

Standardization of procedures and methods in neuroimmunology

Editorial note

The document on “Standardization of procedures and methods in neuroimmunology” faces the requirement for cultural progress of the Italian Association for Neuroimmunology (AINI). Although references studies and expert knowledge assisted the preparation of the document, and the strength of recommendations is clearly stated, they do not intend to represent a predetermined apodictic description of “good laboratory practice”.

Knowledge is rapidly advancing and, accordingly, the documents will advance. All those interested in updating and improving the document are invited to send their opinions to the AINI editorial board (redazione@aini.it). Future revisions will report each of the comments and replies sent for possible modifications of the document.

Preface

The main purposes of AINI in promoting the present standardization are: a) to increase the quality of analytical performances; b) to give the basis for clinical guidelines; c) to facilitate the composition of a laboratory network and of research collaborations; d) to facilitate the procedures for certification.

To standardize is basically to follow procedural and methodological models. Complying with standardization is only an option up to now. However, procedures and methods when standardized need to be followed step-by-step, whereas discretionary points must have been clearly identified. AINI has also the task of controlling that each laboratory complies with procedures and methods. To this end, AINI periodically promotes external quality control schemes. At the beginning at least, this quality control will be “educational”, and laboratories that result “discrepant” will be invited to check procedures and methods.

The document is composed of four sections: each of them covers important aspects of the diagnostics of neurological diseases. The four topics are:

“Cerebrospinal fluid examination”

“Diagnostics of paraneoplastic neurological syndromes”

“Diagnostics of dysimmune peripheral neuropathies”

“Determination of anti-acetylcholine receptor antibodies”.

The four documents share a “point-by-point” structure that facilitates the identification of single points and comments.

The content is the product of a “consensus-based procedure” that lasted one year and involved the participating centres. Information and opinions circulated by e-mail and were discussed during two ad hoc AINI workshops (in 2002 and in 2003). The standardization process was coordinated by pre-identified leading groups. Each document reports the names of participating centres and people involved in the process. Coordinating leading groups are also responsible for the external quality control schemes, consulting advices, and specific training.

Revision

The document will be updated on March 2005.

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Cerebrospinal fluid examination

1.0 GROUP COMPOSITION

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2.0 INTRODUCTION

2.1 General section. The results of cerebrospinal fluid (CSF) examination have produced – and will obtain – data by exploiting non-standardized procedures and methods, either in routine use, or research. A clear example of this condition is the recently reported association between the number of oligoclonal bands and disease prognosis in multiple sclerosis [1]. This finding has a purely “local” significance, as no procedure did standardize the enumeration of CSF oligoclonal band. Moreover, the only study dealing with inter-laboratory reproducibility suggested the need for definite procedural and interpretative criteria and, eventually, standardization [2]. The results did not face the problem of oligoclonal band numbering. A program for procedure and method standardization in the CSF examination should address a series of points that are difficult to face and neglected in the literature. The European *consensus* on CSF examination in multiple sclerosis [3], which also contains general indications, has not been integrally translated into the routine practice so far, and does not thoroughly deals with the topic of procedure and method standardization. The lack of such standardization is among the most important causes underlying the failure of new CSF markers in entering clinical routine, although single, retrospective studies on small and selected patient populations would suggest that such markers could work [4]. These studies generally have “weak points”, given that cutoff values and reference ranges are firstly determined in selected populations of patients and controls, and

then used for the same, or similar populations. This process artificially increases test sensitivity and specificity. Moreover, the multitude of these tests is always evaluated singularly, test-by-test, in different patient populations, without pursuing the application of groups of tests on a single patient population. This is the only way by which the real diagnostic/prognostic power of single tests can be extrapolated.

2.2 Special section. CSF examination suffers from the competition of neuroimaging techniques, which are becoming more and more sophisticated and able to give detailed information on central nervous system pathological processes. In the last decade, CSF examination has not improved substantially. However – at least in Italy – the general quality of the routine examination improved, as the comparison between a past [5] and the present survey indicates. For example, ten years ago fifty percent of the Italian laboratories participating in the survey used the determination of serum and CSF albumin to calculate the degree of blood-brain barrier permeability damage, and measured serum and CSF proteins on different analytical runs [5]. Today, all the laboratories participating in the present survey use the albumin quotient, and analyse serum and CSF samples on the same run. Ten years ago, only 8 out of 33 (26%) laboratories used isoelectric focusing for oligoclonal band determination, whereas today all the laboratories currently use this technique, and immunoaffinity blotting for IgG is used by almost all of them as the detecting system. A recent paper formally demonstrated that isoelectric focusing is the most sensitive technique for oligoclonal band detection [6].

The evaluation of the analytical performances of tests in CSF examination is complex: the lumbar puncture is not usually repeated, the CSF samples are limited in volume, CSF samples from control populations are not easily available, and no test is disease-specific. Diagnostic sensitivity and specificity have relative values in the CSF areas, as they are strongly influenced by the prevalence of neurological diseases (Bayes theorem). Accordingly, CSF reports should be necessarily interpreted taking into account diagnostic hypotheses.

Although clinical indications of lumbar puncture should not be included in the present document, we report the principal pathological conditions for which CSF examination is indicated. The purpose is to give information to the community of “non-neurologist” readers:

- meningitis and meningoencephalitis
- multiple sclerosis and demyelinating encephalomyelitis
- dysimmune polyradiculonevritis
- carcinomatous meningitis
- neurological involvement in systemic inflammatory/autoimmune diseases
- pseudotumor cerebri
- TC-negative, suspected subarachnoidal bleeding
- block of the CSF flow
- oto/rhinoliquorrea
- Creutzfeldt-Jacob’s disease
- Alzheimer’s disease (?).

A basic CSF examination should be performed on a urgent basis in the case of suspected meningitis/meningoencephalitis. The basic CSF examination should include: cell count, serum and CSF glucose determination, serum and CSF albumin determination (CSF total protein as an alternative). In Alzheimer's disease, non-definitive experimental evidence indicates that the contemporary determination of CSF protein τ , hyperphosphorilated protein τ , and amyloid β_{1-42} is useful in the early diagnosis of the disease [7].

Last, but not least, economic aspects, which have been defined only recently [8], should be considered: the search for oligoclonal bands – the most technically difficult and high-cost test in this context – is not cost-effective, although able to improve the management of multiple sclerosis.

3.0 PREANALYTICAL PROCEDURES

- 3.1 Lumbar puncture is normally performed in the morning, after overnight fasting. Non-fasting blood and CSF samples are also acceptable, but the time of sampling should be reported.
- 3.2 The use of the CSF sample (types of requested tests, aliquots, how to send an aliquot to external centres, or to store it etc.) should be decided by both clinicians and laboratorists before sampling.
- 3.3 A site of sampling different from the lumbar site (ventricular, cistic) should be reported.
- 3.4 CSF specimen is sampled in sterile, glass-syliconated/polypropilene tubes (glass is not allowed, as monocytes adhere to it).
- 3.5 The volume of extracted CSF should be standardized (4-5 mL), and aliquots obtained starting from a single tube, every time it is possible:
 - i) The concentration gradient causes differences in the protein content between the first millilitre of extracted CSF, and the last ones [9, 10]. Volume standardization allows accurate comparison when applied to repeated samples of the same patient, and to samples of patients that share the same disease.
 - ii) Aliquoting from a single tube minimizes a possible source of preanalytical error, and allows harvesting the highest number of CSF cells (by centrifugation and recovery of a cell pellet that is, in turn, used for cytocentrifugation, or sedimentation chamber). If the CSF sample is destined to microbiological analysis, the absolute priority is for sample sterility: accordingly, sample manipulations, or aliquoting should be avoided.
- 3.6 A blood sampling is always performed concomitantly to lumbar puncture.
- 3.7 Paired blood and CSF samples are sent to the laboratory as soon as possible. Samples coming from external hospitals should reach the laboratory by two hours from sampling, without freezing.
- 3.8 CSF samples that are sent to external hospitals follow procedures that are established by each institute that will receive the samples.

