Developing Biomarkers for MS

Sharmilee Gnanapavan and Gavin Giovannoni

Abstract Existing clinical outcomes of disease activity, including relapse rates, are inherently insensitive to the underlying pathological process in MS. Moreover, it is extremely difficult to measure clinical disability in patients, which is often a retrospective assessment, and definitely not within the time frame of a clinical trial. Biomarkers, conversely are more specific for a pathologic process and if used correctly can prove invaluable in the diagnosis, stratification and monitoring of disease activity, including any subclinical activity which is not visible to the naked eye. In this chapter, we discuss the development of neurofilaments as surrogate outcomes of disability in MS. The validation and qualification are vital steps in biomarker development and to gaining acceptance in scientific community, and the pitfalls leading up to this are also discussed.

Keywords Biomarker · Neurofilaments · Validation · Qualification · Networks · Biobank

Contents

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1 Introduction

Despite the various iterations of the McDonald criteria for the MRI diagnosis of MS (McDonald et al. 2001; Polman et al. 2005, 2011) and immunological parameters suggestive of inflammation (oligoclonal IgG bands (Davenport and Keren 1988) and IgG index (Tourtellotte et al. 1984), MS remains to a large extent a clinical diagnosis. The McDonald criteria can only be applied in cases where MS is the most plausible explanation for the clinical presentation. This is largely owing to the lack of disease specificity of the biomarkers in question, since the aetiology of MS is either unknown or multifactorial. Likewise, it is this heterogeneity that makes the determination of the future disease course for the individual patient quite challenging to predict. This underlying heterogeneity also extends to the pathology of the disease, with disruption in multiple molecular pathways resulting in different pathological phenotypes.

When faced with such complexity, the solution may seem an intractable one. However, a keen appreciation of the principal factors involved may permit reframing of the complexity in a new light and a way to manage them. For example, although there are different clinical phases to MS, some patients convert to progressive disease and then progress continuously, whereas others progress from the outset, and a seemingly lucky few have a benign disease course. The important question to pose here is not what causes disease progression in MS but what leads to the disease progression, i.e. what is more important, finding the answer to the target or the bull's-eye? The former is either simply unknown or linked to myriad of varying or at times multiple possibilities, whereas the latter is because of axonal degeneration, which is a more tangible or objective and a finite possibility in experimental terms. Measures of axonal breakdown such as neurofilaments therefore have the potential to be good surrogate measures of disease progression in MS by this reasoning alone; in other words, they have face validity as a biomarker.

2 Choosing Biomarkers to Study in MS

A biomarker is "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to therapeutic interventions" (Group 2001). Not all biomarkers make good surrogate markers by virtue of correlating with a clinical end point. For a biomarker to reach the eponymous surrogate status, it should be able to substitute for a validated clinically meaningful end point; allow conclusions to be drawn on the effects on a clinical end point; and also reasonably predict clinical benefit. A long list of characteristics therefore have to be established before a biomarker can be considered a surrogate marker, including technical validation, demonstration of biological feasibility, if possible translation across species and across related disorders with similar pathophysiology, and correlations with other measures of a

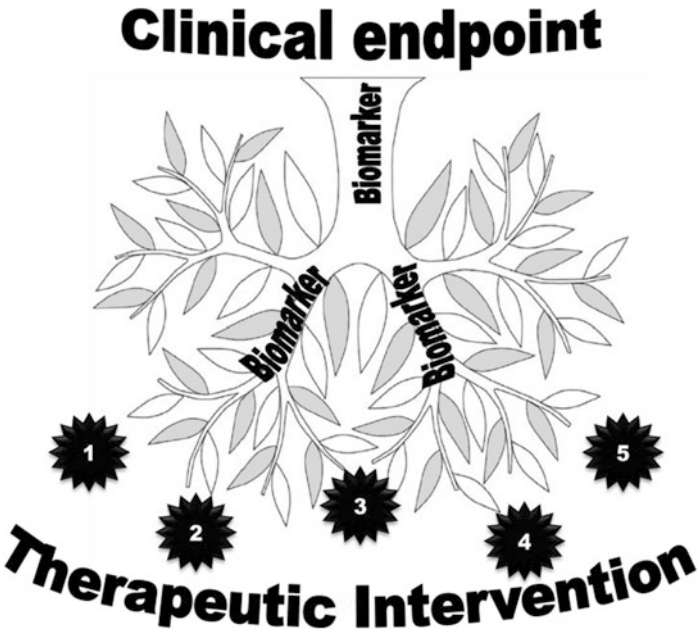

Fig. 1 The process of evaluating therapeutic interventions using biomarkers. Interventions *1–5* should be evaluated by process-specific and treatment-specific biomarkers (near the leaves) as well as by biomarkers most representative of the clinical end point (at the stump)

