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Tesi di dottorato in Scienze Biomediche – corso di Neuroscienze

AUTOIMMUNITY, ENVIRONMENT AND GENETICS IN INFLAMMATORY DISORDERS OF CENTRAL AND PERIPHERAL NERVOUS SYSTEM

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2. STUDY OUTLINE

Failure of the normal mechanisms of self-tolerance results in reactions against one's own cells and tissues that are called autoimmunity. Autoimmune diseases are estimated to affect at least 2-5% of the population in the developed countries and incidence of these disorders is steadily rising.
Inflammatory demyelinating diseases are a broad group of disorders characterized by immune-mediated myelin damage of suspected autoimmune aetiology, leading to conduction abnormalities, and often accompanied by axonal loss. The demyelinating lesions are usually either limited to the central nervous system (CNS) or to the peripheral nervous system (PNS).
Many of these diseases are chronic and debilitating and frequently affect young adults, hence their enormous medical and economic burden. Although in recent years many new effective approaches were developed in order to treat these disorders, the pathologic mechanisms underlying their onset and progression, despite treatment, are still largely unknown. Clinical observations and studies in models have provided evidences to support the general concept that environmental microbial and non-microbial triggers may lead in genetic susceptible individuals to disruption of self-tolerance, including activation of self-reactive lymphocytes and generation of autoantibodies.
However, the environmental triggers remain in most cases elusive, there is no easy way to assess the polygenic susceptibility trait in affected individuals, and we often fail to demonstrate the presence of circulating antibodies or other markers of an auto-immune signature. These limits ultimately result in diagnostic uncertainty and possible misdiagnosis. Finally, many of these disorders show a chronic progressive phase, which is clinically paralleled by increasing disability and resistance to treatment, and for which the ultimate cause is still unknown.

This work tried to address some of these issues in the field of immune-mediated inflammatory disorders of the central and peripheral nervous system, with focus on multiple sclerosis, acute demyelinating encephalomyelitis and chronic inflammatory demyelinating polyradiculoneuropathy.

In the first part "***Environment***" we investigated the role of air pollution as a novel environmental factor impacting on Multiple Sclerosis disease course. We found an association between respiratory exposure to particulate matter and occurrence of contrast-enhancing lesions on brain Magnetic Resonance Imaging in MS patients. We showed *in vivo* in MS patients and *in vitro* that PM exposure can lead to up-regulation on peripheral blood lymphocytes of adhesion molecules and chemokine receptors and to amplification of Th17 cells response.

The second part "***Infections***" encompass two studies on a population of adult-onset acute demyelinating encephalomyelitis (ADEM), another inflammatory disorder of the central nervous system, with heterogeneous clinical presentation and frequent preceding infection. The demyelinating lesions in ADEM are usually limited to the central nervous system (CNS), however, a significant proportion of cases showed contemporary involvement of the peripheral nervous system (PNS). Firstly, we reported on the clinical features, outcome and response to treatment of a large multi-centre cohort of patients with combined central and peripheral demyelination (CCPD) (Cortese et al. 2016). The data suggested that CCPD manifests with heterogeneous features, frequent post-infectious onset, primary PNS or CNS involvement, monophasic or chronic disease course, unsatisfactory response to treatments, and generally poor outcome. Secondly, we investigated the role of Neurofascin-155 as a putative antigen in CCPD and found that, differently from a previous study on a cohort of Japanese patients, NF155 does not represent a common target of the immune response of CCPD in Caucasian patients (Cortese et al. 2016).

The following section "***Genetics***" briefly reviews current evidences on the cross –talk between environment and genetics in the immune-pathogenesis of ADEM (Berzero et al. 2016) and outlines the design and the preliminary results of a study currently in progress which aims to compare the genetic susceptibility profile in individuals with MS *vs* patients with other auto-immune

inflammatory disorders of the CNS, including ADEM, looking at common and distinctive genetic traits.

