How to approach a patient with neuropathy: From diagnosis to therapy - Level 1

Laboratory tests in neuropathies: Genes, CSF and antibodies. What and when?

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Peripheral nerve diseases (PND) are diverse in their phenotypes and pathogenesis. An accurate clinical diagnosis is one element of the clinical formulation that determines whether or not a patient might be suitable for a particular treatment. The diagnostic skills of a neuromuscular specialist still reside in the clinical history and examination. However testing of CSF and serum may assist the diagnostic process by supporting, refuting or providing biomarker data to follow the clinical course of a disease. Examination of fluids should not be forgotten, but also should not be overly relied upon as occasionally it may mislead. In this lecture we will discuss both general and specific issues of serum and CSF testing. In particular we will explore antibodies and biomarkers in the CSF and serum available in many diagnostic laboratories, comparing their utility with their limitations.

CSF and serum are the two primary body fluids with relevance to testing in peripheral neuropathies (PN). At the present time urine is used largely for the detection of light chains (Bence Jones Protein) and both urine and tears may be useful in the future as a noninvasively sourced body fluids as detection methods increase in sensitivity.

Testing of serum and CSF in PND aims to detect

- antibodies which are thought to be linked directly or indirectly to causation, or
- proteins and other molecules that may act as biomarkers for diagnosis, natural history or response to treatment.

A biomarker is defined as ‘a distinctive biological or biologically derived indicator (as a biochemical metabolite in the body) of a process, event, or
condition (as aging, disease, or exposure to a toxic [or therapeutic] substance’) (Webster Medical Dictionary). Biomarkers are most commonly used as indicators of damage or disease, often in a state of transition, and are useful in diagnosis, treatment and prognosis. Biomarkers should always and only be measured in an appropriate context. They should also have as many possible qualities as possible including:

- Specificity: they should differentiate pathologies preferably with values of >0.9
- Sensitivity: there should be a zero baseline from which the biomarker deviates. They should preferably be a marker of ‘early,’ reversible neural damage and rapidly assessed. Once again they should have a sensitivity >0.9
- Predictive: Values recorded should be proportionate to extent of injury, robust and reproducible
- Being rapid, simple, accurate and inexpensive
- Bridging pre-clinical and clinical environments
- Being non-invasive and easily accessible

The serum contains both a greater number of identifiable markers of disease including many antibodies which are fairly easily detected in 2017. In addition, they may be in greater concentration compared to the CSF in PND (except where there is intrathecal production). Diagnostic serum testing measures proteins and antibodies with antibodies to novel targets being of increasing interest.

When a clinician first sees a patent with a peripheral neuropathy it is useful to consider a simple hierarchy of testing that assists in the
algorithmic diagnosis of a patient [1] (see Table 1). Most of these markers are entirely routine to most clinicians so we will concentrate here on a few specific and useful biomarkers and antibodies in relation to inflammatory disease.

The first of these is the detection of serum paraproteins. 10% of neuropathies with no other identifiable cause are associated with a paraprotein [2]. Some of these are chance associations but the presence of a serum paraprotein should not be overlooked, and neither should it be immediately discounted as irrelevant. Anti-MAG antibodies are part of the normal CD5+B-cell repertoire but in demyelinating neuropathies with accentuated peripheral nerve slowing they are likely to be pathogenic. They are often part of a monoclonal gammopathy of uncertain significance (MGUS) or Waldenström’s macroglobulinaemia (WM). In MGUS the actual level of the paraprotein may be very low (<3g/l the detection threshold for many lab systems). Because there is no immunoparesis of other Ig subclasses, this small amount of paraprotein can be overlooked in serum testing that relies on SPEP alone with a sensitivity of only 66% [3]. Furthermore, in the presence of a pathogenic anti-MAG Ab both the absolute levels of light and heavy chains can be normal as can their ratio, making serum free light chain estimation less than 100% sensitive. A serum immunofixation with a urine Bence Jones protein estimation is recommended in all patients; abnormalities here may lead to other investigations.
Table 1 - a hierarchy of serum and other tests in peripheral neuropathy