- 3.9 Paired blood and CSF samples are identified with a form reporting patient's demographic information, diagnostic suspect, and the name of the reference clinician.
- 3.10 Blood samples should not be hemolysed, or lipemic.
- 3.11 Differential diagnostics between traumatic lumbar puncture and subarachnoideal bleeding.
- i) CSF is sampled into three consecutive tubes.
 - ii) Determinations are performed on the third tube, i.e. the less "hematic" in the case of traumatic lumbar puncture.
 - iii) If the degree of "red colour" (at visual inspection) is similar in the three tubes, subarachnoideal bleeding is possibly the underlying cause. However, a traumatic lumbar puncture cannot be excluded.
- 3.12 CSF sample with red cells from a traumatic lumbar puncture:
- i) The sample should be analysed, if another sample from a non-traumatic lumbar puncture is not obtainable.
 - ii) The correction of the CSF cell number in the sample, and the calculation of blood-brain barrier damage, on the basis of the red cell number, is not precise. Resulting reports should be critically interpreted by both the laboratorist and clinician. Particularly, the absence of CSF abnormalities suggests that the CSF sample is "normal", whereas the presence of abnormalities does not exclude a normal sample.
 - iii) The CSF sample should always be excluded from research protocols.

4.0 ANALYTICAL PROCEDURES

4.1 Visual evaluation and spectrophotometric analysis

- 4.1.1 The CSF sample should be evaluated for its appearance and colour before and after centrifugation (1500-3000 rpm/500 g, for 10 min).
- 4.1.2 The best harvesting of CSF cells is obtained with centrifuges with fixed-angle rotors.
- 4.1.3 Appearance and colour are expressed with qualitative scales (for example, "crystal-clear", "sublimpid", "cloudy" for appearance, and "colourless", "xanthochromic", "erythrochromic" for colour).
- 4.1.4 If the CSF sample is collected into three tubes, the report should indicate the tube used for the analysis (usually, the third tube).
- 4.1.5 Spectrophotometric analysis should be performed only in the suspect of subarachnoideal bleeding [11]. Computerized tomography (CT) is almost always diagnostic, but a normal CT in the presence of symptoms suggesting subarachnoideal bleeding does not exclude the bleeding. CSF examination is also useful to diagnose a preceding subarachnoideal bleeding.
- i) Spectrophotometric analysis should be done after CSF sample centrifugation (see point 4.1.1).

- ii) Peaks that are typical for proteins (415 and 460 nm) indicate blood-brain barrier damage.
- iii) Peaks that are typical for degraded hemoglobin products (oxy- and meta-Hb, namely, precocious metabolites that persist up to 4-8 days: 415, 540 and 580 nm; bilirubin and bilirubin compounds, namely, late metabolites that persist up to 15-20 days: 350, 400 and 460 nm) indicate a subarachnoidal bleeding that can be, respectively, recent or antecedent.
- iv) Taking into account OD values, a possible, simplified interpretative hint could be: the presence of oxy-Hb and bilirubin is suggested by a) $OD > 0.04$ at 415 and 450 nm if CSF albumin concentration is < 150 mg/dL; b) $OD > 0.08$ at 415 and 450 nm if CSF albumin concentration is > 150 mg/dL.

4.2 CSF cells

- 4.2.1 CSF cells should be numbered and processed within two hours from the sampling.
- 4.2.2 At least 100 μ L of the CSF sample should be used for cell numbering, after gentle agitation of the tube.
- 4.2.3 Admitted chambers for cell count are: Fuchs-Rosenthal, Bürker, Nageotte, or any other similar.
- 4.2.4 Turk's liquid may be used, at 1:1 dilution, to lyse red cells, and mildly stain CSF cells, thus allowing their easy counting.
- 4.2.5 Cytocentrifuges or sedimentation chambers should be used for enriching the sample and identifying CSF cells.
- 4.2.6 After a preliminary centrifugation (see point 4.1.1), the resulting cell pellet is destined to cytocentrifugation or sedimentation.
- 4.2.7 The cell pellet is processed immediately after CSF sampling, or may be stored in 10% formaldehyde (a drop, or 50-100 μ L).
- 4.2.8 Cell morphology is routinely studied by staining CSF cells with May-Grünwald-Giemsa.
- 4.2.9 Cell morphology studies are mandatory when carcinomatous meningitis is suspected. Differential count of CSF cells is optional, unless requested by clinicians.
- 4.2.10 Immunocytochemical analysis of CSF cells may be postponed by fixing the smear with a dip in cold acetone, and storing at -20°C .
- 4.2.11 Counting and reporting the number of red cells does not add information in the case of traumatic lumbar puncture.
- 4.2.12 A simple staining with India ink is suggested when cerebral cryptococcosis is suspected.

4.3 Biochemical analysis

- 4.3.1 Biochemical analysis is performed on the supernatant of centrifuged CSF samples (see point 4.1.1).
- 4.3.2 Determinations are on paired serum and CSF samples.
- 4.3.3 Basic analytes (glucose, albumin, IgG).

- 4.3.3.1 Glucose should be determined on paired serum and CSF samples, with the report of the percentage ratio ($\text{glucose}_{\text{CSF}}/\text{glucose}_{\text{serum}} \times 100$; reference value, $> 45\%$).
 - 4.3.3.2 Glucose is measured with colorimetric tests.
 - 4.3.3.3 The determination of serum and CSF albumin – with the calculation of the albumin quotient – is the most accurate index for blood-brain barrier damage, and should replace the determination of CSF total protein.
 - 4.3.3.4 Blood-brain barrier damage may be expressed as whether the ratio between serum and CSF albumin ($\text{Alb}_{\text{serum}}/\text{Alb}_{\text{CSF}}$, reference value, > 130), or albumin quotient ($\text{Alb}_{\text{CSF}}/\text{Alb}_{\text{serum}} \times 10^3$ (reference value, < 7.0), or $\times 10^2$ (reference value, < 0.7) [12].
 - 4.3.3.5 A qualitative reference for the blood-brain barrier damage may also be reported in accordance with E.J. Thompson: $\leq 0.7\%$, normal; $\leq 2.0\%$, mild; $\leq 5.0\%$, moderate; $> 5.0\%$, severe) [13].
 - 4.3.3.6 CSF total protein determination should be restricted to cases when CSF examination is urgent and facilities for albumin determination are not available.
 - 4.3.3.7 Similarly to the previous point, Pandy and Nonne-Appelt assays should be restricted to urgent cases (point-of-care testing).
 - 4.3.3.8 Reporting that the reference value for the blood-brain barrier damage are not applicable to infants, and that the barrier is physiologically more permeable in elderly people, is useful.
 - 4.3.3.9 To decrease the analytical imprecision, serum and CSF albumin and IgG should be determined with the same method and on the same analytical run.
 - 4.3.3.10 A further decrease in analytical imprecision can be obtained with the determination of serum and CSF albumin and IgG on a unique calibration curve – i.e., the CSF curve – which is used to test both CSF and appropriately diluted (1:100/500) serum samples.
 - 4.3.3.11 Intrathecal IgG synthesis should be expressed with the use of non-linear functions, such as Reiber formulae [14] (reference values, see the graph [14], or IgG Quotient $< Q_{\text{Lim}}\text{IgG}$, and $\text{IgG}_{\text{Loc}} = 0$), which take into account the blood-brain damage. The use of other formulae, such as the renowned-among-clinicians Link Index (reference value, < 0.70) [12], is optional.
 - 4.3.3.12 Methods allowed for albumin and IgG determination are: nephelometry, turbidimetry, and radial immunodiffusion.
- 4.3.4 Other analytes.
- 4.3.4.1 The determination of CSF lactate is optional. This determination is particularly useful in suspected meningitis/meningoencephalitis, when antibiotics have been administered before lumbar puncture, with the result of normalizing CSF glucose. Lactate concentration in CSF is independent from the serum concentration
 - 4.3.4.2 The determination of CSF myelin basic protein is to be considered useless (no Italian centre measures it).