The fourth part "***Antibodies***" reports on the results of a multicentre study aiming to investigate the prevalence, clinical significance and pathogenic role of recently identified antibodies against the node of Ranvier, and in particular the single components of the paranodal Neurofascin 155-Contactin1-Caspr1 complex in chronic demyelinating polyradiculoneuropathy (CIDP). Antibodies were found in 11/200 subjects with CIDP (5.5%) but in none of healthy and disease controls and are associated with particular clinical features, a more aggressive disease course and a poor response to immunoglobulins. Moreover we identified that up to 40% of CIDP patients show reactivity to axonal of myelin nerve components, and sera are currently being investigated for identification of novel targets.

The fifth section "***Diagnostic challenges and misdiagnosis***" address the probably underestimated burden of misdiagnosis of inflammatory disorder and in particular of CIDP. Recent studies reported that up to 47% of patients diagnosed with CIDP and receiving immune-therapy failed to meet minimal CIDP diagnostic requirements and showed abnormalities which could be better explained by a process other than CIDP, including different acquired and hereditary neuropathies (Allen and Lewis et al. 2015). To this regard, our study focuses on hereditary transthyretin (ATTR) amyloidosis as mimicker of CIDP. ATTR amyloidosis is a debilitating highly penetrant autosomal dominant disease leading to motor disability within 5 years and generally fatal within a decade without treatment. We found that ATTR amyloidosis is misdiagnosed in 30% of cases and CIDP is the most common alternative diagnosis, which is supported by the electrodiagnostic finding of a demyelinating neuropathy in half of them, together with a frequent mild raise in CSF proteins. This section is completed by an appendix (**APPENDIX 1**) reporting on the results of a multicentre study

assessing the effectiveness and safety of Tafamidis for the treatment of hereditary ATTR amyloidosis in Italy (Cortese et al. 2016).

In the last section "***Immunity and degeneration***" we addressed the topic of the relationship between inflammatory and degenerative pathologic processes in sporadic inclusion body myositis (IBM) and other protein aggregate myopathies. IBM is the most common muscle disease in adults aged older than 50 years and its muscle pathology is characterized the contemporary presence of inflammatory changes and degenerative features, including aggregation of TDP43. In the present study we found that TDP43-dependent splicing is altered in IBM and other myopathies with protein aggregates, including the recently described by us and others HSPB8-related distal hereditary motor neuropathy and protein aggregate myopathy (**APPENDIX 2**) and we suggest that more widespread changes of RNA metabolism due TDP43 loss-of-function may bear pathogenic relevance to immunotherapy-resistant muscle and nerve degeneration.

3. ENVIRONEMENT

AIR POLLUTION AS A NOVEL CONTRIBUTOR TO THE INFLAMMATORY ACTIVITY OF MULTIPLE SCLEROSIS

INTRODUCTION

Multiple Sclerosis (MS) is an inflammatory demyelinating disorder of the Central Nervous System (CNS) which is characterized in its early phase by periods of exacerbation and remission along with progressive neurologic disability.

During MS relapses, inflammatory cells migrate through the Blood-Brain Barrier (BBB) inside the CNS, where autoreactive T cells are reactivated, leading to local inflammatory demyelination, disruption of the BBB and subsequent enhanced extravasation of leukocytes (Sospedra and Martin 2005; Mallucci et al. 2015).

MS relapse occurrence varies during the year and different studies reported an increased relapse rate in either autumn/winter or spring/summer (Spelman et al. 2014). Infections, low vitamin D and low melatonin levels have been associated to increase relapse occurrence (Fitzgerald et al. 2015; Farez et al. 2015). Moreover, dietary habits, by modifying the gut microbiota or as a result of higher salt intake, have also been linked to increase incidence and exacerbation of diverse autoimmune diseases (Kleinewietfeld et al. 2013; Wu et al. 2013). However, none of these factors can fully explain the variability of MS course, thus suggesting that additional factors may play a role.

Environmental air pollution is increasingly recognized as a major health issue in western countries. Numerous studies reported an association between ambient levels of air pollution, as indicated by higher concentration of airborne Particulate Matter (PM), with reduced life expectancy and increased incidence and exacerbation of asthma, chronic obstructive pulmonary disease and ischemic heart disease (Dockery et al. 1993; Pope, Ezzati, and Dockery 2009; Hoek et al. 2002; Brunekreef et al. 2009) .