<table>
<thead>
<tr>
<th>1&lt;sup&gt;st&lt;/sup&gt; line investigation</th>
<th>2&lt;sup&gt;nd&lt;/sup&gt; line investigation (with neurophysiology)</th>
<th>3&lt;sup&gt;rd&lt;/sup&gt; line investigation (chosen as appropriate)</th>
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<tbody>
<tr>
<td><strong>Haematology</strong></td>
<td><strong>Biochemistry</strong></td>
<td><strong>Urine</strong></td>
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<td>Full blood picture</td>
<td>Serum protein</td>
<td>Bence-Jones protein</td>
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<td>ESR</td>
<td>electrophoresis AND immunofixation (SFLC)</td>
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<td>B12</td>
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<td>Biochemistry</td>
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<td>Folate</td>
<td>Serum ACE</td>
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<tr>
<td><strong>Biochemistry</strong></td>
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<td>homocysteine/methylmalonic acid?</td>
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<td>Fasting glucose</td>
<td>Immunology</td>
<td>Cerebrospinal fluid</td>
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<tr>
<td>Renal function</td>
<td>ANA/dsDNA</td>
<td>Cells, protein,</td>
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<td>Liver function</td>
<td>ENA (anti-Ro, anti-La)</td>
<td>immunoglobulin OCB</td>
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<td>TSH</td>
<td>RhF and CCP</td>
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<td><strong>Urine</strong></td>
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<td>Immunology</td>
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<td>Glucose, protein</td>
<td>Other - Chest XR</td>
<td>Anti-HIV Ab</td>
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<td>Anti-neuronal Ab (Hu, Yo, Ma2)</td>
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<td>Anti-gliadin Ab</td>
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<td>Anti-ganglioside Ab</td>
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<td>Anti-myelin associated glycoprotein (MAG) Ab</td>
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<td>Tests for Sjögren’s syndrome</td>
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<td>Search for carcinoma or lymphoma or solitary myeloma</td>
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<td>Pelvic ultrasound, abdominal</td>
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<td>CT, chest CT, mammography, PET</td>
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By far the commonest requests for antibody testing the neuroimmunology lab are those for antibody mediated disease. In paraproteinaemic neuropathy anti-MAG antibodies are present in more than 50% of cases with an IgM paraprotein and conduction slowing on electrical studies. ‘Anti-MAG’ antibodies target a carbohydrate epitope known as HNK-1 which is present on myelin associated glycoprotein (MAG) as well as other peripheral nerve molecules such as the glycoproteins P0, PMP22 and the
glycolipids SGPG and SGLPG. In the early days of detection MAG antibodies were sought by immunofluorescence on tissue sections, micro-complement fixation tests, western blotting and thin layer chromatography. These tests were cumbersome, difficult and had relatively poor sensitivity although in some cases high specificity. Availability of strip blots for glycolipids and developments in ELISA technology for glycoproteins anti-MAG antibody testing has become easier and also more widely available but also more sensitive and possibly less specific. The Bühlmann anti-MAG ELISA is probably now the most common platform used for MAG testing. However it is very sensitive and at low and intermediate levels of positivity may not be associated with typical anti-MAG neuropathy. Different labs use different cutoffs for positivity. Furthermore some labs use entirely different assays (eg SGPG/SGLPG strip blots) which are reported as anti-MAG antibodies when this is not necessarily correct. Care needs to be taken to interpret the assay result in the context of the clinical presentation and not the other way around - the presence of an antibody does not make a diagnosis.

Anti-ganglioside antibodies occur in both chronic and acute neuropathies. In general, IgM antiganglioside antibodies are associated with chronic neuropathies and IgG with acute. Some IgM anti-ganglioside antibodies are paraproteinaemic (e.g. some cases of MMNCB with anti-GM1 antibodies). IgM anti-GM1 are however much more frequently not a paraprotein. In CANOMAD, antidasialosyl antibodies associate with a clinical phenotype of ataxia, opthalmoplegia and a paraprotein to give a rare but distinctive phenotype. Others have looked for anti-ganglioside antibodies in CIDP with little success [4]. In GBS, ganglioside antibodies have been far more interesting. Fisher syndrome is associated in 90% of cases with IgG anti-GQ1b antibodies. Antibodies to GM1 are associated with poor prognosis,
GD1a and GalNac-GD1a with AMAN and GM2 to sensory phenotypes following CMV infection. There is strong evidence that gangliosides are the target for antibodies and complement attack in GBS, and yet many cases did not have any reactivity in the serum. Serendipity and then directed searches [5] [6] [7] have identified that antibodies exist to complexes of gangliosides exist which has led to more complex assays and most recently ‘heat maps’ of antiganglioside activity (see figure). Exactly what the meaning of these complex epitopes is at present remains to be seen; work is ongoing to clarify this and the International GBS Outcomes Study (IGOS1000) will go a long way to answering this question in a large group of well phenotyped patients.