- 4.3.4.3 The determination of CSF total IgA and IgM can be useful for the diagnosis of neurotuberculosis (IgA), or neuroborreliosis (IgM), although these diseases are currently diagnosed with more specific tests. The routine determination of CSF total IgA and IgM is optional, though not cost-effective.
- 4.3.4.4 The CSF determination of IgA, IgM, kappa and lambda light chains, and myelin basic protein for the diagnosis of multiple sclerosis was put “under observation” by the European ad hoc consensus report (optional tests) [3]. After ten years, papers that support the routine determination of such analytes have not been published. All these tests remain thus optional (and not cost-effective).
- 4.3.4.5 The determination of antigen-specific antibodies in paired serum and CSF samples in viral meningoencephalitis and other CNS infectious diseases should be evaluated taking into account the rationale underlying the request: PCR guarantees rapid results, with high sensitivity and specificity, early after the disease onset, whereas indexes for antigen-specific intrathecal antibody synthesis become positive later. In some cases, such as in neuroborreliosis, antibody determinations are fundamental for the diagnosis.
- 4.3.4.6 The determination of anti-measles-rubella-varicella zoster antibodies in serum and CSF in multiple sclerosis, other “evolving optional tests” of the European consensus report [3], could be restricted to the rare cases that are difficult to classify.
- 4.3.4.7 Direct consequence of points 4.3.4.5 and 4.3.4.6 is that the number of antigen-specific antibody determinations per year is likely small. Accordingly, these determinations should be performed in specialised laboratories (i.e., Institutes for Infectious Diseases).
- 4.3.4.8 Other occasional tests that belong to systemic autoimmunity, and the determination of various analytes, such as D-dimers, tumour markers, ACE etc., are not considered for standardization. These tests are usually performed outside the laboratories of neuroimmunology.
- 4.3.4.9 Other neurological tests, such as protein τ , hyperphosphorylated protein τ , protein 14.3.3, amyloid β_{1-42} , NSE, asialo-transferrin, anti-interferon-beta antibodies – tests that are not routinely performed in all the Italian centres – will need standardization in the near future.
- 4.3.5 Calculation of antigen-specific antibody intrathecal synthesis.
 - 4.3.5.1 This calculation can be used for every antigen-specific antibody for which the concentration, or titre, can be determined.
 - 4.3.5.2 The different affinity for the antigen that characterised specific antibodies in serum and CSF, whether in the patients, or in controls, is one of the principal causes of analytical imprecision. This imprecision could affect and it should be considered in studying control reference values.

- 4.3.5.3 One of the simple approach to the topic is here reported:
- i) Determine total IgG concentration in serum and CSF.
 - ii) Dilute the serum sample to the same total IgG concentration in CSF.
 - iii) Test the samples in ELISA, and express the results in optical densities (OD).
 - iv) If the ratio OD_{CSF}/OD_{serum} is $> 1.5-2.0$, there is an indication for intrathecal synthesis (in theory, a ratio $OD_{CSF}/OD_{serum} > 1$ could be per se indicative for intrathecal synthesis, but the value is increased for compensating the imprecision due to the determination of total and specific IgG (see point 4.3.5.2) and the dilution factor).
- 4.3.5.4 For the use of Antibody Index, which has been proposed by H. Reiber, please consult the original protocol [15].
- 4.3.5.5 Typing antigen-specific oligoclonal bands (for example, with isoelectric focusing and immuno-affinity mediated capillary blotting) [16] can be useful as well as quantitative indexes (points 4.3.5.3 and 4.3.5.4), with similar diagnostic sensitivity, at least in herpetic encephalitis [17]. The main shortcoming of these qualitative methods is the scarce availability of antigens and the relatively time-consuming methodologies.

4.4 Determination of oligoclonal bands

- 4.4.1 Oligoclonal bands are the most sensitive index for immunoglobulin intrathecal synthesis.
- 4.4.2 Isoelectric focusing is the most sensitive method for oligoclonal band determination [3, 6], which should be “preferentially” used for the diagnosis of multiple sclerosis, in accordance with the guidelines by McDonald and Colleagues [18].
- 4.4.3 Agarose or polyacrylamide gel for isoelectric focusing, whether *home made* or commercial, are allowed.
- 4.4.4 Commercial gels include: gels with standard dimensions, gels with intermediate dimensions (midi-gels), and gels with small dimensions (mini-gels). Mini-gels allow sparing reagents, and automation, but in the literature there is an indication for not using these gels [19]. Mini-gels could be admitted after acceptable results from external quality control schemes.
- 4.4.5 Paired serum and CSF samples should be run on adjacent lanes.
- 4.4.6 The same serum and CSF IgG amounts are to be sewed. This allows the best comparison between paired serum and CSF samples, different samples, and negative and positive controls. Moreover, it is also a further control for IgG determinations, as the staining intensity is proportional to the amount of sewed IgG.
- 4.4.7 Serum – and, if necessary, CSF – samples should be appropriately diluted taking into account the analytical sensitivity of the staining protocol.
- 4.4.8 To concentrate CSF samples is not allowed.

- 4.4.9 Staining protocols should allow IgG immunodetection (peroxidase, avidin-biotin amplification, chemiluminescence).
- 4.4.10 Published protocols are recommended for blotting and staining procedures [20, 21].
- 4.4.11 The use of a pH marker for monitoring isoelectric focusing run is optional.
- 4.4.12 The standardization of isoelectric focusing runs is almost impossible due to a number of factors that can vary between runs (gel conductivity, electrode imbibition, ampholine quality, electroendosmosis, etc.). High voltages are recommended and, consequently, efficient cooling systems of the migration chamber. The modifications of electrical parameters over time allow deciding when the run should be stopped (for example, when ampere values reach a low level that does not modify for 10 minutes). A run that is stopped early causes the entire, or partial, lack of band focalization, whereas an excessively prolonged run causes distorted bands.
- 4.4.13 If agarose gels are used, the elimination of water that electroendosmosis drain at cathode is recommended by means of absorbent paper.
- 4.4.14 Nitrocellulose sheets (immunoblotting), or films (chemiluminescence) should be filed.

4.5 Interpretation of isoelectric focusing results

- 4.5.1 Densitometers are not required (human eyes are more sensitive).
- 4.5.2 It is preferable that two skilled people interpret isoelectric focusing results.
- 4.5.3 Interpretative criteria are those by the European consensus report [3]:
 - i) Type 1, normal polyclonal IgG distribution (absence of oligoclonal bands).
 - ii) Type 2, oligoclonal bands exclusively in the CSF, which are characteristic of chronic inflammation in the CNS (multiple sclerosis).
 - iii) Type 3, oligoclonal bands exclusively in the CSF, in addition to bands that are equal in serum and CSF, which are characteristic of acute inflammation in the CNS (encephalitis). In these pathological conditions, immune response maintains an important systemic component.
 - iv) Type 4, oligoclonal bands that are equal in serum and CSF (“mirror pattern”), which are characteristic of systemic inflammation, with or without manifest participation of the CNS. In these pathological conditions, systemic bands passively transfer into the CNS compartment (absence of intrathecal IgG synthesis).
 - v) Type 5, bands that are equal in serum and CSF with a “ladder” aspect (regular space intervals between bands, and decreasing band intensity from cathode to anode), which are characteristic of monoclonal gammopathies.
- 4.5.4 Type 2 and 3 only associate with intrathecal IgG synthesis.