PM10 is physically defined by the mass median aerodynamic diameter <10 µm of the particles, which are able to reach the lower respiratory tract, where they can be phagocytized by alveolar macrophages. Chemically, PM10 is a mixture of complex aggregates of elemental and organic carbons, metals, sulphates, nitrates and microbial contaminants whose major source is fossil fuel combustion from heating and road transport in urban areas. It has been held responsible for the majority of adverse pulmonary and cardiovascular effects of air pollution (Kelly and Fussell 2015). Several epidemiological studies have also shown how high levels of PM may be associated to increased incidence or worst outcome of different neurological diseases including stroke (Alimohammadi et al. 2016), Alzheimer's disease (Jung, Lin, and Hwang 2015), Parkinson's disease (Kirrane et al. 2015), migraine (C.-C. Chen, Tsai, and Yang 2015) and autism (Volk et al. 2013). However, little is known about the impact of respiratory exposure to PM10 on MS course. Recently, Odoardi *et al*. have identified in the experimental autoimmune encephalomyelitis (EAE), the animal model of MS, a crucial role of the lung in the pre-clinical phase of the disease. After local stimulation in the lung, autoreactive T cells strongly proliferate, assume migratory properties, and enter the CNS inducing paralytic disease. The authors concluded that the lung could therefore contribute to the activation of potentially auto-aggressive T cells and that their transition to a migratory mode is a prerequisite to entering their final target tissue (Odoardi et al. 2012).

Of note, in MS patients, respiratory exposure to tobacco smoking, but not tobacco snuffing or chewing(Hedström et al. 2009), and exposure to organic solvents (Oddone et al. 2013) have also been associated to higher MS incidence and increased activity.

Here, we investigated the influence of particulate air pollution on MS disease course and found an association between respiratory exposure to PM10 and occurrence of contrast-enhancing (CE) lesions on brain Magnetic Resonance Imaging (MRI). We showed *in vivo* in MS patients and *in vitro* that PM exposure can lead to up-regulation on peripheral blood lymphocytes of adhesion molecules and chemokine receptors, including CCR6, which is known to mediate crossing of the BBB by Th17 cells (Reboldi et al. 2009; Sallusto et al. 2012). To this regard, *in vitro* treatment with

urban particulate matter of monocyte-derived dendritic cells (mdDC) induced a massive release of proinflammatory Th17 polarizing IL1 beta, IL6 and IL23 cytokines, which was followed in mixed mdDC/peripheral blood mononuclear cells (PBMC) cultures, by an amplification of Th17 cells response.

METHODS

Patients

Between October 2003 and April 2010, we collected 226 brain MRI from 53 patients diagnosed with relapsing-remitting (RR) MS at C. Mondino National Neurological Institute in Pavia, Italy. Inflammatory activity of the disease was defined as presence of CE lesions after gadolinium injection.

Venous blood samples were obtained from an independent cohort of 44 prospectively enrolled RR MS patients, who underwent clinical examination and MRI scan on the same day and showed stable clinical and radiological disease without CE lesions, and from 19 healthy controls (HC), having the same residence of MS patients.

Study protocol was approved by the local Institutional Ethic Committee, and all subjects signed informed consent form.

Air pollution measurement

Daily recoding of air pollution was obtained by Regional Environmental Protection Agency (ARPA) based on monitors at 53 different sites across Lombardy depending on the residence of the patients. Daily mean concentration of PM10 was obtained using an algorithm that combined levels reported by multiple monitoring as reported in (Angelici et al. 2016). For patients who worked far from their residence, mean daily PM10 levels at residence and work place were averaged. Of note, a previous study found a high correlation between estimated PM10 exposure with personal exposure measured by mobile devices in the region considered in the present study (Zeger et al. 2000).

Flow cytometry

Blood samples were freshly analysed by lyze-and-wash whole blood staining procedure.

Experiments were performed in collaboration with Dr Luca Lova (BD, Milano) and Dr Elisabetta Volpe and Luca Battistini (Fondazione Santa Lucia, Roma)

Surface staining was performed on ice for 20 minutes and the cells were then analysed on a two laser, six colour FACS Canto flow cytometer.

Multiplexed dilutions of monoclonal antibodies (mAbs) were used to characterize lymphocyte populations.