similar nature and clinical outcomes (Lee et al. 2006; Lee and Hall 2009; Cummings et al. 2010). The Prentice criteria for surrogate measures makes inferences on the superiority and inferiority of biomarkers based on its ability to not only correlate with the clinical outcome but also fully capture the net effect of treatment on the clinical outcome (Prentice 1989). This is most difficult to achieve and also raises the quandary of how much the knowledge of the surrogate may contribute to the selection of the primary end point. Therefore, a process of qualification is preferred when establishing surrogate status in a biomarker and is unlikely to be established based on a single study.

The majority of biomarkers are derived from secondary biological processes, and their relationship to the primary pathological event is not directly causal but due to bystander or associated effects. In order to exemplify what takes place in a disease, a multifactorial all-inclusive approach to selecting biomarkers is needed comprising of biomarkers that are both disease specific and process specific, but also to be practically useful should not be remote from the clinical end point (Fig. 1). They should compare favourably with other well-established clinical and laboratory parameters already in use, which requires translation from in vitro and in vivo animal models into well-controlled clinical trials (Fig. 2). Occasionally, the biomarkers may be so novel that there may be no relevant comparisons, for example in

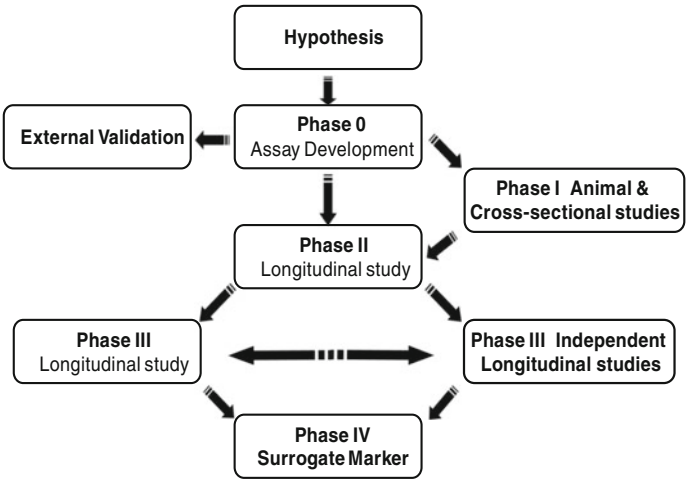

Fig. 2 Validation and qualification of biomarkers

regenerative work where the potential for advancement in the field are yet to be fully realised, in which case qualification at a later stage would be acceptable.

Ultimately, a biomarker has the potential to be fruitful for a longer period of time as a clinically useful biomarker if it is more objective and sensitive than the clinical end point, and can provide meaningful information in a shorter length of time than following the clinical course to its natural predefined end point. Below, we present the qualification steps in establishing neurofilament measures as a surrogate marker of disability progression in MS. The neurofilament assay was first introduced by Karlsson et al. (1989) and later by Petzold et al. (2003), Shaw et al. (2005) for the heavy chain, subsequently in 2005 for the light chain (Van Geel et al. 2005), and it has taken over two decades to establish its potential and acceptance in the scientific community.

3 Neurofilaments as Surrogate Measures of Disability Progression

3.1 What are Neurofilaments?

The physiological function of the axon is very much dependent on the structural layout of the axonal cytoskeleton. This is comprised of a network of interconnected actin microfilaments (6 nm diameter), neurofilaments (10 nm) and microtubules (23 nm) (Fuchs 1996) which are universally responsible for the maintenance of the strength and cross-sectional area of axons. Neurofilaments constitute the most abundant cytoskeletal element in large myelinated axons and to a minor extent in

neuronal cell bodies, accounting for 13 % of total proteins and 54 % of Triton-insoluble proteins in some neurons (Morris and Lasek 1982, 1984; Yabe et al. 2001). Neurofilaments consist of three subunits that differ primarily in their molecular size: light chain (NfL) of 68 kDa, medium chain (NfM) of 150 kDa and heavy chain (NfH) of 190–210 kDa with NfL subunits linking with either hyper-phosphorylated NfM or NfH in an overlapping fashion to give rise to an expanding helical array of a rope-like polymer (Liu et al. 2004). Very little work has been done on NfM, with work on NfL and NfH dominating the field. In proportionate terms, NfL is present in larger quantities at a molar ratio of 4 NfL:2 NfM:1 NfH (Scott et al. 1985).