- 4.5.5 In addition to the 5 canonical patterns, the report could contain the presence of a CSF single band, with or without bands that are equal in serum and CSF. The interpretation of CSF single bands has not been standardized so far, and, above all, it has not been established if the band is possibly associated with intrathecal IgG synthesis. The examination of a subsequent CSF sample, obtained after at least more than six months from the first one, better establish the presence of intrathecal IgG synthesis, if CSF oligoclonal bands are found.
- 4.5.6 The interpretation of the so-called “weak” band cannot be standardized, given that subjective opinion is preponderant. Indeed, “weak” bands are reported when their recognition is critical and uncertain. “Weak” bands do not have impact on the reports when minimum criteria for intrathecal IgG synthesis are satisfied, i.e. when at least two CSF sharp bands are detectable. More critical is the case when the report’s positivity for oligoclonal bands depends on “weak” bands only. A possible recommendation is to test a larger CSF volume, although this procedure increases, in parallel, the intensity of both band and polyclonal background. The analysis of a second CSF sample, which is obtained after at least more than six months from the first one, is indicated if fit the diagnostic procedure.
- 4.5.7 The blood-brain barrier damage, whether real, or as artefact caused by a traumatic lumbar puncture, reduces the chance of detecting oligoclonal bands, as serum-derived IgG increase the polyclonal background. This information should be reported.
- 4.5.8 The European consensus report [3] does not address the possibility that, in the case of oligoclonal bands equal in serum and CSF, some of the CSF bands are more intense than their serum counterpart (after having sewed the same amount of serum and CSF IgG). Theoretically, these bands are possibly the result of intrathecal IgG synthesis. However, the procedures of serum and CSF IgG determination, and of sample dilutions make the comparison between the intensities of serum and CSF bands uncertain. Accordingly, CSF bands more intense than their serum counterparts should not be considered as the product of intrathecal IgG synthesis. The analysis of a second CSF sample, which is obtained after at least more than six months from the first one, is indicated if fit in the diagnostic procedures.
- 4.5.9 The determination of non-IgG oligoclonal bands – i.e., IgA, IgM, kappa and lambda chains – is basically performed with silver staining.
- 4.5.10 The inter-laboratory reproducibility for oligoclonal bands is low, although the whole report’s interpretation (i.e., presence/absence of oligoclonal bands) has good inter-laboratory agreement [22]. This literature indication has been confirmed by 2003 external quality control scheme promoted by the Italian Association for Neuroimmunology.

5.0 QUALITY CONTROL AND SAMPLE STORAGE

5.1 Biochemistry and proteins

- 5.1.1 Each analytical run should have an internal control (aliquots of pool of CSF samples that are stored at -20°C , preferably at -80°C).
- 5.1.2 The results of internal quality controls (serum and CSF) should be reported on paper and/or disk (for example, control chart in accordance with Shewhart-Levey-Jennings [23]).
- 5.1.3 Acceptable deviations from target values are 25% for serum and CSF albumin, 30% for serum and CSF IgG, and 30% for albumin and IgG quotients.
- 5.1.4 An external quality control should be performed, with at least yearly frequency.
- 5.2 Isoelectric focusing
 - 5.2.1 Each analytical run should have a positive and a negative control for oligoclonal bands. An appropriately diluted serum with monoclonal IgG paraprotein can be used as a positive control.
 - 5.2.2 An external quality control should be performed, with at least yearly frequency.
- 5.3 External quality control schemes that are not promoted by AINI are optional.
- 5.4 Other determinations performed with commercial kits: manufacturer's instructions should be applied.
- 5.5 Serum and CSF samples should be stored in aliquots at -20°C , preferably at -80°C .
- 5.6 The storage of mRNA from serum and CSF mononuclear cells, or themselves cells in DMSO and liquid nitrogen is optional.
- 5.7 Paper or disk files should contain patients' essential data and diagnostic suspects.

6.0 REPORTS

- 6.1 Reports should contain the following information:
 - 6.1.1 Site of CSF sampling.
 - 6.1.2 CSF tube used for analysis (unique, third, etc.).
 - 6.1.3 CSF appearance and colour before and after centrifugation.
 - 6.1.4 Methods used for the biochemical and immunological determinations (isoelectric focusing included).
 - 6.1.5 Formulae used for the quantitative expression of the intrathecal IgG synthesis.
 - 6.1.6 Type of chamber used for CSF cell count.
 - 6.1.7 Reference values for each determination, formulae, and CSF cells.
 - 6.1.8 Qualitative description of the results from cytocentrifuge/sedimentation chamber, and isoelectric focusing.
 - 6.1.9 Comments (optional).
- 6.2 Reference values should be calculated on control groups for each laboratory. Alternatively, data from the literature could be used. Age-

dependent reference values should be reported. Particularly, blood-brain barrier permeability depends on age (see point 4.3.3.8). In infants, the degree of barrier maturation is unpredictable, and clinico-laboratory correlations are very difficult to make. In people older than 60 years, mild blood-brain barrier damage – i.e., an albumin quotient up to 2.0% – is physiological.

- 6.3 There is no consensus on how to report on the presence of unique to CSF oligoclonal bands. The proposal is to express the results on the basis of the following 5 conditions:
- i) Absence of bands.
 - ii) Presence of single band.
 - iii) Presence of two bands.
 - iv) Presence of some bands ($n = 3-6$).
 - v) Presence of several bands ($n > 6$).
- 6.4 Reports may contain the sentence: “This laboratory follows procedures and methods that are standardized on behalf of Italian Association for Neuroimmunology (revision, March 2004) and complies with the quality control schemes promoted by the itself association”.

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Diagnostics of Paraneoplastic Neurological Syndromes

1.0 GROUP COMPOSITION

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2.0 INTRODUCTION

2.1 General section. The laboratory for the diagnosis, monitoring and therapy of paraneoplastic neurological syndromes is a specialized laboratory, in which monospecialistic investigations require high-level professional expertise. The techniques used for this purpose can also be used for research. The array of autoantibodies involved in paraneoplastic neurological syndromes widened in the last years, but only a few of these antibodies are well characterized and associated with definite clinical syndromes. Only for these few well-characterized antibodies, diagnostic sensitivity and specificity can be reported.

2.2 Special section. We separated tests with high diagnostic specificity, and tests with undefined diagnostic specificity.

a) tests with high diagnostic specificity:

i) Determination of anti-neuronal nuclei antibodies (ANNA-1/anti-Hu; ANNA-2/anti-Ri)

ANNA-1/anti-Hu, in paraneoplastic sensitive subacute neuronopathy:
sensitivity, 82%

specificity 99% [1]

ii) Determination of anti-Purkinje cell cytoplasmic antibodies (PCA-1/Yo)

PCA-1/anti-Yo in paraneoplastic subacute cerebellar degeneration:
sensitivity, 80%

specificity, 99% [2]

b) tests with undefined diagnostic specificity:

- i) Determination of anti-amphiphysin antibodies (Anti-128kD)
- ii) Determination of anti-Tr antibodies.
- iii) Determination of anti-Ma2 antibodies (anti-Ta).
- iv) Determination of anti-CV2 antibodies.

The test for antibody determinations complies with an internationally agreed protocol (meeting at Rotterdam, 1994), which has been published in form of consensus report [3]. The protocol includes **immunohistochemical techniques** and **dot blots** that are performed with neural proteins. The cut-off for specificity on immunohistochemistry is the positivity of a $\geq 1:500$ diluted serum. However, some laboratorists claim that immunohistochemical techniques alone can be enough for the antibody recognition, on condition that skilled laboratorists interpret the immunohistochemical picture [4]. Immunohistochemical techniques typically are “home made” techniques [3]. However, commercial immunohistochemical kits proved to work when tested in external quality control schemes (INSTAND). On the contrary, commercial dot blots with recombinant antigens are currently used for positivity confirmation of well-characterized antibodies. “Home made” Western blots, which are obtained with cerebellar homogenates, is prevalently used for research purposes. The availability of commercial dot blots has induced some laboratorists to use such blots without previous immunohistochemical screening of serum/CSF samples. If immunohistochemical techniques are able to correctly identify the presence of anti-neuronal paraneoplastic autoantibodies, a positive dot blot alone is not sufficient for a positive report. Therefore, it is recommended that immunohistochemical techniques should be used as screening tests, and dot blots as confirming tests. Antibody reactivities are defined as typical (well-characterized antibodies), and atypical (antibodies to yet-unidentified/uncategorized neuronal antigens, as detected on Western blot of neuronal proteins). Well-characterized antibodies include anti-Hu, Yo, CV2, Ri, Ma2, amphiphysin. A group of partially-characterized antibodies, which include anti-Tr, ANNA3, PCA2, Zic4, and mGluR1, awaits studies that will possibly convert each partially-characterized antibody into a well-characterized antibody.