The following antibodies from BD Biosciences were used: CD69-FITC (Cluster of Differentiation 69), HLA-DR-FITC (Human Leukocyte Antigen - antigen D Related), CD38-FITC (cluster of differentiation 38), CD49d-PE (Very Late Antigen-4), CD11a-PE-Cy7 (Lymphocyte Function-associated Antigen 1), CD4 APC-H7, CD8 APC-H7, CCR6-PerCP-Cy5.5 (C-C chemokine receptors 6), CD183 PE-Cy7 (C-X-C chemokine receptor 3), CD44- PerCP-Cy 5.5 (homing cell adhesion molecule).

Purification, culture and staining of PBMCs from adult blood

PBMCs were separated by Ficoll-Hypaque centrifugation (Amersham Biosciences) from buffy coats of healthy blood donor volunteers (Centro Trasfusionale Policlinico Umberto I, Rome, Italy and Policlinico San Matteo, Pavia, Italy). PBMCs were cultured in 48-well plates (Corning) at a density of 2 x 10^6 per well in RPMI (Sigma) containing 10% fetal calf serum (Lonza) in presence of different doses of particulate matter (1648a, urban particulate matter, National Institute of Reference Material, USA; (final concentration: 10 µg/ml and 40 µg/ml). After 24 hours, cells were harvested, stained with anti-CD4 FITC (Miltenyi), anti-CCR6 PerCP (BD), anti-LFA1 PE-Cy7 (BD) and CD162-PE (BD), washed, and then analysed by flow cytometry (FACS Canto Becton Dickinson).

Monocyte-derived dendritic cells (mdDC) generation and mdDC/PBMC mixed cell cultures

MdDC were generated from peripheral blood monocytes as described previously (Patrizia Comoli et al. 2003). PBMC were suspended at the concentration of $1x10^6$/ml in RPMI 1640 medium, and 1-ml aliquots were plated in 24-well plates. After 90 minutes at 37°C, non-adherent cells were discarded, and human recombinant IL-4 (R&D Systems, Minneapolis, MN) at a final concentration of 500 U/ml and human recombinant granulocyte-monocyte colony-stimulating factor (GM-CSF) at a final concentration of 800 U/ml (Sandoz Pharmaceuticals, Basel, Switzerland) were added. After 5 to 6 days of incubation, cells were recovered, phenotyped to assess the degree of maturity, and pulsed for 24 h with different doses of PM10 (final concentration: 5 μg/ml and 20 μg/ml). Supernatant from DC cultures was collected and tested for cytokine production.

PBMC were cocultured with unmanupulated mdDC, or with mdDC pulsed with PM10 (PBMC:mdDC ratio of 100:1), for 8 days in RPMI medium supplemented with 1% fetal calf serum (Hyclone). Cytokine secretion profile was evaluated on culture supernatant by ELISA and on cultured cells by ELISPOT assay.

