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An exploratory panel of cerebrospinal fluid biomarkers compared to standard diagnostic evaluation in initial demyelinating events suggestive of multiple sclerosis

Tutor: dott.ssa Maria Donata Benedetti

Dottorando: dott. Alberto Gajofatto

CONTENTS

ITALIAN SUMMARY

Premessa: La diagnosi e il profilo prognostico di un primo evento demielinizzante (ED) del sistema nervoso centrale non sono di immediata definizione. La diagnosi di sclerosi multipla e di altre possibili eziologie è basata su una combinazione di criteri che spesso richiedono un lungo monitoraggio per essere soddisfatti, dal momento che singoli test con elevata sensibilità e specificità al momento dell'evento iniziale sono limitati. I fattori predittivi di attività di malattia e disabilità dopo un primo ED sono conosciuti solo in parte.

Scopo dello studio: Indagare il valore di tau, proteina 14-3-3 e cistatina C come marcatori biologici liquorali di un primo ED suggestivo di sclerosi multipla, oltre al significato prognostico dei dati ricavati dalla normale valutazione diagnostica iniziale.

Metodi: Il liquido cerebro-spinale (LCS) di un gruppo di soggetti con un primo ED suggestivo di sclerosi multipla è stato testato per tau, proteina 14-3-3 e cistatina C. Inoltre, sono stati raccolti dati clinici, neurofisiologici, di risonanza magnetica e dell'analisi liquorale standard. Per confronto, sono stati analizzati anche campioni di LCS di pazienti con neuromielite ottica (NMO) o sindromi correlate, mielite trasversa acuta idiopatica (MTAi), malattia di Creutzfeldt-Jacob (CJ) e patologie prive di una componente neuro-infiammatoria o neurodegenerativa nota (non NIND). I marcatori liquorali sono stati testati sia in termini di significato diagnostico che prognostico. Nei casi di primo ED è stato analizzato anche il valore predittivo delle variabili cliniche, neurofisiologiche e di risonanza magnetica. Per l'analisi prognostica sono stati individuati i seguenti esiti clinici di interesse: recupero del primo ED, recidiva, conversione in sclerosi multipla, livello di disabilità neurologica (punteggio EDSS) raggiunto nel corso dell'osservazione clinica.

Risultati: Sono stati inclusi nello studio 46 pazienti con un primo ED, 6 con NMO, 6 con MTAi, 8 con malattia di CJ e 11 con patologie non NIND. I soggetti con NMO o MTAi avevano livelli di tau significativamente più elevati sia rispetto ai casi con primo ED sia rispetto ai controlli con patologie non NIND, anche se inferiori rispetto ai pazienti con malattia di CJ. I casi con primo ED,

NMO e MTAi sono risultati positivi alla proteina 14-3-3 più frequentemente rispetto ai soggetti con patologie non NIND, sebbene una franca positività alla 14-3-3 sia stata osservata molto più spesso nei pazienti con malattia di CJ. La concentrazione liquorale di cistatina C non differiva tra casi con primo ED, NMO, MTAi e patologie non NIND, ma era significativamente più elevata nei pazienti con malattia di CJ rispetto agli altri gruppi, con l'eccezione del gruppo NMO/MTAi. Dopo un'osservazione mediana di 6,7 anni (1,5-15,3), 39 casi con primo ED sono evoluti in sclerosi multipla, mentre 7 sono rimasti monofasici. Tau, 14-3-3 e cistatina C non sono risultate correlate con la gravità e il recupero del primo ED, con il rischio di conversione in sclerosi multipla né con la successiva disabilità e il tasso di ricadute nei pazienti con sclerosi multipla. L'esordio clinico grave era associato in modo indipendente al recupero incompleto del primo ED; la presenza di almeno tre lesioni periventricolari alla risonanza magnetica iniziale era predittiva di recidiva e conversione in sclerosi multipla; mentre il coinvolgimento piramidale all'esordio e il numero di recidive correlavano con la disabilità a lungo termine nei casi di sclerosi multipla.

Conclusioni: La combinazione dei tre marcatori studiati ha fornito indicazioni sui processi patologici alla base di distinte malattie del sistema nervoso centrale. La tau liquorale ha dimostrato una potenziale utilità diagnostica nel differenziare i casi di primo ED suggestivo di sclerosi multipla dai casi di NMO/MTAi. Sebbene il pannello di marcatori proposto non abbia dimostrato un valore predittivo nei soggetti con un primo ED, i dati clinici, neurofisiologici e di risonanza magnetica si confermano in questo studio indicatori prognostici utili in questo gruppo di pazienti.

ABSTRACT

Background: The diagnosis and the prognostic profile of an initial demyelinating event (IDE) of the central nervous system are not straightforward. The diagnosis of multiple sclerosis (MS) and other IDE etiologies stands on a combination of criteria often requiring a long follow-up to be fulfilled, since single tests with high sensitivity and specificity at the time of IDE presentation are limited. Predictors of disease activity and disability after an IDE are only partially known.

Study aim: To investigate the value of tau, 14-3-3 protein and cystatin C as cerebrospinal fluid (CSF) biomarkers of IDEs suggestive of MS, in addition to the prognostic information provided by standard diagnostic evaluation.

Methods: CSF samples of a group of subjects with IDEs suggestive of MS were tested for tau, 14-3-3 protein and cystatin C. Clinical, MRI, neurophysiological, and standard CSF analysis data were also collected. For comparison, CSF from patients with neuromyelitis optica (NMO) spectrum disorders, idiopathic acute transverse myelitis (iATM), Creutzfeldt-Jacob disease (CJD) and non-inflammatory/non-neurodegenerative disorders (NINDDs) was also analyzed. CSF biomarkers were tested for diagnostic and prognostic significance. The predictive value of clinical, MRI, and neurophysiological variables was also analyzed in IDE cases. The outcomes of interest for prognostic analysis were: IDE recovery, relapse occurrence, conversion to MS, and subsequent disability level (EDSS score).

Results: Forty-six patients with MS-like IDEs, 6 with NMO, 6 with iATM, 8 with CJD and 11 with NINDDs were included. NMO/iATM patients showed significantly higher tau levels compared to both NINDDs and IDEs, even if lower compared to CJD cases. IDE and NMO/iATM patients tested 14-3-3 positive more frequently than NINDDs, although frank 14-3-3 presence was seen much more often in CJD cases. CSF cystatin C concentration did not differ between IDE, NMO/iATM, and NINDDs cases, but was significantly higher in CJD compared to other groups, with the exception of NMO/iATM cases. After a median follow-up of 6.7 (1.5-15.3) years, 39 IDE patients converted to MS, while 7 remained monophasic. CSF tau, 14-3-3 and cystatin C were not

correlated to IDE severity and recovery, to risk of MS conversion, or to subsequent disability and relapse rate in MS patients. Severe clinical onset was independently associated with incomplete IDE recovery, the presence of at least 3 periventricular lesions on baseline MRI with relapse risk and conversion to MS, while initial pyramidal involvement and number of relapses with long-term disability in MS cases.

Conclusions: The three combined biomarkers provided indications about the underlying pathological processes in distinct CNS disorders. CSF tau showed potential diagnostic utility in differentiating between IDEs suggestive of MS and NMO/iATM. Although the proposed CSF biomarkers panel seems to lack predictive value for IDEs, clinical, MRI and neurophysiological parameters are here confirmed as useful prognostic indicators in this group of patients.

RATIONALE AND AIMS

An initial demyelinating event (IDE) of the central nervous system (CNS) may be defined as a first-ever episode of acute/subacute neurological disturbance suggestive of primary demyelination in one or more typical anatomical locations, such as optic nerve, spinal cord, brainstem, cerebellum, and cerebral hemisphere. An IDE may be monophasic (as it typically occurs in acute transverse myelitis [ATM] and acute disseminated encephalomyelitis) or may be the inaugural manifestation of a chronic demyelinating disorder of the CNS, such as multiple sclerosis (MS) and neuromyelitis optica (NMO). An IDE with features suggestive of MS is generally indicated as clinically isolated syndrome (CIS) and it represents the most frequent occurrence in the clinical practice.

The differential diagnosis and the prognostic profile of a CIS are not straightforward, since the presenting clinical picture and initial diagnostic evaluation may overlap significantly across different disorders and between patients with good or poor recovery and with mild or severe disease, if MS develops. As a consequence, currently available diagnostic and prognostic tools are often insufficient to draw conclusions in the individual patient. In this view, molecular biomarkers – particularly if analyzed within the cerebrospinal fluid (CSF), which is contiguous to the pathological process – are promising source of information because of their potential to reflect specific biological pathways, to vary according to disease stage, and to be assessed quantitatively. Many molecular markers have been explored and most of them showed poor diagnostic specificity and/or poor prognostic sensitivity, with few exceptions (see below). Among the multitude of candidates, tau, 14-3-3 protein and cystatin C, which have an established diagnostic relevance in selected neurodegenerative disorders, have been studied in CIS and MS, with conflicting results.

Tau is a microtubule-binding phosphoprotein, which plays an important role in axonal locomotion and intraneuronal transport. Tau proteins include six isoforms, with Mr ranging from 45 to 65 kDa, differing for the presence of either three or four repeats in the carboxy-terminal region of the molecule and the presence of one or two inserts in the amino-terminal domain. In normal

conditions, the CSF expresses low levels of tau protein (up to 250 pg/mL), while tau concentration is elevated in neurological disorders characterized by ongoing neuronal and axonal degeneration, such as CJD, stroke, and encephalitis. In AD and other tauopathies, hyperphosphorylated tau accumulates within the neurofibrillary tangles, and is released in the CSF, representing a valuable diagnostic and prognostic indicator of cognitive dysfunction (*Mattsson et al., 2009*). Previous studies generally found higher CSF tau concentration in MS patients with both progressive and relapsing forms of the disease (including CIS), compared to controls (*Kapaki et al., 2000; Martinez-Yelamos et al., Neurosci. Lett. 2004; Bartosik-Psujek and Archelos, 2004; Brettschneider et al., 2005; Terzi et al., 2007; Frederiksen et al., 2011*), although other researchers did not replicate this finding (*Jiménez-Jiménez et al., 2002; Guimaraes et al., 2006; Teunissen et al., 2009*). A correlation between CSF tau and poor short-term outcome of CIS/early MS has been found (*Martinez-Yelamos et al., Neursoci. Lett. 2004; Brettschneider et al., 2006; Frederiksen et al., 2011*), but no predictive value for disability has been shown at the time of initial event.

14-3-3 protein belongs to a family of low Mr (30 kDa) polypeptides, existing in seven known human isoforms (β, γ, ε, η ζ, σ, and θ), which are expressed ubiquitously and are abundant in the human brain, where they represent about 1% of total cytosolic proteins. 14-3-3 protein coordinates adaptation in protein-protein interactions, acts as activator or suppressor, and regulates the subcellular localization of proteins, intervening in important cellular processes such as signal transduction, cell division, growth, adhesion, differentiation, apoptosis, stress response and malignant transformation (*Obsillova et al., 2008*). Owing to their abundant expression, damage to CNS tissue may potentially cause leakage of 14-3-3 proteins into the CSF following cellular disruption. It is largely established that the ε and γ 14-3-3 isoforms are highly expressed in the CSF of patients with CJD, for which 14-3-3 protein detection represents a valuable diagnostic marker (*Hsich et al., 1996; Chohan et al., 2010*), but are also increased in the CSF of subjects with other neurological disorders characterized by active axonal degeneration, such as amyotrophic lateral sclerosis, encephalitis and stroke (*Steinacker et al., 2011*). Notably, in a number of neurodegenerative disorders,

increased CNS expression and/or tissue deposition of 14-3-3 proteins occur in the absence of concurrent CSF release. In Alzheimer's disease (AD), the γ and ε isotypes are overexpressed in several brain regions, 14-3-3ζ is bound to neurofibrillary tangles, and 14-3-3η is a component of amyloid plaques (*Layfield et al., 1996*). However, 14-3-3s are essentially negative in the CSF of AD, with the exception of one study reporting a positive 14-3-3η essay in a small number of patients and one case report (*Wiltfang et al., 1999; Jayaratnam et al., 2008*). In neurodegenerative disorders with sequestration and aggregation of α-synuclein, such as Parkinson's disease and dementia with Lewy bodies, 14-3-3 γ, ε, ζ, and θ isoforms are complexed with α-synuclein, but not expressed in the CSF (*Kawamoto et al., 2002; Berg et al., 2003*). CSF 14-3-3 protein has been also investigated in MS with a wide range of results. In 1999 Satoh et al. initially reported the presence of 14-3-3 protein in the CSF of a patient affected by relapsing-remitting MS in an active stage (*Satoh et al., 1999*). Subsequently, 14-3-3 protein has been detected in the CSF of a certain proportion of subjects with CIS/MS by several (*de Seze et al., 2002; Martinez-Yelamos et al., 2004; Bartosik-Psujek and Archelos, 2004; Colucci et al., 2004; Hein Née Maier et al., 2008*) but not all research groups (*Frederiksen et al., 2011*). Presence of CSF 14-3-3 protein has been associated with a higher risk of MS conversion and subsequent disability in patients with CIS (*Martinez-Yelamos et al., 2001*).