Figure 2 – top left and example of thinlayer chromatography overlay (Lunn). Gangliosides are run out from different sources and developed with resorcinol (A1-4). Overlay with a monospecific anti-GD1a antibody demonstrates detected in ganglioside samples 1, 3 and 4 (B). Multispecific antibody detects GT1b, GT1a and GD1a where present in all ganglioside samples. Bottom left (Kusonoki and Greenshields) an overlay of GD1a and GD1b gangliosides run apart (A1, 2)) and together (A3). Antibody with complex ganglioside reactivity only sees the complex epitope of both gangliosides together (B1-3). Right side (Brennan) a complex heat map of reactivities of sera from MS, healthy control and other neurological disease showing multiple complex antiganglioside activities against combinatorial antigen pairs.
CIDP lags behind with serum tests that act as diagnostic or therapeutic biomarkers. This may be because CIDP is a predominantly T-cell mediated disease with little humoral involvement, or may represent the fact that we are still looking for the wrong thing. Antibodies to peripheral nerve proteins for example P2 and PMP22, were seldom and inconsistently seen outside paraproteinaemic anti-MAG neuropathies. In recent years the concept of nodal and paranodal pathologies has been realised with better understanding of the molecular makeup of these structures. Devaux, Querol and others identified small cohorts of CIDP patients with antibodies to protein components of the nodal complex [8, 9] [10-12]. Identifiable phenotypes of IVIG treatment resistance who had either aggressive motor, or ataxic tremulous phenotypes who had IgG4 antibodies to contactin-1 or neurofascin-186 were recognised and some of these patients respond to rituximab, as is common to other patients with IgG4 diseases. These antibodies remain responsible for only a small cohort of CIDP patients but open the door to further exploration as well as avenues for treatment for some.

Despite its ‘gin clear’ normal appearance the CSF is a relatively complex fluid. In 1916 Guillain Barre and Strohl described the 3 index cases of GBS in a paper entitled ‘*Sur un syndrome radiculo-néurite avec hyper-albuminose du liquide céphalo-rachidien sans reaction cellulaire...*’ [13]. The characteristic raised CSF protein without a cellular reaction was the backbone of laboratory support in GBS and CIDP for many years. There is far more to CSF analysis than a total protein however. The major proteins of the CSF are albumin and IgG, but many other large and small proteins are present in lower amounts as a result of transport, production or release.
The CSF and serum IgG and albumin can be used to calculate indices of their relative levels between the two compartments. Albumin is not made in the thecal space and so its level can only rise when it is allowed to leak through a dysfunctional blood brain barrier. IgG can be synthesised within or outside the thecal space and be transported either way. There is only about 1% of the level of IgG in CSF than in the serum so IgG made in the CSF is seldom detectable in the serum. However IgG moving inward is easily measured. Even more useful then is the Q_{alb} also known as the IgG index [Reiber H et al Restorative Neurology and Neuroscience 21 (2003) 79-96]. This differentiates between breakdown of the blood brain barrier and intrathecal IgG synthesis. Raised IgG indices may correlate with the presence of oligoclonal or monoclonal bands detected by isoelectric focussing. In PN disease the presence of a raised IgG index and oligoclonal bands as opposed to non specific BBB dysfunction might alter one’s index of suspicion from an idiopathic radiculoneuritis to lymphoma, infection or paraneoplastic disease.