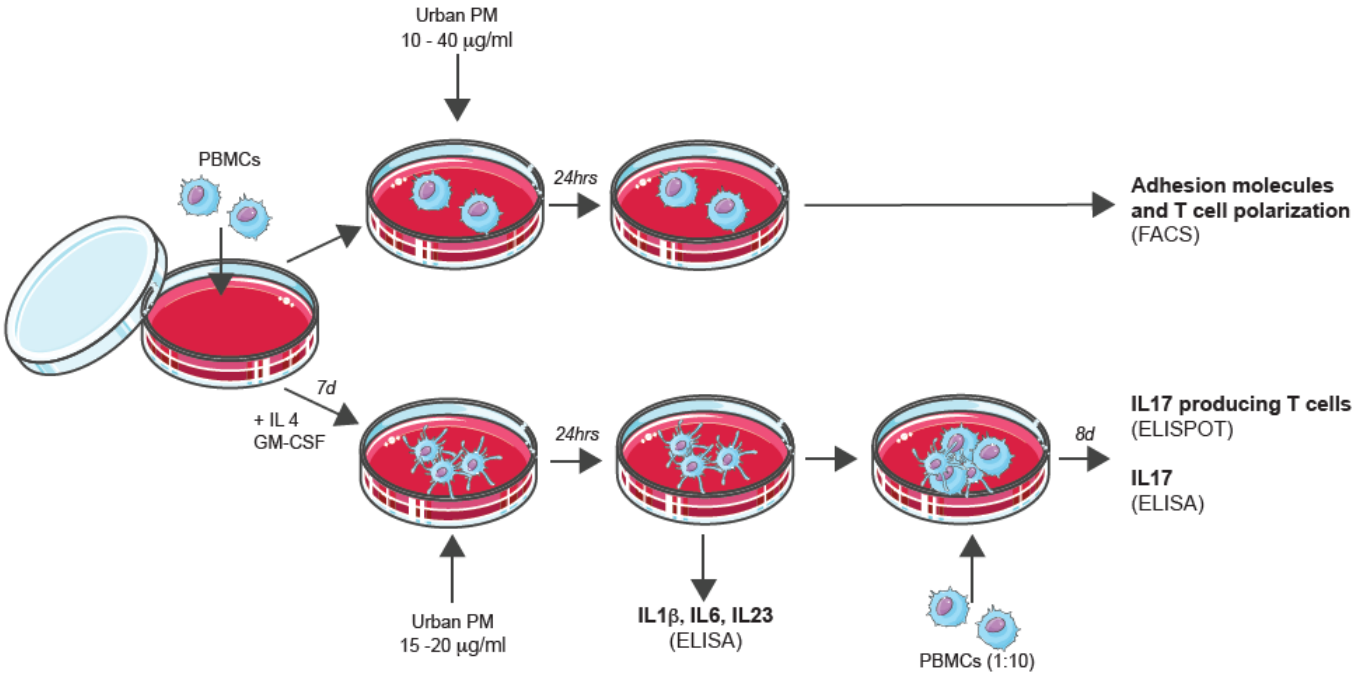
ELISPOT and ELISA assays

ELISPOT assays were performed according to a previously described method (P. Comoli et al. 2007). Briefly, 96-well multiscreen filter plates (MAIPS 4510, Millipore, Bedford, MA) were coated with 100 μl of primary antibody (IL17; Mabtech, Nacka, Sweden) at 2.5 μg/ml, and incubated overnight at 4°C. Cultured cells were plated at $1x10^5$ cells/well in triplicate. After incubation for 24 hours at 37°C, 100 μl of biotinylated secondary antibody (Mabtech, 0.5 μg/ml) was added, and plates were then processed according to standard procedure. Cytokine-producing spots were counted using an ELISPOT reader (Bioline, Torino, Italy). The number of spots per well was calculated after subtracting the assay background, quantitated as an average of 24 wells

containing only sterile complete medium, and specific background, quantitated as the sum of cytokine spots associated with responders alone.

Cytokine levels in the supernatant of mdDC or mdDC/PBMC cocultures were measured using monoclonal antibody pairs (IL1 beta and IL17: Mabtech; IL6: Endogen Tema, Castenaso, Italia; IL23: U-Cytech Biosciences, Utrecht, The Netherlands). Plates were coated with purified antibodies at the appropriate concentrations. Standard curves were prepared with recombinant human cytokines. Biotin-labeled antibodies were added and HRP-conjugated streptavidine was used to develop the reactions. Plates were read at 450 nm (Titertek Plus MS 212M). Results were reported as pg/ml. Procedures followed for *in vitro* studies are summarized in **figure 1**.

Figure 1



Illustrative methods for in vitro procedures. This part of the study was performed in collaboration with Dr Patrizia Comoli (Policlinico San Matteo, Pavia)

Statistical analyses

The sample was described by means of mean and standard deviation for continuous variables and proportions for categorical ones.

In order to investigate the relationship among PM10 levels, time of detection prior MRI, and presence of CE lesions on brain MRI was carried out a three way analysis of variance mixed model. The analysis has taken into account the principal effects of time of detection prior MRI, presence of CE lesions on brain MRI and patients and also of interaction between time of detection prior MRI and presence of CE lesions on brain MRI and between time of detection prior MRI and patients. . The analysis was carried out on ln-transformed data view of the asymmetric frequency distribution of the PM10 variable.

A logistic regression, adjusted for patients, was performed to quantify the risk of presence of CE lesions on brain MRI associated with PM10 levels in the previous 15 days, treatment, smoker status and seasons.