Cystatin C is a protease inhibitor expressed in the CNS by choroidal, leptomeningeal, glial and neuronal cells; it predominantly counteracts the action of cathepsins, a family of lysosomal proteins released by activated microglia and macrophages, and is thought to have a major role in modulating immune cell activation and inflammation-driven cell death (*Reed, 2000*). Cystatin C has been found to be downregulated in the CSF of a number of inflammatory conditions affecting the central nervous system and the peripheral nervous system (*Yang et al., 2009*). There is increasing evidence of a possible role of cystatin C in the pathogenesis of MS. Decreased levels of cystatin C, coupled with increased activity of cathepsin B, were found by Nagai and coworkers in the CSF of MS patients on relapse compared to controls (*Nagai et al., 2000*). In 2006 a study reported that the detection of a 12.5 kDa fragment of cystatin C was 100%

specific for CIS/MS (*Irani et al., 2006*), however other research groups later showed that the truncated peptide was the result of inadequate temperature storage of CSF samples (*Hansson et al., 2007; Del Boccio et al., 2007*). Sladkova and coworkers recently reported lower CSF cystatin C levels in patients with relapsing-remitting MS compared to CIS and control subjects (*Sladkova et al., 2011*). In a previous study our group found increased cystatin C levels in patients with CIS, MS and idiopathic myelitis in remission compared to an internal control reference, using a semi-quantitative immunoblot method. In addition, we observed a significant correlation between CSF cystatin C level and long-term disability in a subgroup of patients with recurrent myelitis and spinal onset MS (*Fiorini et al., 2007; Gajofatto et al., 2010*). In an animal model of CNS demyelination, an increased expression of cystatin C and cathepsin B was found in white matter astrocytes and microglia, respectively, suggesting that during active stages of demyelination, microglia produce cathepsins while astrocytes release their inhibitor, cystatin C; dominance of cathepsins over cystatin C levels may contribute to myelin disruption, persistence of myelin debris, and impairment of remyelination (*Ma et al., 2007*).

In an attempt to clarify the above mentioned issues, the present study aimed at investigating the diagnostic and prognostic significance of CSF tau, 14-3-3 protein and cystatin C, as compared to clinical, radiological and neurophysiological variables in subjects with a CIS.

BACKGROUND

DIFFERENTIAL DIAGNOSIS

As diagnostic tests with sufficient sensitivity and specificity are limited, the definition of distinct IDEs etiologies may remain undetermined – e.g. idiopathic ATM – or require a long follow-up to fulfill current diagnostic criteria of chronic demyelinating disorders, such as MS and NMO. Other types of inflammatory and non-inflammatory disorders including infectious diseases, systemic autoimmune disorders, paraneoplastic syndromes, CNS tumors, and vascular disorders, may mimic an IDE (*Miller et al., 2008*). The clinical picture, medical history, imaging and laboratory tests usually distinguish such conditions one from the other and from inflammatory demyelinating disorders; in this latter group, however, distinct entities (e.g. MS, NMO, idiopathic ATM, etc.) may be difficult to separate, particularly at the time of initial symptoms. Additionally, systemic autoimmune diseases in which CNS manifestations are the initial symptoms can be extremely difficult to differentiate from demyelinating disorders. This is important because these various inflammatory conditions show relevant differences in terms of prognostic profile and treatment response.

Multiple sclerosis

MS is the cause of most IDE cases, being the most common chronic demyelinating disorder of the CNS. Pathogenesis is characterized by a complex interplay between genetic, autoimmune and environmental factors, but etiology is unknown. An IDE with presenting features suggestive of MS (CIS) is the type of onset in patients with the relapsing-remitting course of the disease (about 85% of initial cases), although not all CIS cases necessarily convert to MS. Subjects with primary progressive MS (about 15% of all cases) show insidious neurological deterioration which does not fit CIS definition. MS affects women twice as frequently as men and typically appears between 20 and 40 years of age, although an earlier or later onset may well occur. Typical CIS presentation includes acute partial myelitis (30-50% of cases), brainstem/cerebellum syndromes (25-30%) unilateral optic neuritis (20-25%), and cerebral hemisphere syndromes (5%); more than 20% of CISs present with symptoms and/or signs of more than one

anatomical location (multifocal presentation) (*Confavreux et al., 2000; Tintoré et al., 2005; Mowry et al., J Neurol Neurosurg Psychiatry 2009*). CISs generally present with mild to moderate neurological symptoms, which often recover significantly in few weeks with or without treatment. An IDE with severe clinical features and poor recovery should prompt physicians to also consider diagnosis alternative to MS. Magnetic resonance imaging (MRI) of the brain and spinal cord showing at least one T2-hyperintense, well defined, ovoid, >5 mm diameter signal abnormality in at least 2 typical locations including periventricular, juxtacortical, infratentorial, or spinal cord regions, is strongly suggestive of MS (*Montalban et al., 2010*). Negative or atypical MRI findings warrant further investigations to exclude other disorders (see below). Cerebrospinal fluid (CSF) findings typical of MS include normal biochemical parameters and protein concentration, normal or slightly increased cell count (≤10 cells/μl, predominantly lymphocytes), and evidence of oligoclonal bands or elevated IgG index (demonstrated in around 70% of CIS case, but not specific for MS) (*Sandberg-Wollheim et al., 1990*). Neurophysiological studies support MS diagnosis in case of delayed conduction time with generally preserved amplitude and morphology of visual, somatosensory, motor, and/or brainstem evoked potentials (VEPs, SEPs, MEPs, and BAEPs, respectively), particularly if found within pathways that are not involved clinically, suggesting an ongoing multifocal demyelinating process of the CNS (*Leocani and Comi, 2008*). A diagnosis of MS requires the demonstration of CNS demyelinating lesions dissemination in space and time for which there is no better explanation; dissemination may be proved clinically and/or with the support of paraclinical evidence, particularly brain and spinal cord MRI. Standardized MRI parameters have been incorporated within worldwide accepted diagnostic criteria since 2001 (*Poser et al., 1983; McDonald et al., 2001; Polman et al., 2005*). The most recently published criteria simplified MRI requirements in order to facilitate early diagnosis (dissemination in space and time may be demonstrated with a single scan in some cases) and uniform their application across different populations, apparently preserving sensitivity and specificity (*Polman et al., 2011*). Pathological studies of MS reveal several areas of demyelination, mostly located in the white matter of cerebral hemispheres and in corpus callosum but also

frequently in the brainstem, spinal cord and cerebral cortex. MS plaques are generally centered by a vein or venule and show variable degrees of inflammatory infiltrates, demyelination, axonal loss, and gliosis, depending on disease stage and clinical course. Active plaques are characterized by reduced density of myelinated fibers and irregular ensheathment of axons and are infiltrated by lymphocytes, macrophages and activated microglia, containing intracytoplasmic granules of myelin debris. Four distinct demyelination patterns have been proposed in MS, all characterized by prominent lymphocytes/macrophages infiltrates, plus immunoglobulin and complement deposition (pattern 2), oligodendrocytes apoptosis with preferential reduction of myelin-associated glycoprotein immunoreactivity (pattern 3), or oligodendrocytes loss with even reduction of various myelin proteins reactivity and no sign of apoptosis (pattern 4) (*Lucchinetti et al., 2000*). However, these findings have been independently replicated from other research groups only in part (*Raine, 2008; Hu and Lucchinetti, 2009*).

Neuromyelitis optica

NMO was initially described by Devic in 1894 as the simultaneous occurrence of severe transverse myelitis and bilateral optic neuritis with a fulminant monophasic clinical course, which is currently recognized as the type of onset of rare aggressive forms of the disorder. In fact, NMO typically is a relapsing-remitting condition, in which optic neuritis and acute myelitis occur separately – several years apart from each other in some cases – and tend to be more severe and with poorer recovery, compared to MS. Bilateral involvement of the optic nerve has been described in several cases, and repeated attacks may lead to blindness (*Merle et al., 2007*). Acute transverse myelopathy is the typical clinical presentation of spinal cord involvement and it may cause a significant fixed disability even at the first episode; patients with good initial recovery often experience further spinal relapses with accrual of severe ambulatory disability (*Collongues et al., 2010*). A secondary progressive course is uncommon in NMO (*Wingerchuk et al., 2007*). While it had been thought for long that symptoms implicating other CNS regions excluded a diagnosis of NMO, in the last years a variety of neurological symptoms have been described that prove possible extra-

opticospinal involvement, including neuroendocrine disturbances (hypothyroidism, galactorrhoea and diabetes insipidus due to hypotalamic involvement), intractable nausea and vomiting, refractory hiccup, and reversible posterior encephalopathy (*Matà and Lolli, 2011*). Although it is relatively rare in Europe and North America, NMO is the typical form of demyelinating disease in Asia and Africa. NMO pathogenesis is explained by an inflammatory autoimmune process predominantly against optic nerve and spinal cord tissue. At the pathological level, the lesions show extensive demyelination associated with cavitation, necrosis, and acute axonal injury of both white and gray matter, along with a pronounced loss of oligodendrocytes. The inflammatory infiltrates are composed of macrophages, eosinophils, neutrophils, and rare CD3+ and CD8+ T cells. In addition, there is a prominent perivascular deposition of immunoglobulin (mainly immunoglobulin M) and complement antigens, associated with vascular fibrosis, suggesting the relevance of humoral immunity mechanisms (*Lucchinetti et al., 2002*). In 2004, the identification of a specific immunologic serum marker was a significant step forward in the confirmation of this model. Lennon and coworkers reported that 73% of a group of patients with NMO were seropositive for an antibody named NMO-IgG; this antibody was found in less than 10% of patients with opticospinal MS and no patients with other neurologic or systemic autoimmune diseases (*Lennon et al., 2004*). Shortly after that discovery, NMO-IgG was characterized as an autoantibody selectively binding to the aquaporin 4 (AQP4) water channel, a component of the dystroglycan protein complex located in astrocytic foot processes at the blood-brain barrier and highly expressed in the spinal cord and optic nerve (*Lennon et al., 2005*). Roemer and others noted an early and selective absence of AQP4 immunostaining in NMO lesions, compared with its persistence in acute MS plaques (*Roemer et al., 2007*). Thus, the binding of antibodies to AQP4 may be the initial pathogenic event in NMO. Currently, several NMO-IgG assays exist yielding similarly high specificity (around 100%) but different sensitivities (48-77%). The fluorescence-activated cell sorting assay has the highest sensitivity (77%), followed by visual fluorescence-observation cell-based assay (73%), ELISA (60% with cut-off value of 5.0 U/ml and 70% with cut-off value of 1.6

U/ml), fluorescence immunoprecipitation assay (53%), and indirect immunofluorescence assay (48%) (*Waters et al., 2012*). On MRI acute spinal cord lesions are characterized by central swelling typically extending over three or more vertebral segments. Brain MRI shows signal abnormalities in up to 60% of NMO cases; however, an MS-like pattern is seen very rarely. Brain lesions are generally ill-defined and predominantly located in periventricular areas of brainstem (fourth ventricle, periaqueductal region, and hypothalamus) and cerebral hemispheres, likely reflecting sites of preferential AQP4 expression and explaining symptoms other than spinal cord or optic nerve dysfunction (*Pittock, Weinshenker et al., 2006*). The CSF of patients with NMO often reveals > 50 white blood cells/μl or > 5 neutrophils/μl, typically in the absence of oligoclonal bands and/or an increase in the IgG index. Current diagnostic criteria for NMO require: 1) optic neuritis, 2) acute myelitis, and 3) at least two of the following supportive criteria: a) a contiguous spinal cord MRI lesion extending over ≥ 3 vertebral segments; b) onset brain MRI scan that is not diagnostic for MS; c) NMO-IgG seropositivity. These criteria appear to be an excellent diagnostic tool; however, differentiating between NMO and opticospinal MS is sometimes difficult. For example, some patients present with "incomplete" forms of NMO, such as isolated longitudinally extensive transverse myelitis (LETM), recurrent optic neuritis, or recurrent acute transverse myelitis with or without NMO-IgG positivity and no demonstration of MRI dissemination for a diagnosis of MS.