Neurofilaments are released into the cerebrospinal fluid (CSF) following injury and are useful in monitoring ongoing neuroaxonal damage (Giovannoni and Nath 2011). Elevated CSF neurofilaments have been found in neurodegenerative disorders including ALS (Petzold et al. 2003; Tortelli et al. 2012), multiple sclerosis (Lim et al. 2005), brain injury after stroke (Nylen et al. 2006) or cardiac arrest (Rosen et al. 2004) and CNS infections (Gisslen et al. 2007). Although assessment of the CSF compartment may be more specific for CNS-related injury, neurofilaments can also be measured in the blood, making them more suitable for clinical practice or when a lumbar puncture is contraindicated (Rundgren et al. 2012). Although NfH, unlike NfL and NfM, plays an important role in the development of large-diameter axons (Elder et al. 1998), both NfL (Kuhle et al. 2013b) and NfH (Kuhle et al. 2011) were higher in spinal cord relapses versus brain relapses, suggesting that the site of performance of lumbar punctures to obtain the CSF in close proximity to the pathology may be more relevant. A direct comparison between NfL and NfH in the CSF reveals a good correlation between the two ($r = 0.492$, $p < 0.0001$), suggesting that the two could be used interchangeably (Teunissen et al. 2009a). The only caveat to this is that NfL is easier to measure (picogram quantities *vs* nanogram quantities of NfH), but NfH unlike NfL is significantly raised in SPMS patients independent of the contribution by relapses to disability progression (Semra et al. 2002; Teunissen et al. 2009a; Khalil et al. 2013, 2013b).

3.2 Neurofilaments Predicting Disability

3.2.1 Neurofilament Heavy Chain (NfH)

Serum and CSF NfH levels have been shown to be elevated in both humans with MS and animals with experimental allergic encephalomyelitis (EAE) that resembles some aspects of MS pathologically (Gnanapavan et al. 2012). This is even apparent early on in the disease course as evidenced by elevated levels of CSF NfH (evaluated by ELISA with the monoclonal antibody clone SMI34) in optic neuritis patients (Lim et al. 2004), with further evidence of high serum levels (SMI35) depicting poor visual outcome (Petzold and Plant 2012). There is a similar relationship with relapse

activity in the CSF (Teunissen et al. 2009a; Kuhle et al. 2011, 2013a), which is further supported by a correlation with the number of gadolinium-enhancing lesions and T2 lesions representing evolving inflammatory activity (Teunissen et al. 2009a). This indicates that NfH can be used to monitor ongoing axonal damage during the early stages of MS, and of direct relevance to current clinical practice as early relapses appear to be predictive of future disability in MS (Scalfari et al. 2010). CSF NfH has also been demonstrated to be predictive of future disability, with a positive correlation with EDSS follow-up at 3 years ($r = 0.54$, $p < 0.01$), with a 70 % positive predictive value of conversion to clinically definite MS (CDMS) from first presentation (compared to 63 % for MRI) (Brettschneider et al. 2006), and on average, 1.5-fold higher levels in progressive MS (secondary progressive and primary progressive MS, SPMS and PPMS, respectively) relative to relapsing–remitting MS (RRMS) (Teunissen et al. 2009a). NfH levels are associated with the level of disability in upper limb (peg hole test) and lower limb function (walking times), cognition (paced auditory serial additions, PASAT), MRI measures of atrophy (grey and white matter) and global disease burden (magnetisation transfer ratio, MTR) (Gnanapavan et al. 2013; Khalil et al. 2013). This would make NfH a useful surrogate measure in neuroprotective trials with therapeutics aimed at reducing axonal injury, and possibly in novel adaptive trial designs which utilise elevated baseline NfH levels for inclusion into the trial (power calculations are presented in Gnanapavan et al. 2013).