Correlation between expression levels (mean fluorescence intensity) of surface molecules tested by flow-cytometry in MS patients and HC and PM10 levels was analysed using Spearman correlation coefficient. Univariate associations were tested in a multivariate linear regression model including age, gender, treatment and smoker status.

For pair-wise comparison of different conditions from the same subject/sample or different subjects/samples we used a non-parametric or parametric two-tailed paired or unpaired Student's t test, respectively.

Statistical significance was taken at the <0.05 level.

All analyses involving data of patients were conducted using STATA/SE for Windows, version 12.1 (StataCorp, College Station, TX, U.S.A.).

For analyses of data from in vitro studies Graphpad was used.

Graphics were generated using Graphpad.

Images were created using Adobe Illustrator.

RESULTS

1. High PM10 exposure is associated with increased occurrence of CE lesions on brain MRI

Fifty-three patients were enrolled: mean age was 36±11 years, 16 (30%) were males, 24 (46%) were smoker and 16 (31%) were on first line-disease modifying treatment. Among 226 brain MRI considered in the study (average MRI/patients: 4.2±2.4, range= 1-12), 77 (34%) showed T1-enhancing lesions, indicating inflammatory activity of the disease. The mean PM10 exposure level in 15 days prior MRI, estimated according to the residence of the subjects, was significantly higher in patients with CE lesions compared with patients without active lesions (50.45 μg/m^3 95%CI 44.99-56.56 vs 42.39 μg/m^3 95%CI 38.81-46.31; f= 5.151, p=0.024) and this difference, even if of lower entity, is maintained at 30 days prior MRI (**Figure 2A**). Average PM10 levels varied by time of the year and decreased in summer, when MS relapse rate was also lower (**Figure 2B**).

The logistic regression showed a significant association between the presence of CE lesions on brain MRI and PM10 level in the 15 days prior MRI (OR=1.02, 95%CI 1.00-1.03, p= 0.019) and this association is independent of treatment, smoker status and season (**Table 1**).

Table 1. Particulate matter level is associated with MS disease activity

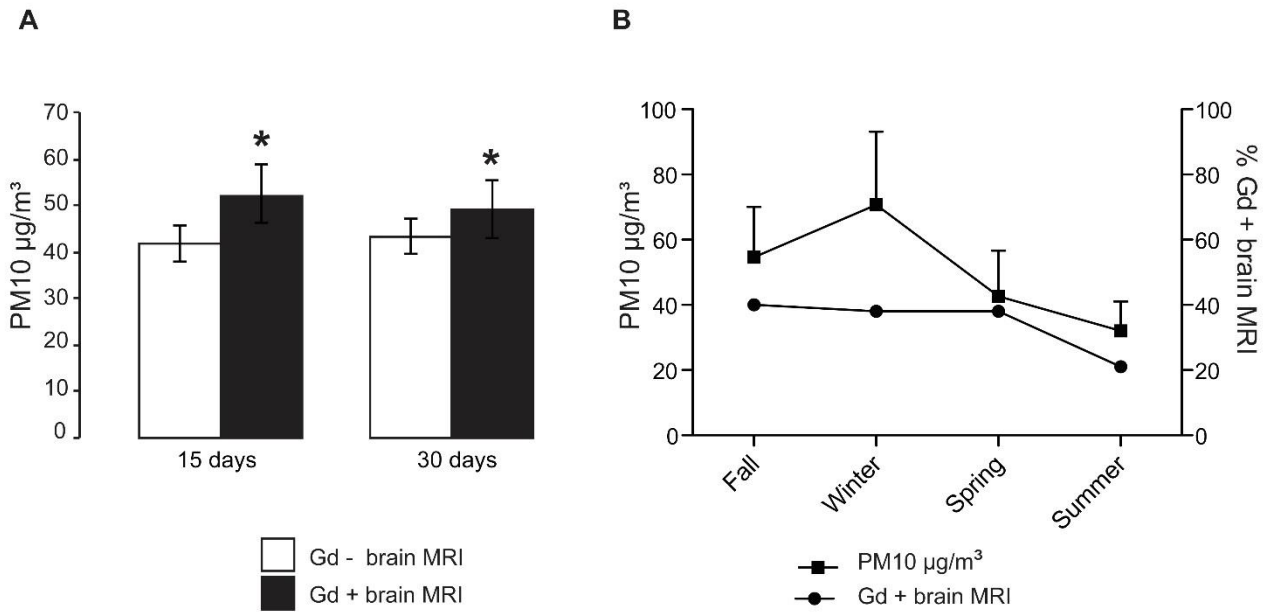
	OR*	95%CI	P value
Mean PM_{10} (1-15 days prior MRI)	1.02	1.00-1.03	0.019
DMT (yes vs no)	0.84	0.47-1.50	0.570
Current smoker (yes vs no)	0.57	0.30-1.07	0.083
Season			
Winter	1		
Spring	1.81	0.68-4.80	0.229
Summer	0.87	0.30-2.53	0.808
Fall	1.31	0.58-2.93	0.506