Idiopathic acute transverse myelitis

Patients presenting acute myelitis not attributable to MS, NMO, systemic autoimmune disease, or infection may have idiopathic ATM. This is largely an exclusion diagnosis, although structured diagnostic criteria have been proposed and applied in research and clinical settings (*Transverse Myelitis Consortium Working Group, 2002; de Seze et al., 2005*). They require: 1) acute bilateral sensory, motor, and/or sphincter spinal cord symptoms, 2) a well defined sensory level, and 3) prove of inflammation by CSF parameters (pleocytosis and/or IgG synthesis) and/or gadolinium-enhancing spinal cord lesion on MRI. Additionally, other conditions (e.g. spinal cord compression or infarct, infection, optic neuritis, MS,

connective tissue disease, arteriovenous malformations, etc.) need to be excluded. A considerable proportion of idiopathic ATM cases experience a preceding vaccination or infectious illness, although the specific etiological microorganism is identified only occasionally. There is no general consensus whether post-infectious transverse myelitis is to be classified as idiopathic ATM or whether it is a distinct disorder. Epitope similarity between microorganisms and CNS proteins (molecular mimicry) is one of the most valid hypothesis to explain the pathogenetic mechanism of idiopathic ATM (*Kerr and Ayetey, 2002*). Idiopathic ATM is frequently severe and may determine significant residual disability. The clinical course is typically monophasic; however, cases of recurrent ATM of unknown cause with no clinical and radiological dissemination outside the spinal cord, absence of CSF oligoclonal bands and NMO-IgG seronegativity have been reported (*Kim, 2003; Ravaglia et al., 2009*). A subset of these patients have serum anti-Ro antibodies (Sjögren syndrome antibody – SSA) in the absence of systemic autoimmune disorder symptoms (*Hummers et al., 2004*).

Acute disseminated encephalomyelitis (ADEM)

ADEM is a severe inflammatory condition of the CNS, which may occasionally be difficult to distinguish from idiopathic ATM, NMO and MS, particularly in the pediatric population. It is characterized by acute or subacute onset of encephalopathy (consciousness alteration, behaviour changes and/or seizures), associated to various combinations of focal symptoms due to involvement of optic nerve, cerebral hemispheres, brainstem, cerebellum, and/or spinal cord. A preceding infectious illness is frequently reported and fever may anticipate or accompany the neurological manifestations. Typical laboratory findings in ADEM include elevations in the CSF protein and white blood cell count (lymphocytes), which are unusual in MS. Elevations of white blood cell count > 50 cells/μl can be seen in both ADEM and NMO, in which however, more than 5 neutrophils/μl are usually revealed. Oligoclonal bands are less frequently observed in ADEM compared to MS, but occasionally can be present. Brain MRI reveals large (>1 to 2 cm in size) confluent T2-hyperintense lesions that are multifocal, located in the supratentorial or infratentorial white matter regions, and

frequently gadolinium-enhancing; gray matter, especially basal ganglia and thalamus, is frequently involved. In rare cases, brain MRI shows a large single lesion, predominantly affecting white matter. Spinal cord MRI may show confluent intramedullary lesions or a single longitudinally extensive lesion with variable enhancement. ADEM pathology is characterized by ill defined areas of demyelination that surround venules and contain inflammatory infiltrates dominated by macrophages. Lesions are dispersed at multiple locations throughout the brain and spinal cord and appear of similar histological age (*Hu and Lucchinetti, 2009*). Although it is typically monophasic, ADEM may relapse either with the same symptoms and anatomical locations as in the initial event (recurrent ADEM) or with involvement of different areas (multiphasic ADEM) (*Krupp et al., 2007*). The response to steroid treatment is generally good and prognosis favorable, with complete clinical and radiological recovery in most cases. Patients with ADEM are at increased risk of developing MS later in life compared to the general population, suggesting a potential pathogenetic link between the two conditions (*Menge et al., 2005*).

Systemic autoimmune disorders

CNS involvement of systemic autoimmune disorders may represent a big challenge for IDEs differential diagnosis, since it can occur as the initial manifestation of the disorder itself (i.e. before appearance of systemic symptoms) and also because autoimmune conditions and inflammatory demyelinating diseases of the CNS may coexist in the same patient. Clinical, imaging and CSF features of neurological complications of rheumatic disorders may be undistinguishable from those of MS, NMO, or idiopathic ATM, although the pathogenetic mechanism is generally driven by vasculitis and/or small arteries thrombosis in the first group rather than demyelination as in the latter. Patients with systemic lupus erythematosus (SLE), antiphospholipid antibody syndrome, Sjögren syndrome, and immune-mediated vasculitides may experience transverse myelitis, optic neuritis, and other symptoms mimicking a demyelinating event, associated with presence of brain MRI abnormalities. Peripheral neuropathy, headache and psychiatric disturbances are frequently observed among or in

association with the neurological manifestations of the above mentioned disorders, while are uncommon in chronic demyelinating diseases such as MS and NMO (*Theodoridou and Settas, 2006*). The predominance of MRI lesions located at the cortical junction and static over time, as well as the concomitant finding of brain infarcts, calcification, or haemorrhages, suggesting small-vessel vasculitis/thrombosis, should always raise the suspicion of systemic immunomediated diseases. In these latter disorders, enhancing lesions and T1 black holes are much less common than in MS. Asymptomatic spinal cord MRI lesions are rare in systemic autoimmune disorders, while lesions spanning over 3 or more vertebral segments can be found in patients with ATM secondary to such conditions and can completely disappear after steroid or immunosuppressive treatment (*Provenzale et al., 1994; Charil et al., 2006*). CSF oligoclonal bands are relatively infrequent in systemic autoimmune diseases compared to MS, and they may be found in association with corresponding bands in the serum (a profile known as "mirror" pattern). Evoked potentials are normal in rheumatic disorders without clinical involvement of the underlying anatomical pathway, and when abnormal, evoked responses are more frequently reduced in amplitude rather than delayed in conduction as in MS (*Ferreira et al., 2005*).

Although subacute meningoencephalitis accounts for the vast majority of its CNS manifestations, Behçet's disease may occasionally include symptoms and signs resembling those of IDEs, such as brainstem syndromes, acute myelitis, optic neuritis, and hemiparesis. Brain MRI abnormalities commonly consist of brainstem and basal ganglia lesions that can be extensive and associated with swelling and enhancement in the acute phase and that can shrink or disappear at follow-up. These lesions are usually associated with regional atrophy of the brainstem. Meningeal enhancement may be observed. In 20–40% of patients with Behçet's disease and CNS involvement, cerebral white matter is diffusely involved. Cerebral venous sinus thrombosis is another manifestation of CNS involvement and can be detected on MRI and/or MRI angiography (*Lee et al., 2001*). Spinal cord imaging may show various degrees of involvement, ranging from one isolated small lesion to a longitudinally extensive abnormality or

multiple cervico-dorsal lesions (*Yesilot et al., 2007*). CSF analysis often reveals modest protein and moderate white blood cell count increase (mostly neutrophils in the acute phase, replaced by lymphocytes in the chronic stage); oligoclonal bands are usually absent, although it has been reported that their presence may be transient (*Al-Araji and Kidd, 2009; Serdaroglu, 1998*). The neuropathology of Behçet's disease in the acute phase is characterized by meningoencephalitis with an intense inflammatory infiltration including neutrophils, eosinophils, lymphocytes, and macrophages, with areas of necrosis and apoptotic neuronal loss. The brainstem, thalamus, basal ganglia, and white matter are all seen to be affected. In the progressive phase, the inflammatory infiltrates remain, although immunohistochemical staining for lymphocytes is less prominent (*Hirohata, 2008*).

Sarcoidosis is a multisystem granulomatous disease of unknown cause. The presence of CNS involvement is reported in 5–10% of patients. The specific manifestations depend on the anatomical location of the granulamatous lesions, although a diffuse cerebral and meningeal involvement is possible. Cranial nerve mononeuropathies (including optic neuritis), spinal cord, brainstem, and cerebellar syndromes are the most challenging differential diagnosis with IDEs. Brain and/or spinal cord MRI is abnormal in about 80% of patients with neurosarcoidosis and may reveal periventricular T2-hyperintense areas, multiple supratentorial and infratentorial brain lesions, a solitary intra-axial mass, optic nerve enhancement, and one or more spinal cord intramedullary lesions. Patients with neurosarcoidosis usually present with a simultaneous enhancement of multiple white-matter lesions; meningeal and punctiform parenchymal enhancement along Virchow-Robin spaces are additional features for differential diagnosis with MS. Another common feature of neurosarcoidosis is the presence of hydrocephalus (*Christoforidis et al., 1999*). Patients with spinal cord and/or optic nerve involvement associated with pituitary/hypothalamic dysfunction may be misdiagnosed as having NMO, especially in case of unremarkable brain MRI. CSF examination in neurosarcoidosis shows a nonspecific pattern of pleocytosis and elevated protein, particularly if meninges are involved; oligoclonal bands may be present and IgG index elevated. CSF levels of angiotensin-converting enzyme,

lysozyme, and beta2-microglobulin can be increased in over half of the patients. At the histological level, the diagnostic hallmark of sarcoidosis is the presence of granulomas in the involved tissue; granulomas are predominantly noncaseating, discrete, and surrounded by epithelioid cells and nodular inflammatory infiltrates consisting of multinucleated giant cells, macrophages, and lymphocytes (*Hoitsma et al., 2004; Terushkin et al., 2010*).

PROGNOSTIC FACTORS

Most of what is known about IDEs prognosis derives from several large studies on CIS patients, while predictive factors of non-MS like IDEs have been mostly explored in relatively small case series. This is greatly due to MS being much more common than other demyelinating disorders, but also to the extreme variability of its clinical picture and natural history and to the availability of several treatment options, compared to other conditions. When patients are diagnosed with an IDE, clinicians have to deal with many questions and challenges regarding not only differential diagnosis, but also the risk of subsequent relapses and disability and decision about disease-modifying therapy, which is likely to be effective in certain patients subgroups but could be superfluous in others (*Roach, 2006*). The following paragraphs will focus on prognostic factors of IDEs, particularly in relation to MS.

Demographic and clinical predictors

Studies of the natural history of IDEs use different groups of patients, either from observational studies or from placebo arms of therapeutic trials.

Recovery of IDEs has been reported to be poorer in patients with older age, severe initial event, spinal cord presentation, and polyregional involvement (*Beck et al., 1992; West et al., 2006; Mowry et al., J Neurol. 2009; Hirst et al., 2012*). In the context of ATM, patients with shock-like symptoms and/or back pain at onset fail to recover in most cases (*Lipton and Teasdall, 1973; Ropper and Poskanzer, 1978*). ADEM usually recover in most cases; however, it has been reported that severity, recovery and mortality is worse in adult compared to pediatric cases (*Ketelslegers et al., 2011*).

After a CIS the rate of conversion to MS may vary greatly in relation to several factors. In a natural history study of a group of CIS patients in London (UK) the percentage who developed clinically definite MS (CDMS) was 43% at 5 years, 59% at 10 years, and 68% at 14 years (*Brex et al., 2002*). The presenting symptoms did not seem to affect the rate of conversion to definite MS. A study of 308 patients from the Gothenburg (Sweden) database found that 45 of 220 patients with a CIS still had a CIS at the 25 year follow-up (*Eriksson et al., 2003*). Patients with efferent lesions – mostly involving pyramidal and/or cerebellar systems – had twice the risk of a later diagnosis of MS than those without efferent lesions (optic neuritis and/or sensory symptoms). Another natural history study of 1215 patients with MS in Lyon (France) found a longer period to the second episode with optic neuritis presentation than with either brainstem or spinal cord presentations (*Confavreux et al., 2003*). In another study, non-Caucasian ethnicity (HR 2.39) and younger age (HR 1.51 for each 10-year decrease in age) were reported to be strongly associated with an increased risk of having a second event within one year of CIS onset (*Mowry et al., J Neurol. 2009*). Data on medium-term outcome in untreated CIS are also available from five major clinical trials: the North American Optic Neuritis Treatment Trial (ONTT) (*Beck et al., 1993*); the Controlled High risk subjects Avonex Multiple sclerosis Prevention Study (CHAMPS) (*Jacobs et al., 2000*); the Early Treatment Of Multiple Sclerosis trial (ETOMS) (*Comi et al., 2001*); the Betaferon/Betaseron in Newly Emerging multiple sclerosis For Initial Treatment trial (BENEFIT) (*Kappos et al., 2006*), and the early glatiramer acetate treatment in delaying conversion to clinically definite multiple sclerosis in subjects Presenting with a Clinically Isolated Syndrome study (PRECISE) (*Comi et al., 2009*). The probability of a second episode during the study period was 17% in ONTT, 38% in CHAMPS, 45% in ETOMS, 45% in BENEFIT, and 43% in PRECISE. According to the results of some of these trials, the chance of developing clinically definite MS was greater in patients with a polyregional onset than in those with a unifocal onset.