3.0 PREANALYTICAL PROCEDURES

- 3.1 Blood is collected in tubes without anticoagulant. No food restrictions are required.
- 3.2 Tubes should be centrifuged, after clotting, as soon as possible (10 min at 1500 g).
- 3.3 Serum samples are stored at 2-8°C until analysis for one month, or at -20°C for longer periods.
- 3.4 Frozen serum samples should not be thawed and frozen again.
- 3.5 Serum samples may be decomplexed at 56°C for 30 min (optional).
- 3.6 Grossly hemolyzed or lipemic samples should be discarded.

4.0 ANALYTICAL PROCEDURES

4.1 **Immunohistochemistry.** This is a screening technique that, when positive, should be followed by dot/Western blot on neural proteins. It has been established that: i) only the merged results from immunohistochemical morphology and dot/Western blot guarantees certain and definite reports; ii) dot blot-positive cases that are negative on immunohistochemistry represent false positive; iii) immunohistochemistry allows the identification of neural antigens that are not represented on dot blots (recombinant proteins), and could be studied with Western blot.

4.1.1 Preparation of the tissue

- 4.1.1.1 Rat cerebellum is currently used (primate cerebellum in commercial kits). When the presence of anti-Ma2 antibodies is suspected, the serum sample should be tested on human cerebral cortex. The optimal procedure for obtaining rat cerebellum implies Lewis rats' perfusion, but the use of cerebellum from non-perfused rats is allowed.
- 1.1.1.2 Rats are perfused with intracardiac injection of 4% paraformaldehyde. Alternatively, post-fixation of cerebellum/cerebral slices with acetone/methanol at 4°C for 5-10 min is allowed.
- 1.1.1.3 Cut 10 um-thick slices of cerebellum and let them dry on glass slides.
- 1.1.1.4 Slides can be used, or stored at -80°C.

1.1.2 Immunoperoxidase

- 1.1.1.1 Reagents: Acetone, 33% peroxydase hydrogen, Triton X-100, NaCl, NaH₂PO₄, Na₂HPO₄, diaminobenzidine (DAB), peroxydase-conjugated rabbit antiserum anti-human IgG, normal rabbit serum (in any case, the serum from the animal from which the anti-human antiserum is obtained should be used), distilled water.
- 1.1.1.2 Preparation of the reagents (reagents to points i-v can be stored for about one month):
 - i) Diluting serum: normal rabbit serum, 2 mL; 10% Triton X-100, 0.36 mL; phosphate buffer 240 mM (pH 7.4); NaCl 4M, 1.350 mL; distilled water, 1.290 mL; final volume, 6 mL. Store at +2/+8°C.
 - ii) Phosphate buffer (240 mM, pH 7.4): Dissolve NaH₂PO₄, 28.8 g in 800 mL of distilled water, and then up to a final volume of 1000 mL. Dissolve Na₂HPO₄, 34.08 g in 800 mL of distilled water, and then up to a final volume of 1000 mL. Add NaH₂PO₄ to 900 mL of Na₂HPO₄ up to a pH 7.4. Store at room temperature.

- iii) Triton 10%: Dissolve Triton X-100, 10 mL in 90 mL of distilled water. Stir with a magnetic bar for 15 min. Store at room temperature.
- iv) NaCl 4M: Dissolve NaCl, 233.6 g in 800 mL of distilled water up to a final volume of 1000 mL.
- v) Washing solution: Mix NaCl 4M, 112.5 mL; Triton 10%, 30 mL; phosphate buffer, 83 mL.
- vi) Chromogen substrate: DAB should be prepared immediately before use. Add a drop of liquid DAB to phosphate buffer, 1 mL.

1.1.1.3 Samples and controls:

- i) Dilute 1:500 samples and positive and negative controls with the dilution serum that, in turn, has been previously 1:2 diluted with distilled water. Cerebrospinal fluid samples are 1:10 diluted.

1.1.1.4 Procedures:

- i) Prepare the tubes that should be used, and mark them with a progressive number.
- ii) If frozen, let slides thaw.
- iii) Inhibit endogen peroxydase by putting the slides in acetone and 0.33% H₂O₂ for 5 min. Let the slides dry.
- iv) Put the slides into a humid chamber, and add dilution serum (blocking serum); incubate for 30 min.
- v) Discard the dilution serum, and add the patient's serum (100 uL); incubate for 1 hour in a humid chamber.
- vi) Wash the slides twice with washing buffer (5 min each), and remove the buffer.
- vii) Incubate with anti-human IgG for 30 min that has been previously 1:50 diluted with the dilution serum.
- viii) Repeat point vi.
- ix) Incubate the slides with DAB and 0.03% H₂O₂ for 5-15 min.
- x) Wash the slides twice with phosphate buffer (5 min each).
- xi) Put the slides into distilled water for a few minutes before mounting with aqueous mounting medium.

1.1.1.5 Detecting techniques allowed:

- i) Indirect immunoperoxydase.
- ii) Immunoperoxydase with avidin-biotin amplification.

1.1.1.6 Titers:

- i) Positive samples should be titered.

1.1.3 **Indirect immunofluorescence**

1.1.3.1 Reagents: PBS (phosphate buffer) 1x, Acetone, Triton X-100, fluoresceinated goat anti-human IgG, goat normal serum (in any case, the serum from the animal from which the anti-human antiserum is obtained should be used), bovine serum albumin (BSA) 1% in PBS.

1.1.3.2 Samples and controls:

- i) See point 4.1.2.3.
- 1.1.3.3 Procedure:
 - i) Prepare tubes that should be used, and mark them with a progressive number.
 - ii) If frozen, let slides thaw.
 - iii) Wash the slides with PBS for 5 min.
 - iv) Put the slides into 1% BSA in PBS for 5 min.
 - v) Put the slides into a humid chamber, and add 1:10 PBS-diluted goat normal serum plus 1% BSA for 30 min at room temperature.
 - vi) Discard the serum and incubate the slides with sera and controls, which have been previously 1:100 diluted with 1% BSA in PBS for 1 hour at room temperature.
 - vii) Wash twice with 1% BSA in PBS for 5 min.
 - viii) Incubate with fluoresceinated anti-human IgG, which has been diluted 1:50/1:30 in PBS, for 30 min.
 - ix) Wash twice with PBS for 5 min.
 - x) Mount with aqueous mounting medium.
- 1.1.3.4 Titers:
 - i) Positive samples should be titered.

1.2 **Dot blot.** Commercial kits are available for confirming the presence of typical autoantibodies in paraneoplastic neurological syndromes. Recombinant proteins are fixed onto nitrocellulose stripes. The typical antigens that can be identified are: CDR62 (Yo), Nova-1 (Ri), HuD (Hu) and amphiphysin. Human IgG represent the internal control antigen. Neuronal antigens are purified by affinity, separated with SDS-PAGE, and immobilized onto nitrocellulose. Autoantibodies are detected with an indirect immunoenzymatic reaction. These commercial kits are routinely used.