The CSF does not contain very much protein (normal levels usually <0.4g/l), most of which is actively and passively transported albumin and IgG. The detection of ultra low levels of specific CSF proteins is increasingly realistic. A number ultrasensitive platforms now exist in specialist and more routine laboratories with very low detection thresholds and high dynamic ranges (e.g. MSD platforms and SIMOA). In PND biomarkers are beginning to be described that might have important prognostic utility. Axons contain a number of cytoskeletal and transport proteins that with axonal damage are measurable in fluids into which they are released. Periaxin, peripherin, GFAP and neurofilaments all show promise in PN disease. Levels of CSF a-beta 42, tau, phosphotau, S100b and 14-3-3 protein have been used for some years in the diagnosis of
Alzheimer’s Disease, frontotemporal dementia, prion and other neurodegenerative conditions as increasingly specific and sensitive diagnostic markers. Neurofilament (NF) light, medium and heavy are not significantly different in the CNS or PNS. In theory, significant proximal axonal damage in nerve roots should release NF into the CSF. More significant damage along the length of nerves should release neurofilament systemically that might be detectable in the serum. In 2003 Petzold et al published a study that demonstrated NF-H was detectable in the CSF of patients with GBS, and also MND, subarachnoid haemorrhage and other CNS diseases, but not in normal controls [14]. He later showed that in GBS levels of CSF NF-H only increased in axonal GBS ad that the rise correlated with the functional outcome score [15]. Furthermore there was a longitudinal relationship of NF release over time which correlated with transformation to an axonal phenotype and poor outcome [16]. However this assay was difficult and levels of NF were at the lower end of detection with ELISA. Promising data from animal studies indicate now that with ultrasensitive methodology dynamically changing levels of NF-L can be detected in serum and CSF at pg/ml levels. This should also hold true for proteins at even lower concentration, previously undetectable and which may provide important clues to pathogenesis and outcome in the future.
A number of other proteins are potential biomarkers for PN disease. These include tau protein for which there are existing (difficult) commercial assays [17] where raised levels correlate with poor prognosis, GD1a antibodies, diarrhoea and axonal transformation. However tau is not specific to the PNS and may have limited utility.

Both limitations in levels of detection and PN specificity also limit the utility of cytokines as biomarkers of inflammation or more specific pathologies. IL6 may be revealing going forwards but early excitement over TNF-alpha, II2 and other soluble interleukins has not translated to the clinical arena as levels are highly variable between individuals, sensitive to change from other cormorbid conditions and are often below the levels of accurate detection in most assays. Vascular Endothelial Growth Factor (VEGF) is proving extremely useful in the diagnosis of POEMS. Watanabe [18, 19] originally described very raised levels of VEGF in POEMS over 20 years ago. In the context of a paraprotein (lambda light chain) and an appropriate neuropathy, when iron deficiency and hypoxia are excluded, VEGF has a specificity of 96% and sensitivity very near to 100% for diagnosis. (see Figure 3 - Pihan unpublished). A raised level of VEGF is now included as one of the 5 major criteria in the diagnosis of POEMS [20]. However anti-VEGF monoclonal antibodies are ineffective and possibly dangerous in the treatment whereas anti-IL6 and stem cell therapies to turn off the production of VEGF and other cytokines by lymphoid stroma are very effective.
Figure 3: VEGF levels in patients presenting with POEMS syndrome or inflammatory neuropathy of other causes. The ‘normal’ cutoff is 717pg/ml. With a neuropathy and a lambda light chain paraprotein and with a VEGF of >1000pg/ml sensitivity and specificity are nearly 100%.

Serum and CSF testing has come far since the descriptions of CSF protein by Gullain and his colleagues, and these observations remain as relevant today as they did 100 years ago. We still have some way to go however. Greater understanding of the micromolecular biology of the peripheral nerve in terms of proteins, their distribution and immunological availability is giving us new targets to explore. The discovery of complex antigenic targets gives us many decades of work understanding how far this complexity might stretch and what it may mean for patients. Hugely greater sensitivity for detection of low levels of protein has brought within our reach an understanding of low level biomarkers released into the CSF and serum by the very low tissue burden of damaged nerves even in a florid disease, as well as the changes in inflammatory molecules
previously at limits of detection. And yet there is more to explore in front of our eyes; T-cell receptor targets, CSF LDH, IgM and flow cytometry, and many other CSF molecules are ripe for investigation and will potentially greatly aid diagnosis going forwards.

Disclosure:
M Lunn has accepted offers of travel assistance, conference registration and hotel charges to medical conferences relevant to his clinical work from CSL Behring for the PNS in 2017 and PNS meeting in 2015. He has attended Advisory Boards for UCB Pharma (2016) and a further Advisory Board for a clinical trial in which he was local PI with CSL Behring (2017). He has also performed advisory board roles and given teaching seminars for Grifols, CSL Behring, Baxter and LfB over the last decade. He has been local PI on trials of therapy for IVIG and SCIG with company sponsorship. He is joint Co-ordinating Editor of Cochrane Neuromuscular. He is on the Board of the Peripheral Nerve Society, and Treasurer of the British Peripheral Nerve Society and is on the medical advisory board of the GAIN Charity. He has no financial interest in any of these organisations. He performs clinical neurology service within the UK National Health Service and the UK Private Sector.
References