Female sex, older age at onset, and less severe motor impairment with the sentinel myelitis event have been reported as predictors of relapse in NMO (*Wingerchuk and Weinshenker, 2003*). The occurrence of a second attack after

ADEM in a pediatric population was associated with optic nerve involvement at onset, family history of CNS demyelination, and complete recovery of the first event (*Mikaeloff et al., 2007*).

The likelihood of long-term irreversible disability is probably the most relevant concern for patients who develop or are at risk of developing a chronic demyelinating disease after an IDE. Irreversible disability may be the result of accumulation of fixed sequelae after one or more attacks or may be due to transition to a secondary progressive phase, in which insidious neurological deterioration substitutes a preceding relapsing-remitting stage of the disease. Secondary progression is common in MS (up to 75% of relapsing-remitting cases), rare in NMO and not observed in other demyelinating conditions such as ATM and ADEM (*Runmaker and Andersen, 1993; Wingerchuck et al., 2007*). Demographic and clinical predictors of disability in CIS patients who later develop MS include male gender, older age at onset, multifocal symptoms, efferent systems involvement, and incomplete remission of the initial event, although not all studies replicated the same findings (*Weinshenker et al., 1991; Runmaker and Andersen, 1993; Confavreux et al., 2003; Tremlett et al., 2006; Debouverie et al., 2008; Koch et al., 2008*). The relevance of age at MS onset as a prognostic factor, for example, is subject to interpretation depending on the temporal frame in which disability levels are captured. Indeed, while older age at onset is associated with a more rapid disability progression – likely due to prevalence of the primary progressive disease course, age-dependent degenerative processes, and dysfunction of repair mechanisms in older subjects – early onset MS patients reach disability milestones at a younger age compared to late onset MS cases, even though in a longer time interval (*Confavreux and Vukusic, 2006; Tremlett et al., 2006*). This has been shown also in the pediatric population (*Renoux et al., 2007*). In this perspective, older age at onset may be viewed as favorable prognostic factor, meaning a longer disease-free interval before MS symptoms occurrence in life and an older age at which significant disability milestones are reached, compared to early onset (*Tremlett et al., 2010*). One single study reported a shorter time to secondary progressive MS in patients with family history of MS (*Hensiek et al., 2007*).

Even though the majority of CIS cases convert to MS at some point, a sizable proportion of MS patients does not accumulate irreversible disability during the entire natural history of the disease. This type of course is known as benign MS, although there is no general agreement on its definition and consequently, on its prevalence in the MS population (*Hutchinson, 2012*). While initial definitions predominantly stressed the absence of significant ambulatory disability (expanded disability status scale [EDSS] score < 3.5 or 2.5) after a reasonable time interval from initial symptoms (10 or 15 years) (*Lublin and Reingold, 1996; Pittock et al., 2004*), more recent studies highlighted the importance of carefully considering cognitive status and quality of life when defining benign MS (*Amato et al., 2008*). Whatever the definition, it has been shown that benign status at 10 years after MS onset persist at 20 or more years in 52-69% of patients, leading to the conclusion that benign MS is a transient condition for a considerable proportion of cases (*Pittock et al., 2004; Sayao et al., 2007; Ramsaransing and De Keyser, 2007*). However, studies addressing this topic are generally limited by the clinic-based design which determines the possibility that MS patients with mild disease who are not seen on a regular basis in the neurology practices, are not included in the analysis falsely reducing the proportion of benign cases. There are no diagnostic tools or validated markers to identify CIS patients who will develop a mild form of MS; however, female gender, younger age, and absence of motor symptoms at onset have been indicated as predictors of a benign course (*Costelloe et al., 2008*).

Long-term prognosis of subjects who develop NMO after an IDE is generally worse than in MS. NMO observational studies are mostly retrospective or cross-sectional and include clinic-based treated populations. Clinical features associated with a poor outcome (severe disability or death) are blindness and/or sphincter involvement at onset, lack of recovery of the first attack, and high relapse rate in the first year of disease (*Wingerchuk and Weinshenker, 2003; Cabre et al., 2009*). A benign course of NMO – defined as EDSS score < 3.5 after at least 10 years from onset – has been described in a small subgroup of the population of an observational study (11 out of total 175 subjects, 41 with disease duration of 10 years or longer); a good outcome was associated with Caucasian ethnicity and lower relapse rate. However, 3 of the 11 patients had a disabling attack later than

15 years from onset, questioning the existence of a truly benign form of NMO (*Collongues et al., 2011*).

Imaging predictors

As mentioned earlier, the presence, number, location, and size of signal abnormalities on brain and spinal cord MRI provide fundamental information for the differential diagnosis of IDEs etiologies. Additionally, baseline MRI features may predict the subsequent clinical evolution of IDEs, serving as a prognostic tool.

In three observational prospective studies with a follow-up of 7, 15 and 20 years, CIS patients with mixed clinical syndromes (one study with optic neuritis only) and an abnormal brain MRI had a risk of relapse – an event that identifies such patients as having CDMS – of 65%, 72%, and 80%, respectively, while in CIS subjects with a normal scan the risk of conversion to CDMS was 8%, 25%, and 20% (*Tintoré et al., 2006; Optic Neuritis Study Group, 2008; Fisniku et al., 2008*). Interestingly, there is a correlation between the number/location of MRI lesions at CIS onset and the risk of a subsequent relapse, allowing to classify patients as at low, medium, or high risk of developing MS. Over a follow-up period of 5 years, CIS patients with 1-3, 4-9, and 10 or more lesions on initial brain MRI have a hazard ratio of converting to CDMS equal to 4.3, 7.4, and 19.3, respectively, compared to patients with no lesions. Moreover, initial MRI fulfilling 1-2, and 3-4 Barkhof's criteria is associated with a hazard ratio of CDMS equal to 6.1 and 17.0, respectively, compared to MRI fulfilling none of the criteria (*Tintoré et al., 2006*). The Barkhof's criteria, which were included in the 2001 and 2005 versions of the McDonald's diagnostic criteria for MS, are: 1) at least one gadolinium enhancing lesion or nine T2-hyperintense lesions; 2) one or more infratentorial lesion; 3) one or more juxtacortical lesion; 4) three or more T2 periventricular lesions. Independent of other MRI features, patients who have at least one infratentorial lesion at onset have an increased risk of MS conversion, the risk being slightly higher in those with a lesion in the brainstem than in those with a lesion in the cerebellum (*Tintoré et al., 2010*). The number of spinal cord lesions at presentation of acute partial transverse myelitis has been associated

with the number of relapses in patients who subsequently develop MS (*Cordonnier et al., 2003*). Among non-conventional MRI measures, the occurrence at presentation or early development of global or regional grey matter atrophy in patients with CIS has been associated with conversion to MS (*Raz et al., 2010; Calabrese et al., 2011; Jenkins et al., 2011*).

The correlation between MRI lesions at CIS presentation and long-term disability in cases who develop MS is less robust than it is between MRI and risk of MS conversion. Some studies have shown that the number of baseline lesions is correlated with the confirmed EDSS score reached up to 20 years later (*Optic Neuritis Study Group, 2008; Fisniku et al., 2008*), while at least another study did not find such a correlation (*Tintoré et al., 2006*). In a study examining patients with initial optic neuritis, disability at 6 years was predicted by the presence at baseline of gadolinium enhancing lesions, spinal cord lesions, and number of infratentorial lesions (*Swanton et al., Neurology 2009*). In a different study including mixed CIS types, brainstem lesions on baseline MRI were associated with higher disability after 7 years (*Tintoré et al., 2010*). A high rate of lesion load increase in the short term after a CIS (particularly in the first 5 years) contributes to the development of subsequent disability (*Li et al., 2006*), but this is not predictable at CIS presentation and may be monitored only in the follow-up phase. Increase of MRI lesion load in the first few years after a CIS has also been associated with conversion to secondary progressive MS over a 20 years follow-up period (*Fisniku et al., 2008*). Overall, the correlation between MRI measures and disability in CIS/MS patient tends to be weak, likely due to conventional MRI detecting both clinically relevant and silent lesions, lacking specificity for distinguishing between the various biological substrates of lesions (edema, demyelination, gliosis, etc.), and lacking sensitivity for identifying potentially important tissue damage within normal appearing white and grey matter (*Barkhof, 2002; Goodin, 2006*).

Non-conventional MRI techniques – such as white/grey matter atrophy quantification, magnetic resonance spectroscopy, magnetization transfer, diffusion tensor, and functional imaging – have shown the ability to detect brain and spinal cord abnormalities in CIS and MS patients, which are not captured by

the classical lesion-based framework of conventional MRI. Findings reported in CIS patients include regional atrophy in cortical grey matter, deep grey matter and spinal cord (*Calabrese et al., 2011; Henry et al., 2008; Audoin et al., 2010; Brex et al., J Neurol Neurosurg Psychiatry 2001*), decreased N-acetyl aspartate in whole brain (*Filippi et al., 2003*) and normal-appearing white matter (*Audoin et al., 2007; Wattjes et al., 2008*), increased myoinositol in normal-appearing white matter (*Fernando et al., 2004*), decreased magnetization transfer ratio in white and grey matter (*Fernando et al., 2005; Rocca et al., 2008*), abnormal diffusion parameters in white matter (*Henry et al., 2009; Raz et al., 2010*), and abnormal functional MRI activation (*Pantano et al., 2002; Filippi et al., 2004; Roosendaal et al., 2010*). Low magnetization-transfer ratios in normal-appearing brain tissue of patients with CIS was reported to be an independent predictor of subsequent disease progression in one study (*Iannucci et al., 2000*), while other studies – with region-of-interest or whole-brain-histogram analysis of magnetization-transfer MRI data – did not confirm such finding (*Kaiser et al., 2000; Brex et al., Am J Neuroradiol 2001*). A functional MRI study found that CIS patients who had an MS-defining relapse over the subsequent year of follow-up showed at baseline a widespread recruitment of contralateral and ipsilateral cerebral areas not normally involved in a motor task compared to patients who did not develop clinically definite MS over the same period (*Rocca et al., 2005*). In patients with a CIS presenting as optic neuritis, it has been shown that voxel-based diffusion connectivity is abnormal in the optic radiation bilaterally (*Ciccarelli et al., 2005*) and magnetization transfer ratio is decreased bilaterally in the visual cortex (Brodmann's area 17) when compared with healthy individuals (*Audoin et al., 2006*), suggesting post-synaptic changes and/or degeneration within visual pathways. Interestingly, one study found that magnetization-transfer ratio was lower in the optic nerves of patients with MS without recovery than in those with clinical recovery (*Inglese et al., 2002*). Additionally, Jenkins and coworkers reported that greater baseline functional MRI responses in higher visual areas (i.e. lateral occipital cortex) are associated with better visual outcome at 12 months in patients with acute optic neuritis, indicating neuroplasticity as an important mechanism for demyelinating events recovery (*Jenkins et al., 2010*). A recent study showed that decreased axial

diffusivity within the optic nerve predicted incomplete visual recovery in patients with a first event of optic neuritis (*Naismith et al., 2012*). The above mentioned findings show that non-conventional techniques have a high potential of providing quantitative measures that correlate with axonal/neuronal damage and neurological disability. However, most studies addressing this point have a cross-sectional design and there is insufficient prospective data showing a robust correlation between such measures and long-term disability in patients with an IDE (*Miller et al., 2012*).

Little is known about the prognostic significance of MRI in non-MS-like IDEs. Although a variety of brain and spinal cord MRI abnormalities are found in NMO spectrum disorders using both conventional and non-conventional techniques (*Pittock, Lennon et al., 2006; Pichiecchio et al., 2011*), none clearly predict the outcome of the initial event or the subsequent disease course. A retrospective study on 41 patients with NMO revealed no effect of the presence of brain MRI lesions both on relapse rate and progression of disability over more than 4 years of follow-up (*Bichuetti et al., 2009*). More recently, Collongues and coworkers reported that a high number of MRI brain lesions at NMO diagnosis was predictive of a residual visual acuity ≤1/10 after optic neuritis (*Collongues et al., 2010*). In ADEM patients MRI does not seem to have a predictive value in the short-term recovery (*Tenembaum et al., 2002*); however, fulfillment of Barkhof's and/or KidMUS (≥1 lesion perpendicular to long axis of corpus callosum; sole presence of well defined lesions) criteria on initial MRI in pediatric cases are associated with an increased risk of a second attack, suggestive of recurrent/multiphasic ADEM or MS (*Dale et al., 2009*).