**Adjusted for patient's ID DMT: disease modifying treatment, PM10: particulate matter 10*

The OR=1,02 indicates that, for PM10 increases of 10 μg/m^3 in the previous 15 days, the risk of CE lesions increases of 22%.

Together these data suggest a possible role for PM10 in inducing MS activity.

Figure 2.



Average and 95%CI PM10 exposure levels in 15 and 30 days prior MRI in MS patients with (Gd+ brain MRI) and without (Gd- brain MRI) contrast-enhancing lesions on brain MRI ***[A].*** *Seasonal variation of the frequency of Gd+ brain MRI scans (%) in MS patients and mean + standard deviation airborne PM10 levels* ***[B].*** *OR: Odds Ratio. Gd: Gadolinium, PM10: particulate matter 10. * $p<0.05$*

2. In MS patients PM10 boosts the expression of adhesion molecules and chemokine receptors on circulating lymphocytes

Based on our clinical findings, which link respiratory exposure to air pollution with MS activity, and given the recent evidences of a role in EAE of the lung in licensing auto-reactive lymphocytes to enter CNS by boosting their migratory properties (Odoardi et al. 2012), we decided to study in MS patients the correlation between PM10 exposure and expression of adhesion molecules (AMs) and chemokine receptors (CCRs) on circulating CD4+ and CD8+ lymphocytes.

We tested by flow-cytometry the expression of AMs and CCRs in an independent cohort of 44 RR MS patients with clinically and radiologically stable disease and 19 healthy controls (HC), who were prospectively enrolled from December 2013 to February 2015. HC were recruited among people having the same residence of enrolled MS patients and thus most likely exposed to similar environmental conditions. Mean age of patients was 43 ± 10 years, 19 (43%) were males, 11 (25%) were smoker, 23 (52%) were on first line-disease modifying treatment. Mean EDSS was 1,7 ± 1,3 and mean disease duration was 11 ± 9 years. HC had a mean age of 46 ± 12 years, 9 (47%) were males and 5 (27%) were smoker.

Expression level of tested molecules did not significantly differ between MS patients and HC, except for the expression of CD44, which was significantly higher in MS patients *vs* HC, on both CD4+ and CD8+ T cells (**Table 2**).