3.2.2 Neurofilament Light Chain (NfL)

Similar to NfH, CSF NfL has been found to be elevated in early stages of MS with optic neuritis (Modvig et al. 2013), but is a general feature throughout MS disease course with increased levels in RRMS and SPMS without significant differences between the two (Malmestrom et al. 2003). Levels peak to almost 10 times higher during acute relapses (Lycke et al. 1998; Malmestrom et al. 2003) and correlate with other biological markers of inflammation, such as CXCL13, chitinase-3-like-1 and osteopontin (Khademi et al. 2013; Modvig et al. 2013), as well as exacerbation rates (Lycke et al. 1998). CSF NfL determination may therefore be an objective means of supporting a relapse in the clinical setting where there might be some uncertainty. Despite there being a lack of a step rise in NfL in SPMS, there was a positive correlation with EDSS up to 3 and 3.5, suggesting that NfL may be a predictor of early disability (Teunissen et al. 2009a; Madeddu et al. 2013). This is further supported by data that NfL correlates significantly with the multiple sclerosis severity score for cases with recent relapse ($r = 0.60$, $p < 0.001$) than for all cases after a median of 14 years ($r = 0.30$, $p = 0.005$), suggesting that raised levels are more predictive of disability in the short term (Salzer et al. 2010). It also alludes to a fundamental point about relapses, that is that they cause greater axonal damage acutely, and in all likelihood make a significant contribution to the accrual of disability in MS. This corroborated by a significant correlation with MRI T2 lesion load ($r = 0.347$, $p < 0.024$) and an even better correlation with gadolinium-enhancing lesions

($r = 0.496$, $p < 0.001$) (Teunissen et al. 2009a), a marker of active disease, and Kaplan–Meier analysis where conversion to SPMS was more likely when NfL >386 ng/L, increasing the risk of severe MS by fivefold (odds ratio 5.2, 95 % confidence interval 1.8–15) (Salzer et al. 2010).

3.3 Neurofilaments as Biomarkers in Clinical Trials

3.3.1 Neurofilament Heavy Chain (NfH)

Lamotrigine, a sodium channel blocker and putative neuroprotectant, was found to reduce serum NfH levels in subjects on lamotrigine based on serum treatment compliance compared to placebo (Gnanapavan et al. 2013). The trend for reduction was only apparent in the 12–24 months of the trial, suggesting a lag in the treatment effect, an important point to consider when designing neuroprotection trials in progressive disease. This lag was not seen with CSF NfL levels in relapsing MS patients treated with either natalizumab (Gunnarsson et al. 2011) or fingolimod (Kuhle et al. 2013c), in both studies levels were seen to come down within 12 months. A similar trend was noted in the CSF using mass spectrometry, in addition to other putative biomarkers of neurodegeneration, including 14-3-3, tau and osteopontin (Jia et al. 2012). Measurement of NfH levels have also proved useful in interpreting the potential neurotoxicity of chemotherapy agents; in one study of bone marrow transplant recipients undergoing chemotherapy as part of preconditioning regimen, serum NfH (SMI35) levels rose >100-fold within a month post-chemotherapy (29.73 ng/ml versus 0.28 ng/ml at baseline, $p < 0.0001$), with an increase in EDSS with persistently high levels at 3 months and an acute increase in brain atrophy rate (-2.09, $p < 0.05$) (Petzold et al. 2010b).

3.3.2 Neurofilament Light Chain (NfL)

Highly active anti-relapse treatments, such as natalizumab and fingolimod which reduce annualised relapse rates by over 50 %, demonstrate a reduction in NfL levels as well, twofold to threefold reduction depending on the study (Gunnarsson et al. 2011; Kuhle et al. 2013a), while the reduction in NfH levels was less obvious suggesting that NfL is better suited in measuring neuroaxonal damage secondary to relapses (Kuhle et al. 2013a). Conversely, CSF NfL levels were found to be reduced only in a small proportion of patients in the MBP8298 study in SPMS, which may be a reflection of the negative study outcome or that NfL generally remains unchanged in SPMS (Romme-Christensen et al. 2013). The latter is corroborated from findings in the mitoxantrone treatment study in progressive MS wherein CSF NfL reduction was generally confined to those patients with gadolinium-enhancing lesions on MRI prior to study entry and untreated with immunosuppressants beforehand (Axelsson et al. 2014).