Neurophysiological predictors

The relevance of neurophysiological assessment as a diagnostic tool in demyelinating disorders of the CNS progressively decreased in the last two decades, as MRI became largely available as a more sensitive technique. However, evoked potentials still maintain a prognostic significance, which have been especially demonstrated in MS. This is probably because evoked potentials are expression of the functional integrity of specific anatomical pathways and

consequently tend to better correlate with neurological disability than conventional MRI, which provide purely morphological information. Several cross-sectional and observational studies established that the degree of evoked potentials abnormalities is significantly associated with the EDSS score at the time of neurophysiological evaluation and up to 14 years later in patients with established MS (*O'Connor et al., 1998; Fuhr et al., 2001; Leocani et al., 2006; Kallmann et al., 2006; Invernizzi et al., 2011; Schlaeger et al., 2012*), while less is known about the predictive value of evoked potentials in patients with CIS. Evoked responses suggestive of demyelination at sites not clinically involved at CIS presentation raise the suspicion of MS, but the overall predictive value for relapse occurrence is low (*Gronseth and Ashman, 2000*). Hickman and coworkers reported that higher VEP amplitude predicted a better visual recovery after a first episode of acute unilateral optic neuritis (*Hickman et al., 2004*). A recent study found that CIS patients with at least three abnormal evoked potentials at baseline have an increased risk of reaching moderate disability over a mean follow-up period of six years, independent of initial MRI features (*Pelayo et al., 2010*).

Absent cortical responses at motor and somatosensory evoked potentials and signs of denervation on electromyography have been associated with poor prognosis in patients with ATM (*Kalita J et al., 1998*). Watanabe et al. reported that the absence of VEPs P100 component was significantly related to the development of severe visual impairment (OR=35.4, $p<0.001$) in patients with NMO-like syndromes from a Japanese population (*Watanabe et al., 2009*).

Molecular biomarkers

Considering that clinical and paraclinical tools show a limited immediate capability of identifying the etiology and predicting the disease course in the individual patient with an IDE, biomarkers are promising source of information with a good potential of quantitative measure, sensitivity, and reliability. Ideally, reliable prognostic studies of biomarkers should fulfill some key methodological requirements, particularly: (1) prospective design; (2) sufficient follow-up duration; (3) adequate biomarker and outcome measurement; (4) clinical significance of the biomarker (i.e., good correlation and consistency with relevant

clinical outcomes); (5) reproducibility (*Bielekova and Martin, 2004*). Currently, very few biomarkers in the field of CNS demyelinating disorders have shown all of such characteristics. Although lumbar puncture is a relatively invasive procedure, CSF is the optimal biologic sample to analyze in CNS disorders. Considering the complex pathogenesis of demyelinating diseases, no single CSF molecule is expected to have absolute prognostic significance. However, families of biomarkers representative of specific pathogenetic pathways – particularly those related to axonal/neuronal damage – may correlate with irreversible neurological dysfunction and be used as prognostic indicators to identify patients at risk of a more aggressive disease course. Furthermore, such biomarkers may help in deciding treatment initiation and monitoring therapeutic efficacy.

According to their biological role, molecules of potential prognostic significance for IDEs may be classified as follows:

- markers of immune activation (e.g., cytokines, chemokines, antibodies, complement factors, adhesion molecules, etc.)
- markers of blood-brain barrier disruption (e.g., matrix metalloproteinases)
- markers of demyelination (e.g., myelin basic protein [MBP], myelin oligodendrocyte glycoprotein [MOG], proteolytic enzymes, proteases inhibitors etc.)
- markers of axonal/neuronal and glial damage (e.g., neurofilaments, tau, 14-3-3 protein, glial fibrillary acidic protein, etc.)
- markers of remyelination/neural repair (e.g. nerve growth factor, brain-derived growth factor, Nogo-A, etc.).

A striking amount of papers have been published addressing the identification of molecular markers of CIS, MS, NMO and related disorders, but only a few have demonstrated a robust diagnostic value and even less have shown a clear predictive significance both in IDEs and during the course of established demyelinating disorders (*Teunissen et al., 2005; Awad et al., 2010*). Several CSF and blood markers have been associated with an increased risk of relapse after a CIS (i.e. conversion to clinically definite MS), including but not limited to: CSF oligoclonal IgG/IgM bands (*Tumani et al., Neurobiol Dis. 2009; Garcia-Barragàn et al., 2009*); CSF IgG index (Freedman et al., 2005); serum antibodies to MBP and

MOG (*Berger et al., 2003*); blood T cells differentiation transcription factors (*Corvol et al., 2008; Frisullo et al., 2008*); CSF immunoglobulins VH2 and VH4 sequences (*Bennett et al., 2008*); CSF 14-3-3 protein (*Martinez-Yélamos et al., J. Neurol. 2004*); CSF tau (*Frederiksen et al., 2011*); CSF neurofilament heavy chain (*Brettschneider et al., 2006*); CSF neurofilament light chain (*Teunissen et al., 2009*); CSF serin peptidase inhibitor; low CSF fetuin-A (*Tumani et al., Neurosci Lett. 2009*); CSF chemokine CXCL13 (*Brettschneider et al., 2010*); CSF chitinase 3-like 1 (*Comabella et al., 2010*); and low serum vitamin D (*Mowry et al., 2010*). However, for most of the candidates evidence is limited and/or results across studies are conflicting, and it remains unclear whether molecular markers may improve or even overcome the sensitivity and/or specificity of MRI in terms of prediction of MS development. CSF oligoclonal IgG bands represent an exception since their presence significantly and consistently increases the risk of relapse after a CIS, independent of MRI lesions dissemination (*Tintoré et al., 2008*), and in some cases proved to be superior to MRI in predicting such an outcome (*Tumani et al., Neurobiol Dis. 2009*). CSF IgG oligoclonal bands do not influence the long-term risk of disability after a CIS, although a contrasting observation has been described in patients with established MS (*Joseph et al., 2009*). CSF oligoclonal IgM reactive against myelin lipids have been associated with a poor CIS outcome in terms of time to conversion to MS, frequency of relapses and disability progression (*Garcia-Barragàn et al., 2009*). Vitamin D deficiency is implicated as a risk factor for MS; however, its role as an outcome predictor for patients with a CIS still awaits further confirmation (*Ascherio et al., 2010; Mowry et al., 2010; Miller et al., 2012*).

Since axonal loss is regarded as the biological determinant of irreversible neurological disability in demyelinating disorders, CSF and blood markers of neuronal or CNS tissue injury are the most promising candidates for predicting disease progression. While several studies have explored the correlation between such markers and disability in established MS, few have addressed this topic in CIS (*Teunissen et al., 2005*). Neurofilament heavy and light chain proteins (NfH and NfL, respectively) concentrations are increased in the CSF of MS patients compared to age-matched normal controls, although they do not reach the levels

observed in neurodegenerative and more disruptive CNS disorders. Furthermore, CSF NfH and NfL levels seem to correlate with MS clinical course (higher levels in relapsing-remitting MS compared to CIS and in progressive compared to relapsing MS) and with disease activity (higher levels in CIS patients who convert to MS and during relapse compared to remission phase). Finally, it has been shown that CSF NfH and NfL concentrations may correlate with disease severity both at the time of lumbar puncture and at subsequent follow-up time points (*Malmeström et al., 2003; Teunissen et al., 2009; Salzer et al., 2010; Kuhle et al., 2011*).
Tau and 14-3-3 protein have been already discussed in the previous section. A recent study showed an inverse correlation between CSF α-cleaved soluble amyloid precursor protein concentration and neurological disability in patients with CIS, MS, and NMO, although no significant differences emerged between the three diseased groups and healthy controls (*Mai et al., 2011*).
In the context of IDEs suggestive of NMO, serum anti-aquaporin-4 antibody has mainly a diagnostic value, although it may also have a prognostic importance. NMO-IgG positivity predicts recurrence of attacks after a first event of isolated LETM (*Weinshenker et al., 2006*), is associated with poor recovery after optic neuritis (*Matiello et al., 2008; Jarius et al., 2010*), and correlates with long-term disability (*Akman-Demir et al., 2011*). Although there are no validated CSF prognostic markers for NMO at the time of initial event, increased CSF levels of glial fibrillary acidic protein and interleukin 6 may be predictive of a more severe disease course (*Takano et al., 2010; Uzawa et al., 2011*).
The presence of 14-3-3 protein and increased levels of interleukin 6 in the CSF of patients with ATM have been proposed as potential predictors of permanent neurological disability (*Irani and Kerr, 2000; Kaplin et al., 2005*).
Peculiar CSF chemokine and citokine profiles in patients with ADEM have been described; however, the prognostic significance of the biomarkers that have been identified is unknown (*Franciotta et al., 2006; Ishizu et al., 2006*).

METHODS

STUDY CASES INCLUSION

Patients with an IDE suggestive of MS (CIS) were retrospectively identified by means of medical charts review among patients seen at the Section of Clinical Neurology at Verona University Hospital. For this purpose, study subjects had to fulfill the following requirements: (1) Inclusion criteria: a) occurrence of a CIS, defined as the appearance of one or more neurological symptoms lasting at least 24 hours, suggestive of MS-type acute or subacute demyelination in one or more of the following locations: optic nerve, cerebral hemisphere, brainstem, cerebellum, and spinal cord; b) follow-up duration 18 months or longer starting from symptoms onset; c) CSF sample collected within 6 months from symptoms onset or before a relapse occurred. (2) Exclusion criteria: a) history of previous episodes of CNS demyelination; b) CIS explained by a specific etiology other than MS, e.g. NMO (*Wingerchuk et al., 2006*), idiopathic ATM (*Transverse Myelitis Consortium Working Group, 2002*), or systemic autoimmune disorder, according to currently available diagnostic criteria; c) other concomitant CNS disorders.

CLINICAL AND LABORATORY DATA

CIS patients were seen at our site within one year from symptoms onset and then prospectively followed with follow-up visits not less than once a year. Initial evaluation included: medical history, physical and neurological examination, blood work-up including autoimmunity screening, brain and spine MRI, lumbar puncture (see CSF analysis section), and EPs. All follow-up visits included at least medical history and neurological examination. In patients who had a LETM or predominant optico-spinal involvement serum NMO Ig-G was assessed in our laboratory by tissue-based indirect immunofluorescence.

The maximum degree of neurological impairment caused by the CIS was defined based on the expanded disability status scale (EDSS) functional systems scores as: (1) mild, one or more functional system with a score of 1, others 0; (2) moderate, one or two functional systems with a score of 2, others 0 or 1; (3) severe, at least one functional system with a score equal to or greater than 3 or at least three functional systems with a score of 2 (*Kurtzke, 1983; Panitch et al., 2002*).

Brain and spine MRI were performed at different sites with a 1 or 1.5 T machine within 6 months from CIS symptoms onset or before a relapse occurred. All patients had at least one follow-up MRI scan obtained at various time intervals to assess dissemination of lesions. Radiological data were obtained unblindly from original MRI scans of the brain and spinal cord. When scans were not available, data were collected from reports. Brain and spinal cord MRI were considered positive if there was at least one T2-hyperintense signal abnormality compatible with an MS lesion (*Montalban et al., 2010*). Lesion number, location and extension as well as gadolinium enhancement were assessed whenever possible. Dissemination in space was determined according to Polman's criteria (*Polman et al., 2005*).

EPs were performed at different sites within 6 months from CIS symptoms onset or before a relapse occurred. EPs were considered abnormal if the central conduction time was increased or significantly asymmetric, showed a significant morphologic alteration, or was not recognizable. The normative values were those used in the laboratory in which the electrophysiological evaluation was performed.

For all CIS cases the following outcomes were considered: recovery from initial event, relapse occurrence, conversion to MS (Polman's criteria), annual relapse rate, and disability level (EDSS score) at various follow-up time points (1.5, 3, 5 years and last visit). A relapse was defined as an episode of new or recurring neurological symptoms suggestive of an inflammatory CNS lesion, lasting for at least 24 hours in the absence of increased body temperature or infection, after a remission of 30 days or more. Recovery from initial event was determined at 6 months from symptoms onset or before a relapse or disability progression occurred and was scored as: (1) full, EDSS=0 and no residual symptoms; (2) with mild residual disability, EDSS=1.0-2.0 or EDSS=0 with residual symptoms; (3) with moderate to severe residual disability, EDSS=2.5 or more.