1.2.1 Procedure: Follow the manufacturer's instructions. Do not use after the expiring date.

1.3 **Western blot.** Western blot of cerebellum/neuronal cell homogenates can be routinely used as an alternative to dot blot, although Western blot can be standardized and interpreted with difficulties [5]. Therefore, it is preferably used when a serum sample is positive on immunohistochemistry screening and negative to commercial dot blots, or for research purposes. Current diagnostics may only include immunohistochemistry/immunofluorescence and dot blot.

1.3.1 **Preparation of cerebellum homogenate.** Many and non-standardized methods for Western blots of cerebral homogenate are described in the literature: here we cite one of them [6], with the classical references for SDS-PAGE [7] and blotting [8].

1.3.2 **Preparation of neuronal cell homogenate.** Reference methods are those by Blomstrand and Hambeger [9], and Yanagihara and

Hamburger [10], for the preparation, respectively, of homogenates of cortical neurons and Purkinje cells. It is recommended that the procedures be performed at 4°C, no more than 16 hours after death.

5.0 QUALITY CONTROL AND SAMPLE STORAGE

5.1 Immunohistochemistry

- 5.1.1 In every analytical run, a positive and a negative control should be used.
- 5.1.2 If the positive control gives no staining, the analytical run should be repeated. If the negative control gives staining, it is useful to repeat the analysis.
- 5.1.3 External quality control schemes should be at least yearly performed.

5.2 Dot blot

- 5.2.1 Kits include an internal control (for anti-human IgG).
- 5.2.2 In every analytical run, a positive control is included.
- 5.2.3 External quality control schemes should be at least yearly performed.

5.3 Samples are stored in aliquots at –20°C, preferably at –80°C.

5.4 Samples used as positive controls should be stored at –80°C to obtain a constant degree of reactivity.

5.5 Do not store diluted sera.

6.0 REPORTS

- 6.1 The presence/absence of autoantibodies in the biological sample (serum, or paired serum and cerebrospinal fluid) should be reported.
- 6.2 Reports include immunohistochemistry, dot blot and, whenever the case, Western blot.
- 6.3 Reports should contain the following information:
 - i) Animal from which the tissue is obtained.
 - ii) How the tissue is obtained (perfusion of the animal, or post-fixation).
 - iii) Method for antibody detection (peroxydase, fluorescence, or variants).
 - iv) Dilution factor of serum/cerebrospinal fluid samples.
 - v) Name of the manufacturer of dot blot.
 - vi) Comment (optional).
- 6.4 Positive immunohistochemistry reports should describe the cellular structures against which autoantibodies are directed. The antibody titer should also be reported.
- 6.5 When the interpretation of immunohistochemistry results is difficult, it is useful to send the serum and digital pictures to expert laboratories participating in the network.

- 6.6 Reports may contain the sentence: “This laboratory follows procedures and methods that are standardized on behalf of Italian Association for Neuroimmunology (revision, March 2004) and complies with the AINI quality control schemes”.

7.0 BIBLIOGRAFIA ESSENZIALE

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Diagnostics of Dysimmune Peripheral Neuropathies

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2.0 INTRODUCTION

2.1 General section. The laboratory for the diagnosis of dysimmune peripheral neuropathies is a specialized laboratory, for which monospecialistic investigations require high-level professional expertise. The presence of anti-glycolipid antibodies (S-glucuronyl-paragloboside and S-glucuronyl-lactosaminyl-paragloboside) was firstly described in IgM paraproteinemic neuropathies about 20 years ago. After this first description, autoantibodies against more than 20 different glycolipids have been reported in a wide array of acute and chronic neuropathies. The study of the relationships between anti-glycolipid antibodies and neuropathies has fostered researches that have been mainly addressed clinical and serological observations, rather than experimental investigations. The association between particular clinical phenotypes and anti-glycolipid antibodies produced studies on their possible pathogenetic role, and a rationale for subsequent researches. In parallel with clinical studies, the use of tests for anti-glycolipid antibody determination in serum samples of patients with suspected dysimmune peripheral neuropathies has increased, whether to research, or diagnostic purposes. Gangliosides are part of a large family of glyco(sfingo)lipids that are characterized by a molecule of sialic acid that is bound to an oligosaccharidic core. Gangliosides are widely represented on the bimolecular lipidic layers that characterize all the cell membranes.

2.2 Special section. The determination of serum anti-ganglioside antibodies implies technical difficulties with many possible variations due to antigen extraction and purification, different methods, and reference values. The most widely used method for autoantibody screening is an ELISA, and the above-mentioned factors contribute to increase the inter- and intra-laboratory variability. Indeed, the analysis of the more than 200 papers published on this topic reveals a very high variability in the association between anti-ganglioside antibodies and dysimmune peripheral neuropathies. For examples, anti-GM1 IgM are described in 20-80% of chronic motor neuropathies. On the contrary, anti-GM1 IgG are reported,

with good agreement between authors, in 20-30% of patients with acute neuropathies. Differences in methods and non-homogeneous case series can account for the discrepancies in the associations between anti-GM1 IgM and chronic neuropathies. Many attempts have been made for the standardization of ELISAs. The comparisons were made between: a) each local method vs previously published methods [1-3]; b) each local method in multicentric studies [4-5]; c) each local method vs a standard method that was proposed by Inflammatory Neuropathy Cause and Treatment (INCAT), namely an European network of neurological centres devoted to the study of patients with dysimmune peripheral neuropathies [6]. An important conclusion from these studies is that all the laboratories are able to identify sharply positive or negative sera, whereas sera with intermediate titers produce discordant results. To reduce inter- and intra-laboratory variations, INCAT recommends the use of standardized controls, which could reduce false positive and negative, and optimize the classification of patients with borderline autoantibody positivity.

As many anti-ganglioside antibodies are described in the literature, we feel that the standardization should be restricted to the autoantibodies that more frequently associate with definite clinical phenotypes, and, consequently, are important for the differential diagnosis. Other reasons that support our choice are the test feasibility in all the laboratories – those without hyperspecialistic facilities included – and the cost/effectiveness analysis. Therefore, we focus our attention on the determination of **anti-GM1 IgM and IgG** and **anti-GQ1b IgG**. The determination of serum anti-GM1 IgM is useful in the differential diagnosis of chronic motor neuropathies/neuronopathies, and allows discriminating the patients that should be treated efficaciously. To this purpose, the analytical goal of diagnostic accuracy is primarily important, and diagnostic specificity should be pursued more than diagnostic sensitivity. The determination of anti-GM1 IgM has 100% specificity and 38% sensitivity in multifocal motor neuropathy vs motor neuron disorders [7], and 100% specificity and 50% sensitivity vs motor neuropathies [8]. Anti-GM1 IgG and anti-GQ1b IgG share less analytical shortcomings vs anti-GM1 IgM, and associate with definite acute neuropathies, i.e Guillain-Barré syndrome and its variants (Fisher syndrome). Accordingly, the differential diagnostics is less critical than that for anti-GM1 IgM vs chronic neuropathies.

3.0 PREANALYTICAL PROCEDURES

- 3.1 Blood is collected in tubes without anticoagulant. No food restrictions are required.
- 3.2 Tubes should be centrifuged, after clotting, as soon as possible (10 min at 1500 g).
- 3.3 Serum samples are tested after centrifugation, or store at -20°C until analysis.
- 3.4 Store serum samples at -80°C for long periods.

- 3.5 Frozen serum samples should not be thawed and frozen again.
- 3.6 Grossly hemolyzed or lipemic samples should be discarded.

4.0 ANALYTICAL PROCEDURES

4.1 Enzyme linked immunosorbent assay (ELISA) for anti-GM1 IgG/IgM and anti-GQ1b IgG.

4.1.1 Materials and reagents

- 4.1.1.1 Microplates for ELISA.
- 4.1.1.2 Antigens for microplate coating: Monosialoganglioside GM1 (Sigma) and Tetrasialoganglioside GQ1b (Calbiochem).
- 4.1.1.3 Buffers and antisera: bovine serum albumin (BSA), NaCl, NaH₂PO₄, Na₂HPO₄, 3% H₂O₂, citric acid, o-phenyldiamine (OPD) in 10 mg tabs, H₂SO₄, distilled H₂O, peroxidase-conjugated rabbit anti-human IgG/IgM (Dako), Methanol, absolute ethanol.

