methodology</p> <p>The investigation of a potential CSF biomarker and assessment of its utility as a surrogate end-point in MS requires a rigid methodology similar to that used for drug development. Figure 2 is a schematic diagram of the proposed process. Intrinsic to the process is the development and validation of biomarker assays. Assays have to be standardized and preferably validated in several laboratories. It is important to make sure that the variability of the assays is kept to a minimum to optimize the power of studies to detect meaningful changes. When reporting results of prognostic biomarker stud-</p>
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* Adapted with permission from McShane et al. [2].

ies in MS it is important to publish sufficient information so that other investigators can interpret the paper and reproduce the studies if necessary. Finally, investigators must make a commitment to publish negative results so as to prevent publication bias (see Table 2, adapted from McShane et al. for the subcommittee of the NCI-EORTC Working Group on Cancer Diagnostics [2].)

10. Conclusions

CSF is not necessarily the most ideal body fluid for the monitoring of MS disease activity because of the logistical difficulties in obtaining frequent samples. However bulk markers such as GFAP and possibly NF have distinct advantages over intermittent markers such as MBP. Currently none of the CSF biomarkers studied to date can be used as surrogate end-points. The main reasons for this are methodological; in general studies have been too small, cross-sectional in design or have insufficient follow-up to allow meaningful conclusions to be drawn about their utility. Large multi-center collaborative CSF biomarker studies need to be designed and have to run in parallel with clinical and MRI monitoring to assess the true potential of candidate biomarkers of key pathogenic processes. Novel approaches using other biological fluids need to be developed, to overcome the ethical and practical difficulties of repeat lumbar punctures. Researchers working on CSF and other body fluid biomarkers in MS should respond to the call and address the challenges proposed by new insights into the pathogenesis of MS and the need for a standardized approach.

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