CSF BIOMARKERS ANALYSIS

Lumbar puncture was performed within 6 months from symptoms onset or before a relapse occurred, as requested by study inclusion criteria; timing was

classified as on acute phase if performed within 30 days from CIS symptoms onset. If not immediately analyzed, CSF samples were stored at −80 °C just after collection to ensure stability of biomarkers. All CSF analyses were performed blindly to clinical status of patients. Standard exam included protein concentration, cell count, IgG index, and oligoclonal bands assessment (isoelectrofocusing). Tau protein concentration was measured in duplicate using an ELISA kit (Innogenetics, Ghent, Belgium), according to the manufacturer's instructions. Tau concentrations were estimated from standard curves made for each assay. 14-3-3 protein was assayed by immunoblotting, after sodium dodecyl sulphate polyacrilamide gel electrophoresis (SDS-PAGE). For this purpose, 10 μL of CSF was separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon P; Millipore). Each gel included 10 and 20 pg of recombinant 14-3-3 γ. Membranes were incubated with anti-14-3-3 β polyclonal rabbit IgG cross-reacting with γ, ε, ζ, and η isoforms (Pan 14-3-3 antibody SC-629; Santa Cruz Biotechnology), at 1:500 dilution, then incubated with anti-rabbit immunoglobulin at 1:3,000 dilution. The reaction was revealed by an enhanced chemiluminescence system (ECL; Amersham). The presence of the 14-3-3 band was scored by two independent observers and classified as negative, weak positive, or positive based on comparison with recombinant 14-3-3 protein. Cystatin C concentration was measured in duplicate using a sandwich ELISA kit (Biovendor), according to the manufacturer's instructions. Additionally, cystatin C was detected by immunoblotting, after SDS-PAGE. For this purpose, 10 μL of CSF was separated by SDS-PAGE and transferred to PVDF membranes (Immobilon P; Millipore). Each gel included recombinant cystatin C as positive control (lower detection limit 9.8 ng/ml). Membranes were incubated with an anti–cystatin C polyclonal antibody (Upstate Biotechnology) at 1:1000 dilution and revealed by an ECL system (GE Healthcare). Cystatin C concentration was estimated from standard curves made for each assay based on recombinant protein, using a dedicated software (UN-SCAN-IT GEL 6.1).

For comparison with CIS cases, additional CSF samples were analyzed in patients with: 1) NMO spectrum disorders (n=6) or idiopathic ATM (n=6); 2) Creutzfeldt-Jacob disease (CJD, n=8); 3) non-inflammatory non-degenerative

disorders (NINDDs), including epidural anesthesia for surgical procedure (n=4), psychiatric disorders (n=3), lacunar syndrome (n=1), arteriovenous malformation (n=1), migraine (n=1), and cerebral palsy (n=1). Patients with NMO spectrum disorders included 4 NMO anti-AQP4 antibody positive, 1 NMO anti-AQP4 antibody negative, and 1 recurrent LETM anti-AQP4 antibody positive subjects. Three of the 6 idiopathic ATM cases had a LETM and tested negative for anti-AQP4 antibody.

STATISTICAL ANALYSIS

Differences between groups were analyzed by Kruskall-Wallis (with post-hoc Dunnett's t statistic for multiple comparisons) and Mann-Whitney tests for quantitative variables (due to small and unequal sample size of groups) and by Chi-square or Fisher's exact test for categorical variables, as appropriate. The correlation between quantitative variables and outcomes was analyzed computing either the Pearson's or Spearman's coefficient, as appropriate. To examine the predictive value of variables, multivariate logistic regression model analysis and – for time-to-event outcomes – Cox proportional hazards model analysis were also performed. A two-tailed significance level alfa=0.05 and 95% confidence intervals (CI) were adopted. All the analyses were performed using PASW Statistics 18 (SPSS Inc., Chicago, IL, USA).

RESULTS

Forty-six subjects who experienced a CIS between 1996 and 2010 entered the study. Forty-three patients were prospectively followed at our site. Three patients were followed at other sites after the initial evaluation at our center; for these cases information were provided by the treating neurologist. In addition, 31 control patients were included for CSF analysis as mentioned in the Methods section.

CLINICAL AND LABORATORY DATA

Descriptive characteristics of CIS cases are shown in Table 1. Variables associated with recovery from the initial event are shown in Table 2. At last available follow-up visit, 7 patients were still classified as CIS, while 39 had converted to MS according to Polman's criteria. Ten patients (4 who had a LETM at presentation and 6 with predominantly optico-spinal manifestations) were assessed for NMO-IgG, which resulted negative in all cases. The presence of at least 3 periventricular (PV) lesions and at least 9 total lesions on baseline MRI were each associated with an increased risk of conversion to MS in the univariate Cox proportional hazards analysis (hazard ratio [HR]: 3.3, CI: 1.2-8.9, p=0.017 and HR: 2.1, CI: 1.01-4.4, p=0.047 respectively). Of the 46 CIS patients, 37 had a clinical relapse, which in 34 cases was diagnostic of clinically definite MS. The presence of at least 3 PV lesions on initial brain MRI was the only predictor of the risk of conversion to clinically definite MS, using the Cox model (HR: 3.9, CI: 1.3-11.4, p=0.015). Median annual relapse rate was higher in MS patients with at least 9 lesions on initial MRI compared to patients with a lower lesion count (0.38 [0-1.2] vs 0.14 [0-0.9], p=0.006). The level of disability (EDSS score) reached by MS patients at the various follow-up time points was influenced by different baseline variables (Table 3). Additionally, final EDSS score was significantly correlated to the number of relapses occurring during follow-up (Spearman's rho: 0.52, p=0.001). Moreover, the number of relapses predicted the risk of reaching a final EDSS score 2 or more, adjusting for initial pyramidal tract involvement and CIS recovery (HR: 1.2, CI: 1.1-1.4, p=0.04).

CEREBROSPINAL FLUID BIOMARKERS

Results of biomarkers analysis are summarized in Table 4. Tau concentration was similar in CIS and NINDDs patients, while it was significantly higher in NMO/ATM compared to CIS cases (Figure 1), although not as high as in CJD patients (Table 4). Of the CIS and NMO/ATM patients, a small subgroup tested 14-3-3 protein positive, while the majority had a weak positive assay, compared to NINDDs subjects who tested all negative and CJD cases who tested all positive (Figure 2). CIS patients had similar CSF cystatin C concentration compared to both NINDDs and NMO/ATM cases, but had a significantly lower concentration compared to CJD patients (Figure 3). Cystatin C immunoblotting detected the whole 13.4 kDa protein and no truncated fragments in all cases and controls (Figure 4). Interestingly, cystatin C and tau concentrations were significantly correlated, independent of diagnostic group, as shown in Figure 5.

Within the CIS group, none of the biomarkers showed a statistically significant correlation with recovery, relapse occurrence, conversion to MS, relapse rate, and EDSS scores at the various time points. Among standard CSF analysis parameters, total protein concentration was associated with disability level in MS patients at 3 years (median EDSS score 3.5 [1.5-8.5] for patients with CSF protein >45 mg/dl vs EDSS score 1 [0-2.5] for patients with CSF proteins ≤45 mg/dl; p=0.04) and at 5 years (EDSS score 3.5 [1.5-8.5] for patients with CSF protein >45 mg/dl vs EDSS score 1.5 [0-4] for patients with CSF proteins ≤45 mg/dl; p=0.019) after onset.

DISCUSSION

The present study explored tau, 14-3-3- protein and cystatin C in a group of patients with IDEs suggestive of MS both as potential diagnostic biomarkers – by comparison with other CNS disorders – and as possible predictors of clinically relevant outcomes, in addition to standard evaluation variables.

Most CIS cases presented as acute myelitis, followed by brainstem/cerebellar syndromes. CIS severity was an independent predictor of incomplete recovery, which however, occurred with significant sequelae only in a minority of patients. Nearly 85% of CIS cases converted to MS during follow-up (74% converted to clinically definite MS). Presence of at least three PV lesions on initial brain MRI was the only independent predictor of both MS development defined according to Polman's criteria and occurrence of a relapse. The high conversion rate to MS in this series compared to previous CIS studies may reflect selection bias of the retrospective identification of CIS cases in an MS-specialized center and the application of Polman's criteria in our study. CIS patients had a significantly shorter follow-up duration compared to MS cases and were similar to MS patients in terms of clinical, neurophysiological, CSF and MRI characteristics, suggesting that CIS subjects would probably meet diagnostic criteria for MS with the prosecution of follow-up. In patients who developed MS, incomplete CIS recovery was an independent predictor of EDSS 1.5 or more at 18 months after onset, while pyramidal involvement at CIS and number of relapses during follow-up where associated with the risk of EDSS score 2 or greater at the end of follow-up, in accordance with most previously published data. In some large observational studies, a short interval to the second event and high relapse rate in the first 2-5 years of MS were predictive of subsequent severe disability (*Weinshenker et al., 1991; Confavreux et al., 2003; Tremlett et al., 2006; Debouverie et al., 2008; Koch et al., 2008*); however, one important study did not find this association (*Runmaker and Andersen, 1993).* According to the authors who found a positive correlation between relapses and long-term disability, the long term impact of relapses seems to be stronger in younger patients (<25 years old at MS onset), it diminishes significantly after the first 2-5 years of disease, and it is minimal after

the progressive phase has begun (*Tremlett et al., 2009; Scalfari et al., 2010*). Our findings confirm clinical parameters as useful prognostic indicators in patients with a CIS.

The combined analysis of tau, 14-3-3 protein and cystatin C in the CSF of patients included in this study provided general indications about the underlying pathological processes in well distinct CNS disorders. For the three biomarkers there was an overall trend showing negative/normal values in NINDDs subjects, minimally altered values in CIS, moderately altered values in NMO/ATM, and markedly positive/elevated values in CJD, although statistical significance was not reached at each step. This trend may suggest a "neurodegenerative gradient" across disease groups, from no evidence of cell injury in "negative" controls through demonstration of massive neurodegeneration in CJD cases. Interestingly, we observed a significant correlation between CSF tau and cystatin C indicating the latter as a possible marker of axonal injury as well.

CIS patients had normal CSF tau and cystatin C concentrations (i.e. similar to NINDDs controls who did not have any "active" neurological condition at the time of LP), while several CIS cases tested positive for 14-3-3 protein, suggesting that it may be detectable in the CSF in the absence of relevant neurodegenerative processes, as indicated by tau levels falling within the normal range. This finding is in accordance with several studies that reported normal CSF tau concentration in MS patients at various stages of the disease (including CIS), compared to controls (*Jiménez-Jiménez et al., 2002; Guimaraes et al., 2006; Teunissen et al., 2009*). On the other hand, other studies reported increased tau levels (*Kapaki et al., 2000; Martinez-Yelamos et al., Neurosci. Lett. 2004; Bartosik-Psujek and Archelos, 2004; Brettschneider et al., 2005; Terzi et al., 2007; Frederiksen et al., 2011*); however, they included patients who mostly were in the progressive phase of MS or on relapse, both characterized by a more obvious axonal damage and CSF tau release compared to patients in remission and/or after a single episode. CIS patients included in our study often underwent lumbar puncture well after the acute phase; additionally, they lacked a truly healthy control group to be compared with. Presence of 14-3-3 protein in the CSF of a certain proportion of CIS/MS patients has been consistently reported over the past decade (*de Seze et al., 2002;*

Martinez-Yelamos et al., 2004; Bartosik-Psujek and Archelos, 2004; Colucci et al., 2004; Hein Née Maier et al., 2008). The observation that presence of 14-3-3 protein is generally coupled with normal or slightly increased tau levels challenges the hypothesis that 14-3-3 is a marker of neuronal/axonal loss in MS, also considering that 14-3-3 protein isoforms expressed in CIS/MS patients (predominantly β and ζ) are substantially different from isoforms expressed in patients with neurodegenerative diseases (*Fiorini et al., 2007*). Moreover, no correlation between CSF 14-3-3 and positive OB or increased IgG index was found in CIS patients from the present study, in contrast with the hypothesis that humoral immunity driven inflammation in the CNS is related with 14-3-3 leakage in the CSF (*Bartosik-Psujek and Archelos, 2004*). In vitro studies have shown that 14-3-3 protein interacts with vimentin and glial fibrillary acidic protein in cultured human reactive astrocytes derived from MS demyelinating lesions, and that astrocytes and oligodendrocytes, at the site of demyelinating lesions, show increased immunoreactivity for 14-3-3, suggesting that these cells might be the source of 14-3-3 in the CSF of MS patients (*Kawamoto et al., 2004; Satoh et al., 2004*). Decreased CSF levels of cystatin C were previously reported in MS patients on relapse compared to controls (*Nagai et al., 2000*) and in patients with relapsing-remitting MS compared to CIS and control subjects (*Sladkova et al., 2011*). Our group previously found increased cystatin C levels (measured by Western blot) in patients with CIS, MS and idiopathic myelitis in remission compared to an internal control reference (*Fiorini et al., 2007; Gajofatto et al., 2010*). Since cystatin C is involved in such a dynamic process in CNS demyelinating disorders, it can be argued that its CSF concentration may fluctuate significantly depending on clinical course, disease stage, and treatment (*Haves-Zburof et al., 2011*), indicating the need of taking into account these factors when interpreting the diagnostic and prognostic significance of cystatin C and other biomarkers.