4 Other Biomarkers Associated with Neurodegeneration in MS

Table 1 provides a list of biomarkers which have been associated with neurodegeneration in MS. The findings from the individual markers are not always consistent, which is why they are listed as an association. The biomarkers are listed based on their strength of association and as a result of conflicting results across studies; with osteopontin at the top and complement regulator factor H at the bottom. As a whole, reliability also improves when analysed in the CSF compared to blood as a whole due to the matrix effect in the latter. With respect to MMP9, many researchers have used serum tubes, rather than heparin plasma, which can lead to artificially high results, because MMP9 can be released from platelets and leukocytes where clot activators are present (Jung et al. 2001). Lastly, as a general rule, there is often a weak or lack of correlation between biomarkers measured in the blood compared to the CSF, which is influenced by pre-analytical as well as analytical factors, the sampling volume and a higher contribution source in the blood than in the CSF.

5 The Challenges Faced with Biomarkers Development in MS and Ways Forward

The reproducibility of published findings, Validation (including pre-analytical variables) or verification, and ultimately their usefulness are common hurdles encountered when translating biomarkers from the bench to the bedside. Even the choice of control groups, be it healthy controls or neurological controls owing to the lack of access to the former, or age-matched controls, introduce variability into the mix, making interpretation of the data difficult. The narrative presented here about neurofilaments takes these into consideration before investigators were all aware of these variables and required several reproductions of similar experiments by different groups before the trends related to the biomarker became evident. This is a time-consuming process and results in high attrition of biomarkers at the various stages of development.

The biomarker under scrutiny is also relevant; for example, cytoskeletal proteins are often more robust than enzymatic proteins when exposed to the more prevalent variables such as intra- and inter-assay variation, linearity, recovery, freeze-thaw cycles and bench stability. Even neurofilaments which can prove to be quite robust in the hands of a single laboratory (Koel-Simmelink et al. 2014), can prove to be difficult when a multi-centre approach is utilised (Petzold et al. 2010a), thereby arguing for a more centralised approach to biomarker analysis.

The use of biobank-issued samples may be one way of standardisation at the pre-analytical level (Teunissen et al. 2014). European networks such as the BioMS-eu (http://www.bioms.eu/) have looked at unifying control groups for quality control

Table 1 Other biomarkers associated with neurodegeneration in MS

Biomarker	Sample source	Findings	Reference
Osteopontin (OPN)	CSF, plasma	•↑ In SPMS • Associated with MBP in progressive MS • (+) correlation with cognitive impairment index (CII), while a reduction in CII values correlates with ↓ OPN levels • ↓ MSFC z-score, MRICCV, and grey matter and whole-brain MTR • ↓ by natalizumab/GA treatment	Comabella et al. (2005), Gnanapavan et al. (2013), Modvig et al. (2013), Romme-Christensen et al. (2013), Shimizu et al. (2013), Szalardy et al. (2013), Iaffaldano et al. (2014), Kivisakk et al. (2014)
Glial fibrillary acidic protein (GFAP)	CSF	• ↑ SPMS • (+) correlation with EDSS and MSSS • Baseline levels predict future disability • Unaffected by immunosuppressive treatment	Rosengren et al. (1995), Petzold et al. (2002), Malmestrom et al. (2003), Norgren et al. (2004), Axelsson et al. (2011), Axelsson et al. (2014), Burman et al. (2014)
Chitinase 3-like 1 (CHI3L1)	Plasma	• ↑ In progressive forms of MS (SPMS/PPMS) • ↑ levels in acute ON related to NfL and MBP • Allele C of rs4950928 (polymorphism) is associated with PPMS	Canto et al. (2012), Modvig et al. (2013)
N-acetyl aspartate (NAA)	CSF	• ↓ Disease progression • (−) correlation with EDSS, MSFC • (+) correlation with brain volume, but lower NAA ↑ lesion load	Jasperse et al. (2007), Teunissen et al. (2009a, b)
Matrix metalloproteinases (MMP) and tissue inhibitor of metalloproteases (TIMP) (MMP9/TIMP1) (MMP2/TIMP2)	Serum, CSF	• MMP9/TIMP1 ↑ PPMS • MMP9 related to MBP in progressive patients and predicts new enhancing lesions in SPMS • IFN-B ↓ MMP9 while TIMP1 is unchanged in PPMS • MMP2/TIMP2 ↑ SPMS/PPMS than short-duration RRMS but not different to healthy controls	Avolio et al. (2003), Waubant et al. (2003), Yushchenko et al. (2003), Romme-Christensen et al. (2013)