4.1.2 Reagent preparation

- 4.1.2.1 Antigens for microplate coating. Reconstitute lyophils with methanol: GM1 at the concentration of 1mg/mL, and GQ1b at the concentration of 0.1 mg/mL. Reconstituted antigens can be stored at -20°C for 6 months.
- 4.1.2.2 Non-specific site blocking solution. For 200 mL, dissolve in distilled H₂O: gr 4, BSA; gr 1.2, NaH₂PO₄; 2.4 gr, NaCl (pH 7.4).
- 4.1.2.3 Washing solution. For 500 mL, dissolve in distilled H₂O: gr 1.0, BSA; gr 3.0, NaH₂PO₄; 6.0 gr, NaCl (pH 7.4).
- 4.1.2.4 Stock solutions. Na₂HPO₄ 0.2M: dissolve 2.85 g, Na₂HPO₄ in 100 ml of distilled H₂O (mildly heat to favour salt dissolving). Citric acid 0.1M: dissolve 2.1g, citric acid in 100 ml of distilled H₂O. Solutions are stable at 4°C for 1 month.
- 1.1.1.5 Staining solutions. Use stock solutions. For 25 mL, mix: 6.4 mL, Na₂HPO₄ 0.2M; 12.5 mL, H₂O; 6.1 mL, citric acid 0.1M (pH 5.09. Add an OPD tab 10 min before using (keep in the dark). *Warning!* OPD is carcinogenic: use gloves and chemical hood. Add 100 uL, 3% H₂O₂ immediately before using the solution.
- 1.1.1.6 Staining blocking solutions. Use H₂SO₄ 0.1M: dilute 556 uL, H₂SO₄ 18M in 100 mL of distilled H₂O.

1.1.3 Samples and controls

- 1.1.1.1 Dilute serum samples and positive and negative controls at 1/640 (for anti-GM1), or 1/1280 (for anti-GQ1b) using the blocking solution.

1.1.4 Procedure

- 1.1.4.1 Sew the antigen at the concentration of 1µg/100µL in ethanol, per well, using half microwell plate (48 wells), and letting without antigen the remaining half microwell plate.
 - 1.1.4.2 Incubate the microwell at 4°C overnight, or up to complete ethanol evaporation.
 - 1.1.4.3 Add 200 µL/well of blocking solution, and incubate at 4°C for 4 hours.
 - 1.1.4.4 Remove the blocking solution by aspiration.
 - 1.1.4.5 Add 100 µL/well of samples and controls, each in quadruplicate, i.e., in duplicate in the wells with antigen, and in duplicate in the wells without antigen, and incubate at 4°C overnight.
 - 1.1.4.6 Add 100 µL/well of the blocking solution to two wells with antigen, and to two wells without antigen, to obtain the blank readings.
 - 1.1.4.7 Repeat point 4.1.4.4.
 - 1.1.4.8 Wash the wells with 200 µL/well of washing solution (5 times).
 - 1.1.4.9 Repeat point 4.1.4.4.
 - 1.1.4.10 Add 100 µL/well of anti-human IgG/IgM, which were previously 1/500 diluted in blocking solution, and incubate at 4°C for 1 hour.
 - 1.1.4.11 Repeat point 4.1.4.4.
 - 1.1.4.12 Wash as to point 4.1.4.8.
 - 1.1.4.13 Repeat point 4.1.4.4.
 - 1.1.4.14 Add 100 µL/well of staining solution, and incubate at room temperature for 1 hour.
 - 1.1.4.15 Block staining with 50 µL/well of staining blocking solution.
 - 1.1.4.16 Read with a spectrophotometer at 492 nm, using the wells without sera as blanks.
- 1.1.5 Readings and result interpretation: An optical density (OD) value that expresses samples' absorbance corresponds to each well. The results are obtained by subtracting OD mean values of wells without antigen to OD mean values of the corresponding wells with antigen. OD < 0.1 indicate negative samples, OD between 0.1 and 0.5 positive samples, with the titer that corresponds to the original dilution, OD > 0.5 positive samples that should be titered.

1.2 **Commercial enzyme linked immunosorbent assay (ELISA) for anti-GM1 IgG/IgM and anti-GQ1b IgG.** Commercial kits for the determination of autoantibodies in dysimmune peripheral neuropathies are available (Bühlmann, Allschwil, Switzerland; Grifols-SeraQuest). The microwell plates are ready to use (no antigen coating required), and the array of autoantibodies that can be tested is wider than what we propose for standardization in this document. These kits can be used in diagnostic routine at present, as results from external quality control schemes by AINI are not available.

- 1.2.1 Procedure: Follow the manufacturer's instructions. Do not use after the expiring date.

5.0 QUALITY CONTROL AND SAMPLE STORAGE

- 5.1 Quality control
- 5.1.1 In every analytical run, a positive and a negative control should be used.
- 5.1.2 External quality controls should be at least yearly performed.
- 5.2 Samples are stored in aliquots at -20°C , preferably at -80°C .
- 5.3 Samples used as positive controls should be stored at -80°C to maintain a constant degree of reactivity.
- 5.4 Do not store diluted sera.

6.0 REPORTS

- 6.1 The presence/absence of serum autoantibodies should be reported.
- 6.2 In the case of positive samples, the titer should be reported (the last positive dilution).
- 6.3 Serum samples with OD between 0.1 and 0.5 should be considered as positive at "intermediate" titer.
- 6.4 The report should contain the following general information:
- Type of method (ELISA, with the indication of the source of antigens, or manufacturer of the kit).
 - Reference values: report the dilution at which sera are tested: 1/640 (for anti-GM1), or 1/1280 (for anti-GQ1b).
 - Comment (optional).
- 6.5 Reports may contain the sentence: "This laboratory follows procedures and methods that are standardized on behalf of Italian Association for Neuroimmunology (revision, March 2004) and complies with the quality control schemes promoted by the itself association".

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Diagnosics of Myasthenia Gravis

1.0 GROUP COMPOSITION

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2.0 INTRODUCTION

2.1 **General section.** This document offers indications on laboratory diagnostics and monitoring of myasthenia gravis, and, specifically, deals with the standardization of antibodies to the nicotinic acetylcholine receptor (AChR). Myasthenia gravis is an acquired autoimmune disease that is part of the myastheniform syndromes. These syndromes include neurological disorders that are characterized by muscle weakness and fatigability. Myastheniform syndromes are diagnosed with the support of highly specific serological investigations, such as the determination of anti-AChR and anti-MuSK antibodies for myasthenia gravis, and the determination of voltage-gated calcium channel antibodies in Lambert-Eaton syndrome. Given that these tests are technically complex and require specific expertise, it is advisable that they are performed in specialized laboratories. AChR is located on the post-synaptic membrane of neuromuscular junctions. Serum anti-AChR antibodies are present in 80-90% of patients with myasthenia gravis, and in 40-70% of patients with the ocular form of the disease. Antibody reactivity against the AChR is heterogeneous, as these antibodies recognize each subunit of the receptor. However, they mainly bind the extracellular region of the α subunit, which is called “Main Immunogenic Region”. Antibodies to the γ subunit of receptor’s fetal isoform have also been reported (1-3). Anti-AChR antibody titers do not correlate with the severity of the disease. However, serial determinations in

single patients showed a fairly good correlation between autoantibody titers and worsening of symptoms (4). Anti-AChR antibodies are not detected in 10-15% of patients with myasthenia gravis. In 40-70% of these seronegative patients, the presence of antibodies to the receptor of muscle specific tyrosine kinase (MuSK) has been recently demonstrated (5-7). This receptor is essential in the development of neuromuscular junctions.