In the current study, tau levels were significantly higher in patients with NMO/ATM cases compared to CIS, suggesting for the first time a potential diagnostic utility of this biomarker, to be confirmed in a larger sample including patients with established MS. This finding may be explained by the different

pathogenetic mechanisms underlying the two disease groups, characterized by more pronounced neuronal death and axonal loss in NMO/ATM than in MS. The distinction between CIS/MS and NMO may be extremely difficult in some cases; serum anti-aquaporin antibody exhibits high specificity but has relatively low sensitivity for the diagnosis of NMO. Alternative diagnostic biomarkers have been proposed, such as glial fibrillary acidic protein, which is significantly increased in the CSF of patients with NMO, with sensitivity and specificity approaching 100% when the reference group is limited to MS cases (*Takano et al., 2010; Petzold et al., 2011*). Additionally, increased serum concentration of glial fibrillary acidic protein distinguished optic neuritis related to NMO from those related to MS or other conditions, in a study examining patients with various forms of idiopathic inflammatory optic neuropathy (*Storoni et al., 2011*). In a CSF proteomic analysis of NMO patients, Bai and coworkers showed that the most relevantly altered protein was haptoglobin, which was significantly up-regulated in NMO compared to control subjects with migraine and trigeminal neuralgia (*Bai et al., 2010*), while a previous study reported decreased CSF haptoglobin levels in CIS patients who converted to MS (*Tumani et al., Neurosci. Lett. 2009*). Interestingly, an Italian study reported that CSF and serum levels of N-acetyl aspartate were significantly higher in patients with MS compared to NMO and healthy controls; values of serum N-acetyl aspartate exceeding the 95th percentile of healthy controls were found in 100% of MS and none of NMO patients (*Tortorella et al., 2011*).

None of the biomarkers analyzed in the present study was significantly associated with CIS severity and recovery, risk of MS conversion, subsequent disability, or relapse rate in MS patients. Brettschneider et al. found increased CSF tau levels in CIS patients who later converted to MS compared to non-converters, improving the sensitivity of MRI for the diagnosis of MS (*Brettschneider et al., 2006*); similarly Frederiksen et al. reported higher CSF tau concentration in patients with initial optic neuritis who developed MS compared to patients with monophasic optic neuritis (*Frederiksen et al., 2011*). Martínez-Yélamos et al. showed that a positive CSF 14-3-3 assay at the first neurological event suggestive of MS predicted conversion to clinically definite MS and development of significant neurological

disability over a median follow-up period of 33 months (*Martínez-Yélamos et al., J. Neurol. 2004*). There are discordant studies on the possible prognostic significance of CSF 14-3-3 in patients with established MS at the time of lumbar puncture (*de Seze et al., 2002; Colucci et al., 2004*). Our group previously reported a significant correlation between CSF cystatin C level – assessed by Western blot – and long-term EDSS score in a small subgroup of patients with recurrent myelitis and spinal onset MS (*Gajofatto et al., 2010*), a finding that was not replicated in the current study. However, Western blot may not be appropriate to quantify protein concentration and may show significant discrepancies compared to the ELISA method, which is more sensitive though less specific.

The present study has some limitations, including the retrospective collection of clinical variables and outcomes, the relatively small sample size, and the absence of a completely normal control group for CSF analysis. On the other hand, we are comforted in our findings by the long follow-up of most CIS patients and the wide range of clinical, CSF, MRI and neurophysiological data, collected with relatively homogenous protocols.

Research on biomarkers of IDEs may provide new insights into early pathological changes and pathogenetic mechanisms that might affect the course of the disease. Considering the complex pathogenesis of CNS demyelinating disorders, no single molecule is expected to have diagnostic and/or prognostic significance. However, families of biomarkers representative of specific pathogenetic pathways may support diagnosis and/or correlate with irreversible neurological dysfunction, identifying patients at risk of a more aggressive disease course (*Bielekova and Martin, 2004*). The identification of such biomarkers would be of paramount importance in guiding the approach and management of patients with an IDE.

TABLES AND FIGURES

Table 1. Characteristics of CIS patients according to CIS or MS status at last follow-up

Characteristic	**All cases** n=46	**CIS→CIS** n=7	**CIS→MS** n=39	p
Gender (F/M)	30/16	5/2	25/14	.54
Age at onset (years), mean ± SD	34.6 ± 8.9	36.2 ± 4.7	34.4 ± 9.4	.53
Onset location, n (%)				
spinal cord	31/46 (67.4%)	7/7 (100%)	24/39 (61.5%)	.08
brainstem/cerebellum	12/46 (26.1%)	1/7 (14.3%)	11/39 (28.2%)	.66
cerebral hemisphere	6/46 (13%)	0	6/39 (15.4%)	.57
optic nerve	5/46 (10.9%)	0	5/39 (12.8%)	.99
polyregional	6/46 (13%)	1/7 (14.3%)	5/39 (12.8%)	.99
Impairment at onset, n (%)				.66
mild/moderate	32/46 (69.6%)	4/7 (57.1%)	28/39 (71.8%)	
severe	14/46 (30.4%)	3/7 (42.9%)	11/39 (28.2%)	
Recovery, n (%)				.34
full	17/46 (37%)	1/7 (14.3%)	16/39 (41%)	
with mild disability	28/46 (60.9%)	6/7 (85.7%)	22/39 (56.4%)	
with moderate/severe disability	1/46 (2.1%)	0	1/39 (2.6%)	
Brain MRI at onset, n (%) *				.39
≥1 MS-like lesion	43/46 (93.5%)	6/7 (85.7%)	37/39 (94.9%)	
0 MS-like lesions	3/46 (6.5%)	1/7 (14.3%)	2/39 (5.1%)	
Spine MRI at onset, n (%) **				.27
≥1 spinal cord lesion	35/39 (89.7%)	7/7 (100%)	28/32 (87.5%)	
0 spinal cord lesions	4/39 (10.3%)	0	4/32(12.5%)	
Dissemination in space (MRI), n (%)				
≥9 lesions	20/40 (50%)	1/6 (16.7%)	19/34 (55.9%)	.08
≥1 gad+ lesion	21/32 (65.6%)	5/6 (83.3%)	16/26 (61.5%)	.14
≥3 PV lesions	29/41 (70.7%)	2/6 (33.3%)	27/35 (77.1%)	.05
≥1 infratentorial lesion	39/45 (86.7%)	6/6 (100%)	33/39 (84.6%)	.58
≥1 juxtacortical lesion	27/39 (69.2%)	5/6 (83.3%)	22/33 (66.7%)	.64
≥3 Barkhof criteria	29/41 (70.7%)	5/7 (71.4%)	24/34 (70.6%)	.60
≥2 MRI lesions + positive CSF	30/46 (65.2%)	4/7 (57.1%)	26/39 (66.7%)	.68
EP at onset, n (%)				
VEPs +	11/41 (26.8%)	0/6	11/35 (31.4%)	.27
SEPs +	17/38 (44.7%)	1/6 (16.7%)	16/32 (50%)	.30
MEPs +	16/29 (55.2%)	3/5 (60%)	13/24 (54.2%)	.86
BAEPs +	12/25 (48%)	1/3 (33.3%)	11/22 (50%)	.71
Follow-up (years), median (range)	6.7 (1.5-15.3)	3.4 (2.0-7.2)	7.2 (1.5-15.3)	.02
Annual relapse rate, median (range)	0.2 (0-1.3)	0	0.3 (0-1.3)	
EDSS score, median (range)				
at lumbar puncture (n=46)	2.0 (0-4.0)	2.5 (0-3.5)	2.0 (0-4.0)	.38
at 1.5 years (n=46)	1.0 (0-8.5)	1.0 (0-2.0)	1.0 (0-8.5)	.68
at 3 years (n=41)	1.5 (0-8.5)	1.0 (1.0-2.0) n=5	1.5 (0-8.5) n=36	.52
at 5 years (n=29)	1.5 (0-8.5)	1.0 (1.0-1.0) n=3	1.5 (0-8.5) n=26	.17
at last visit (n=46)	2.0 (0-8.5)	1.0 (0-2.0)	2.0 (0-8.5)	.05

* 29/46: actual scan review; 17/46: data from radiological report

** 24/39: actual scan review; 15/39: data from radiological report

Table 2. Predictors of CIS recovery

Predictor	Cases with complete recovery (EDSS=0)	Cases with incomplete recovery (EDSS>0)	Unadjusted OR (95%CI) for complete recovery*	p	Adjusted OR (95%CI) for complete recovery**	p
Pyramidal involvement	7	23				
No pyramidal involvement	10	6	5.5 (1.5-20.5)	.01	2.7 (0.6-12.3)	.21
Moderate/severe impairment	10	27				
Mild impairment	7	2	9.5 (1.7-53.4)	.01	1.9 (0.2-16.1)	.55
Initial EDSS score *median (range)*	1 (0-3.5)	2.5 (1-4)	0.3 (0.2-0.7)	.002	**0.4 (0.2-0.8)**	**.009**

* Univariate logistic regression model
** Multivariate logistic regression model adjusted for pyramidal involvement and initial EDSS score

Table 3. Baseline predictors of disability level at various follow-up time points (MS cases)

Predictor	EDSS score at 1.5 years from CIS		EDSS score at 5 years from CIS		EDSS score at last visit		EDSS ≥2 (time-to-event)
	median (range)	OR (95%CI) for EDSS >1	median (range)	OR (95%CI) for EDSS ≥2	median (range)	OR (95%CI) for EDSS ≥2	HR (95%CI)
Male gender Female gender	1.5 (0-8.5) 1 (0-3.5) p=.21*	2.4 (0.6-9.0) p=.21	1.75 (1-8.5) 1.5 (0-4.5) p=.29*	1.3 (0.3-6.3) p=.78	1.75 (0-8.5) 2 (0-6.5) p=.92*	0.7 (0.2-2.5) p=.55	0.8 (0.3-1.9) p=.61
Pyramidal system involvement No pyramidal system involvement	1.5 (0-8.5) 1.0 (0-2) p=.04*	4.7 (1.1-20.9) p=.04	2 (0-8.5) 1.5 (0-2) p=.24*	2.3 (0.4-12.1) p=.35	2 (0-8.5) 1.5 (0-3) p=.04*	3.8 (0.9-15.2) p=.06	3.6 (1.3-10.1) p=.02(3)
Full CIS recovery Incomplete CIS recovery	0 (0-2) 1.5 (0-8.5) p<.001*	 13.1 (2.4-72.7) p=.003(1)	1 (0-3.5) 2 (1-8.5) p=.03*	 5.0 (0.8-31.6) p=.09	1.25 (0-3.5) 2.0 (0-8.5) p=.02*	 2.4 (0.7-8.9) p=.19	 3.1 (0.7-14.5) p=.15
Normal MEPs at onset Abnormal MEPs at onset	0 (0-2) 2 (1-3.5) p=.002*	 8.9 (1.4-56.6) p=.02	1 (0-2) 2.5 (1-4.5) p=.04*	 1.9 (0.3-11.6) p=.50	1.5 (0-3) 3 (1-6.5) p=.002*	 14.7 (1.9-109.2) p=.009(2)	 0.7 (0.2-3.0) p=.63
Normal SEPs at onset Abnormal SEPs at onset	0 (0-2) 1.5 (1-8.5) p=.007*	 1.5 (0.6-3.5) p=.39	1 (0-2) 1.75 (1-8.5) p=.04*	 0.7 (0.1-5.5) p=.67	1.5 (0-3) 2 (0-8.5) p=.02*	 0.7 (0.9-4.6) p=.68	 0.6 (0.1-2.3) p=.42
DIS§ on initial MRI no DIS§ on initial MRI	1 (0-8.5) 0.75 (0-2) p=.30*	3.4 (0.3-34.9) p=.31	2 (0-8.5) 1.25 (0-3.5) p=.20*	2.3 (0.2-29.8) p=.54	2 (0-8.5) 0.75 (0-3.5) p=.03*	3.0 (0.4-21.7) p=.28	2.5 (0.2-27.0) p=.46