(continued)

Table 1 (continued)

Biomarker	Sample source	Findings	Reference
Neurofilament light (NfL) antibody	CSF, Serum	• ↑ MS • Anti-NfL index (−) correlates with brain parenchymal fraction	Eikelenboom et al. (2003), Amor et al. (2014)
Tau	CSF	• ↑ In MS, in particular RRMS • RRMS ↑ levels predictive of poor short-term outcome •↓ following lamotrigine treatment (sodium channel blocker) in SPMS	Kapaki et al. (2000), Martinez-Yelamos et al. (2004b), Salzer et al. (2010), Jaworski et al. (2012), Jia et al. (2012)
14-3-3	CSF	• (+) 14-3-3 expression related to neurological disability in acute transverse myelitis • (+) 14-3-3 at CIS may be an indicator of severe neurological disability, and in general, the detection of 14-3-3 is a predictor of severe disease • ↓ by lamotrigine treatment in SPMS	Irani and Kerr (2000), Colucci et al. (2004), Martinez-Yelamos et al. (2004a)
NO*x*	CSF, serum	• ↑ In MS • ↑ Levels in those with disability progression and • (+) Correlation with MRI T2 lesion load and volume of Gd enhancement	Peltola et al. (2001), Yuceyar et al. (2001), Rejdak et al. (2004)
Oligoclonal bands (OCB)	CSF, serum	• OCB+ patients have higher EDSS. • Oligoclonal IgM bands have an early ↑lesion load and brain atrophy	Balnyte et al. (2011), Magraner et al. (2012)
Complement regulator factor H	Serum	• ↑ In progressive forms of MS (PPMS, SPMS)	Ingram et al. (2010)

within biobanks and have proposed the following for uniformity: healthy controls, spinal anaesthesia subjects, symptomatic controls, inflammatory neurological disease controls, peripheral inflammatory neurological disease controls and non-inflammatory neurological disease controls (Teunissen et al. 2013, 2014). The group has also proposed collaborations between the various biobanks to permit studies to be performed on a larger sample size, thereby diluting out the influence of pre-analytical variables in the analysis (Teunissen et al. 2009b, 2011). Not only

should efforts be made to standardise biomarker research at the outset, but also at the methodological and reporting stages to permit interpretation of biomarker data at face value. The REMARK guidance in cancer research for prognostic studies is one such example and uses a reporting format similar to those used by most journals (introduction, materials and methods, results and discussion) to encourage its adoption (McShane et al. 2006). It remains to be seen whether these changes will improve the quality of biomarker data published.

6 Concluding Remarks

Neurofilament proteins have stood the test of time and are now developing into a viable surrogate end point to be utilised in neuroprotection clinical trials in partnership with MRI. Furthermore, their timely response to the effects of treatment makes them an attractive alternative where existing clinical measures are insensitive, or unwieldy. Having said this, some pertinent information has come through where a certain amount of caution is needed, namely that NfL may be more relevant to early disease pathophysiology in MS and more sensitive to relapses than NfH which appears to be more reflective of chronic disability, despite the relative abundance of the former. This needs to be specifically addressed in future studies utilising neurofilaments as biomarkers.

Overall, as a general rule, biomarkers need to get over many hurdles of validation and qualification before correlations with disease processes can take place. Standardisation of methodology and reporting across groups will be a fundamental step in achieving this over a realistic time period. Networks, such as the BioMS-eu consortium, have already started looking into this, and establishment of catalogued biobanks analogous to the brain tissue banks will allow for large-scale biomarker analysis to be performed. Once the requisite studies have been performed, biomarkers can be combined into a paradigm of process-specific, disease-specific and treatment-specific biomarkers to best understand the overall disease process of MS at a snapshot and longitudinal level. The selection of these biomarkers should be hypothesis driven rather than generated by non-directed methods, in order to justify the prima facie aim of the study question posed. Otherwise, the complexity of the derivatives alone will compromise the end result.

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