2.2 **Special section.** The determination of anti-AchR antibodies represents a fundamental laboratory support for the diagnosis and follow-up of myasthenia gravis (8). The laboratory test that guarantees the best diagnostic performances is the radioimmunoassay (RIA), which can be carried out whether with “home-made” protocols, or with commercial kits. The recommended method for anti-AChR antibodies is a RIA that uses ¹²⁵I- α -bungarotoxin-labeled AChR as the source of antigen. The receptor should derive from human muscle, or human cells that are able to express it (TE671 cells) (9-11). RIA develops into two main parts: the incubation of sera with the antigen, during which stable antigen-antibody complexes will form, and then the precipitation of immunocomplexes by anti-human IgG. The amount of specific autoantibodies is proportional to the immunoprecipitated radioactivity. ELISAs and immunofluorescence methods have been proposed as suitable techniques for anti-AChR antibodies determination, but their analytical performances are worse than those by RIAs.

The determination of anti-MuSK antibodies represents a promising laboratory support for the diagnosis of myasthenia gravis in seronegative patients. Given that a few case series have been reported so far, and the “home-made” methods for their determination are quite complex, we feel that the determination of anti-MuSK antibodies should not be considered as a routine test.

3.0 PREANALYTICAL PROCEDURES

- 3.1 Blood is collected in tubes without anticoagulant. No food restrictions are required.
- 3.2 Tubes should be centrifuged, after clotting, as soon as possible (10 min at 1500 g).
- 3.3 Serum samples are stored at 2-8°C until analysis for 3 days, or at -20°C for longer periods.
- 3.4 Frozen serum samples should not be thawed and frozen again.
- 3.5 Before testing, centrifuge the serum to get rid of any material that could be present in the samples.
- 3.6 The test can be alternatively performed on serum, or plasma samples.
- 3.7 Grossly hemolyzed or lipemic samples should be discarded.

4.0 ANALYTICAL PROCEDURES

4.1 Recommendations for laboratorists. The use of radioactive materials requires that laboratorists comply with established radioprotection rules. All the samples should be treated as they can be potentially infective.

4.1.1 “Home made” RIA. This method requires specific expertise, and technologically advanced laboratories. Accordingly, it is restricted to high-specialized centres.

4.1.1.1 Main reagents: Human muscle, ^{125}I - α -bungarotoxin, anti-human IgG.

4.1.1.2 Instruments and materials: Ultracentrifuge, refrigerated centrifuge, homogenizer, -80°C freezer, precision pipettors, Vortex, system for liquid aspiration, system for collecting/wasting radioactive materials, gamma-counter, polystyrene tubes (3 mL).

4.1.1.3 Extraction of AChR from human muscle: The main protocol is by Lindstrom (12). Briefly, the muscle is obtained from surgical parts, stored at -80°C until used, and homogenized in 0.01 M phosphate buffer (pH 7.4). The homogenate is centrifuged at 100'000 g for 30 min, and the pellet, after resuspension in phosphate buffer containing 2% Triton X-100, is incubated for 1 hour with gentle agitation to make the receptor soluble. After a centrifugation at 100'000 g for 1 hour, the receptor is recovered.

4.1.1.4 AChR labeling with ^{125}I - α -bungarotoxin: AChR is incubated with ^{125}I - α -bungarotoxin to obtain the complexes that will be used as antigen.

1.1.1.5 AChR- ^{125}I - α -bungarotoxin complexes: Anti-AChR antibodies are determined in accordance with the protocol by Lindstrom (12). Briefly, patients' sera and controls (5 uL) are incubated, in duplicate/triplicate, with known amounts of AChR- ^{125}I - α -bungarotoxin complexes (0.1 pmol) at 4°C overnight. The immunocomplexes that are composed of the AChR- ^{125}I - α -bungarotoxin complex and anti-AChR antibodies are, in turn, immunoprecipitated with anti-human IgG (incubation time, 4 hour or overnight). After washing with phosphate buffer, a gamma-counter measures the radioactivity of the immunoprecipitate, and the results are expressed in pmoles of AChR- ^{125}I - α -bungarotoxin complexes that are immunoprecipitated per volume of serum (pmol/mL).

1.1.2 Commercial RIA. Commercial kits (IBL, Hamburg, Germany ; DLD, Hamburg, Germany; RSR, Cardiff, UK) use AChR that is extracted from human muscle, TE671 cells that express AChR in the adult form,

or TE671 cells that express AChR in both the adult and fetal form (γ/ϵ TE671 cells).

- 1.1.1.1 Reagents in the kits: Antigen: ^{125}I - α -bungarotoxin-labeled AChR (lyophilized); positive control: a serum sample with known amount of anti-AChR antibodies, or a series of sera with known amount of anti-AChR antibodies (calibration curve); negative control: pool of normal sera/cut-off control; normal serum for sample dilution; washing buffer; buffer for the reconstitution of the labelled receptor; anti-human IgG. All the reagents should be stored at 2-8°C.
- 1.1.1.2 Instruments and materials: polystyrene tubes (5 mL), Pipettors and tips, refrigerated centrifuge, Vortex, System for liquid aspiration, system for collecting/wasting radioactive materials, gamma-counter.
- 1.1.1.3 Procedures: in accordance with manufacturers' instructions. Briefly, reconstitute the labelled AChR. Incubate sera and controls, in duplicate/triplicate, with the labelled AChR at room temperature for 2 hour. The highest analytical sensitivity is reached by using 5 uL of undiluted serum, or 20 uL of diluted serum (depending on different kits). Add anti-human IgG, and incubate in accordance to each kit's instructions. Wash with washing buffer (1 mL), and centrifuge at 1500 g (2-8 °C) for 20 min. Remove supernates by aspiration, and repeat washing after having suspended the pellets with a vortex. Remove the supernates, and count radioactivity with a gamma-counter. The positive control can be used to monitor the results over time.
- 1.1.1.4 Calculating the results: Radioactivity of the pellet is proportional to the immunocomplexes composed of AChR-labeled-bungarotoxin and anti-AChR antibodies. The results are expressed in pmoles of the AChR-labeled-bungarotoxin complexes that are immunoprecipitated by volumes of serum (in mL). To calculate the results, follow each manufacturer's instructions. Each laboratory should calculate reference ranges by testing an appropriate number of sera from non-myasthenic patients. To validate each analytical run, the value of positive controls should fit the range that has been established by each manufacturer, and not exceed the quality control rules (Shewhart-Levey-Jennings). The correlation between autoantibodies and cpm is linear within the limits of positive controls that are supplied in each kit. Values exceeding these limits should be tested again after appropriate dilution of the sample with normal serum.

5.0 QUALITY CONTROL AND SAMPLE STORAGE

5.1 Test at least one negative control for anti-AChR antibodies.

- 5.2 Test at least 4 serum samples that are positive for anti-AChR antibodies.
- 5.3 If the laboratory joins to external quality controls, sera from these control schemes could be used as internal controls, after the result evaluation from the promoter.
- 5.4 Three organizations offer external quality control schemes (two international, and one Italian). The participation to at least one of the schemes is recommended.
 - 5.4.1 EQAS (European Quality Assessment Trial): EQAS for acetylcholine receptor antibody organizes four dispatches/year of three samples each. Payment is required. For information, go to www.immqas.org.uk.
 - 5.4.2 IBL Quality Assessment Trial: three dispatches/year of four samples each. No payment is required. For information, go to www.IBL.hamburg.com.
 - 5.4.3 Quality control "Dosaggio Anticorpi anti-recettore dell'acetilcolina", coordinated by AINI: three dispatches/year of four samples each. No payment is required. For information, go to www.aini.it.

6.0 REPORTS

- 6.1 The following information should be reported:
 - i) Complete patient's data.
 - ii) Methods for autoantibody determination.
 - iii) Results (in pmoles/mL, or nmoles/L)
 - iv) Reference range.
 - v) Result interpretation.
 - vi) Comment (optional).

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