* Mann-Whitney test

(1) OR=9.2 (1.5-57.8) p=0.02 after adjusting for pyramidal system involvement and MEPs (other variables not significant in the model)

(2) OR=12.2 (1.3-117.8) p=0.03 after adjusting for relapse rate and follow-up duration

(3) OR=3.5 (1.2-10.2) p=0.02 after adjusting for relapse rate

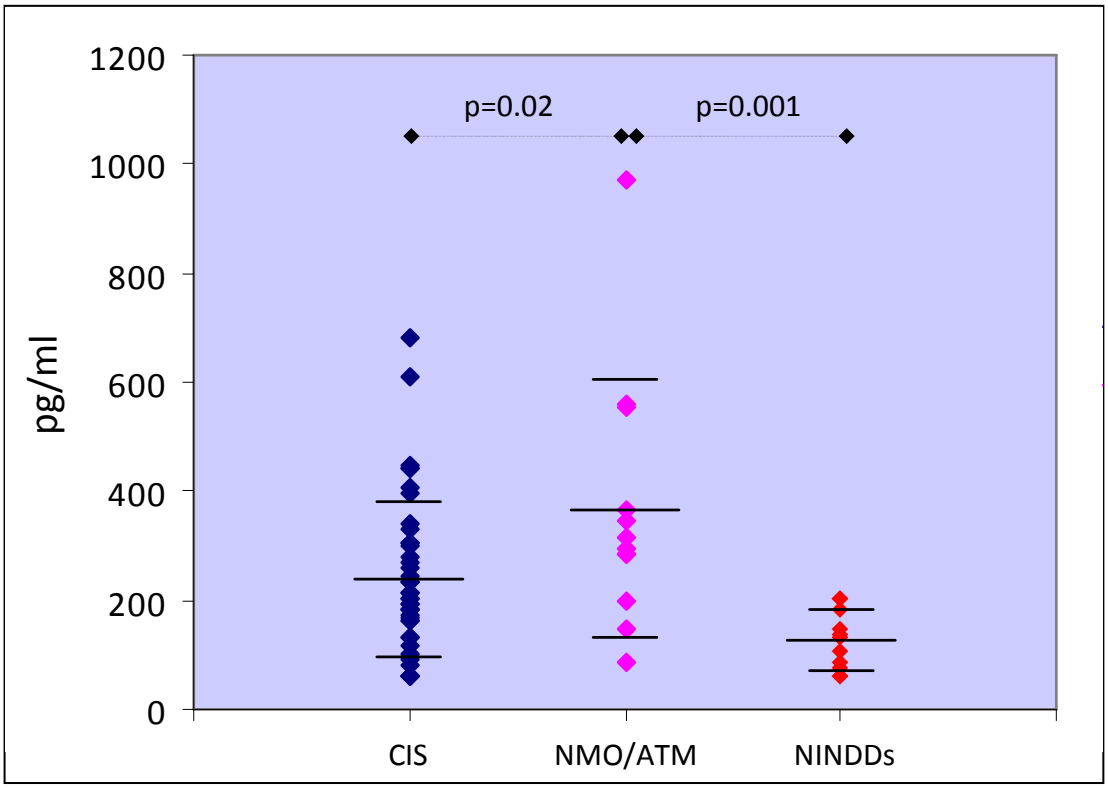
§ DIS: dissemination in space (fulfillment of 3 out of 4 Barkhof criteria)

Table 4. CSF biomarkers analysis

Biomarker	NINDDs	CIS	NMO/ATM	CJD
OB/IgG index	n=8	n=46	n=12	
n positive/increased (%)	0	32 (69.6%)	6 (50%)	N/A
n negative/normal (%)	8 (100%)	14 (30.4%)	6 (50%)	
Tau	n=11	n=42	n=12	n=8
ELISA (pg/ml)				
median (range)	130 (59-204)	204 (59-681)#	306 (86-971)#	>4000#
14-3-3	n=11	n=45	n=11	n=8
immunoblot				
n positive (%)	0	4 (8.9%)	2 (18.2%)	8 (100%)*
n weak pos (%)	0	24 (53.3%)	7 (63.6%)	0
n negative (%)	11 (100%)	17 (37.8%)	2 (18.2)	0
Cystatin C	n=11	n=40	n=10	n=8
ELISA (ng/ml)				
median (range)	2842 (595-9868)	3167 (1000-6922)	4231 (1861-8921)	5337 (3144-8534)§

# $p<0.001$ Kruskall-Wallis' test; $p=0.02$ CIS vs NMO/ATM, $p=0.001$ NINDDs vs NMO/ATM, $p<0.001$ CJD vs each group (Dunnett's t test)
* $p<0.001$ CJD vs each group (χ^2)
§ $p=0.01$ $p=0.01$ Kruskall-Wallis' test; $p=0.004$ CJD vs CIS, $p=0.01$ CJD vs NINDDs (Dunnett's t test)

Figure 1. CSF tau concentration in the different diagnostic groups



CJD patients are not shown because tau concentration was beyond the upper detection limits in all cases in this group

Figure 2. CSF 14-3-3 protein assay in the different diagnostic groups

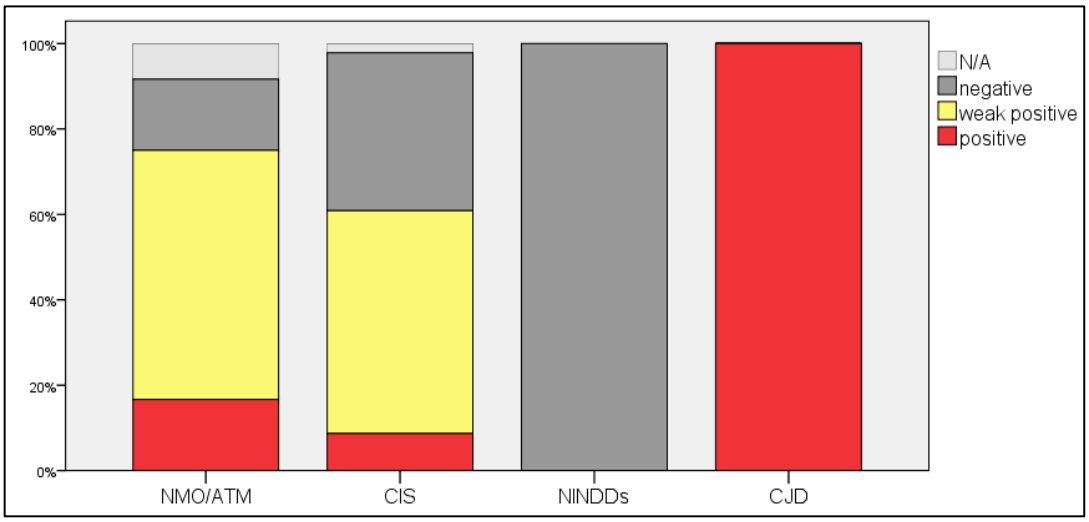


Figure 3. CSF cystatin C concentration in the different diagnostic groups

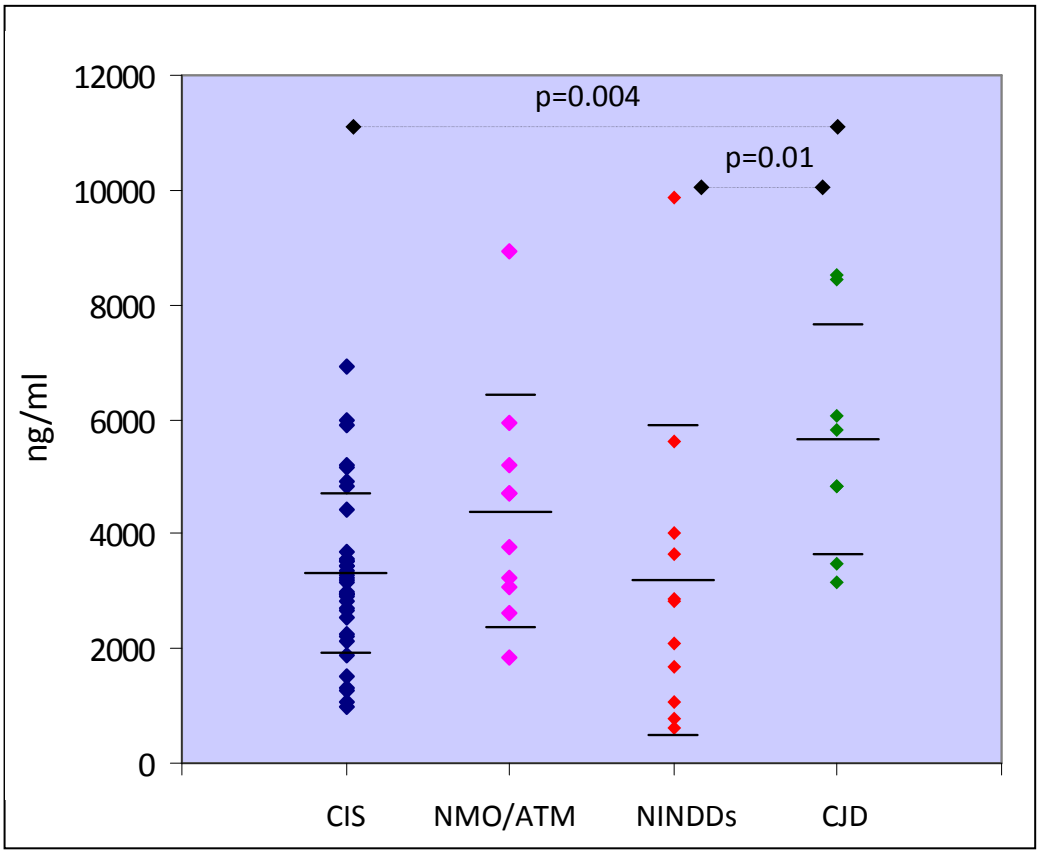
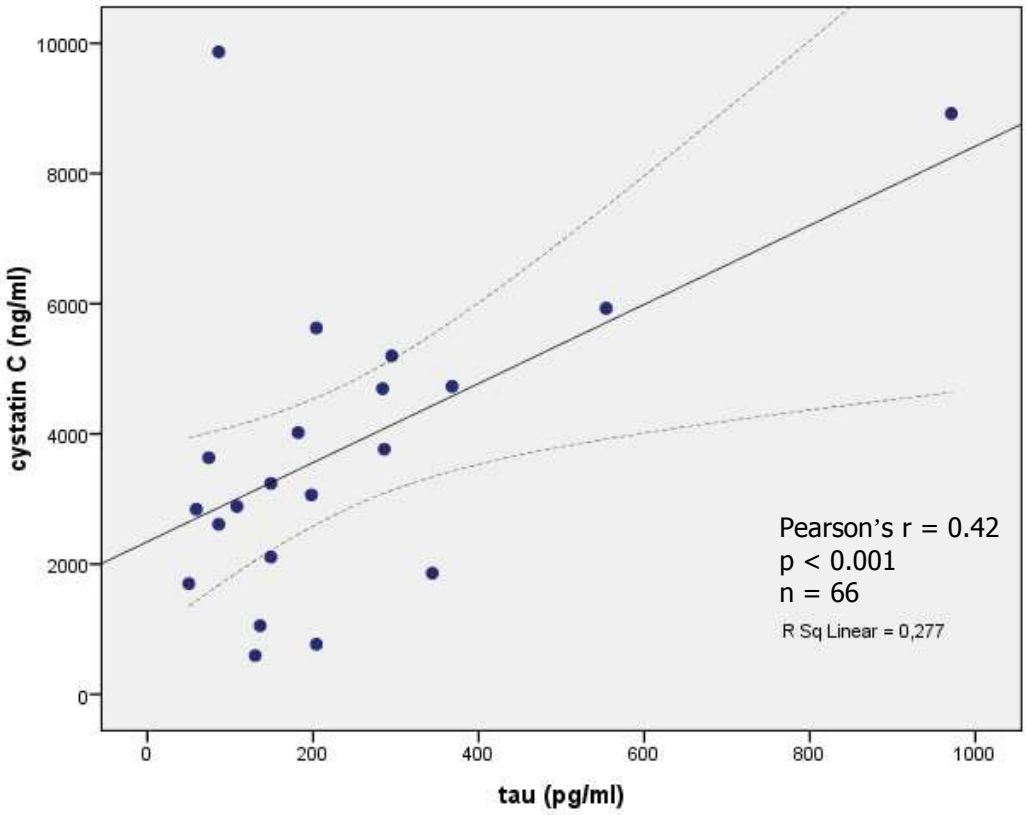


Figure 4. Cystatin C detection by immunoblot in a sample of CIS cases

Figure 5. Correlation between CSF tau and cystatin C in CIS and control subjects



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