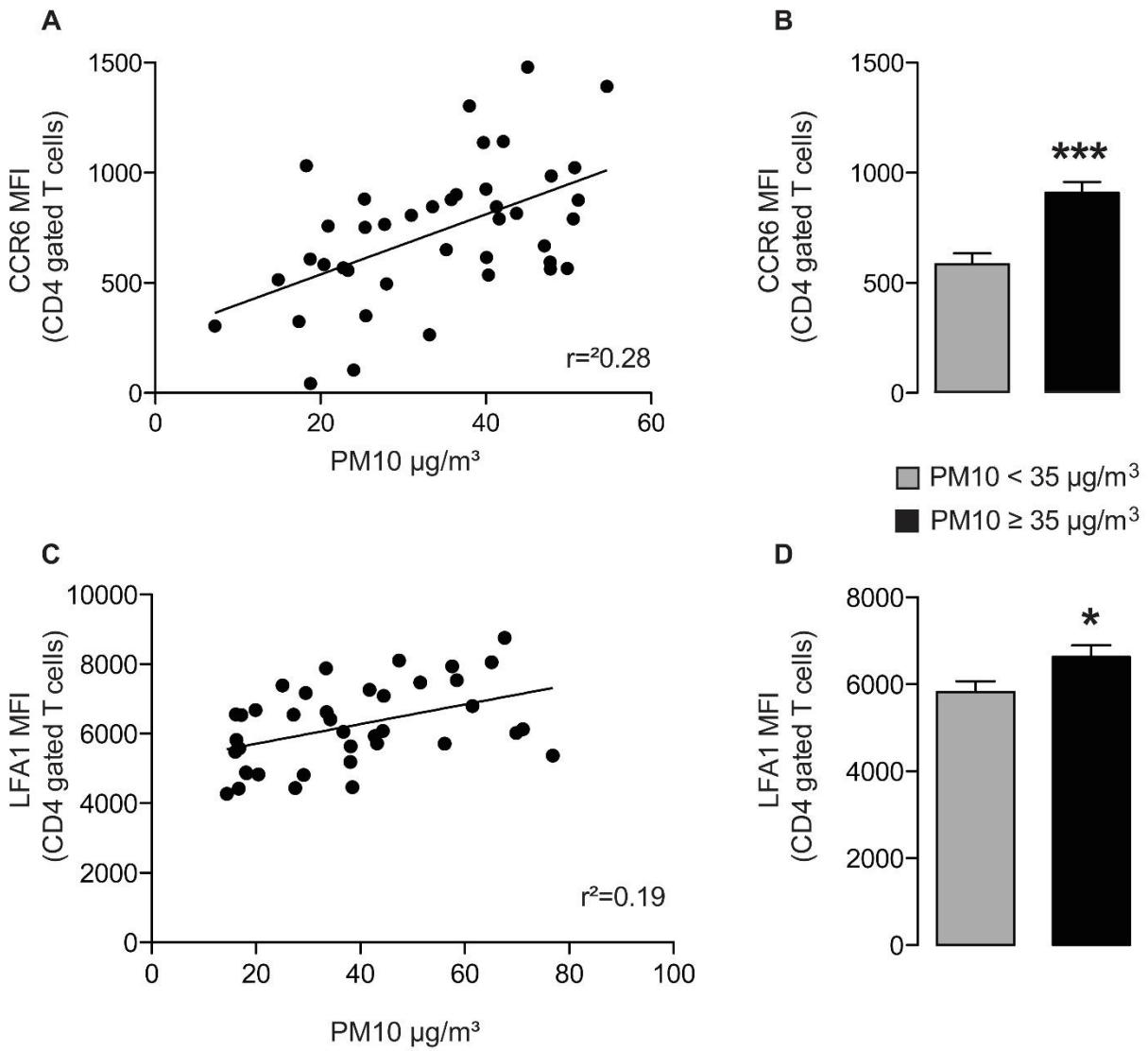
Table 2: Mean of expression level, expressed as mean fluorescence intensity (MFI), of all tested molecules on CD4+ and CD8+ T cells in MS patients and HC.

MFI	**MS**	**HC**	***p***	**MS**	**HC**	***p***
Activation molecules	**CD4**			**CD8**		
CD69	221.1 ± 8.1	205.9 ± 7.7	0.32	277.3 ± 11.6	285.2 ± 13.1	0.41
HLA DR3	1052.9 ± 125.5	1375.6 ± 392.3	0.41	1290 ± 137	1406.1 ± 379.8	0.63
CD38	532.4 ± 88	476.1 ± 94	0.89	689.1 ± 118.7	519.3 ± 110.2	0.59
Adhesion molecules						
CD44	32469.6 ± 1451.3	23701.7 ±3122.4	0.01	26900 ± 1414.9	17791.6 ± 2288.3	0.002
VLA4	2248.4 ± 92.5	2518.5 ± 184.6	0.43	3043.3 ± 122.5	3214.2 ± 161.3	0.37
LFA1	6218.3 ± 187.8	7139.5 ± 561.4	0.37	10470.2 ± 403.9	12436.3 ± 1041.1	0.22
Migration molecules						
CCR6	733.8 ± 48.1	807.6 ± 72.1	0.57	462.3 ± 33.2	574.6 ± 52.5	0.10
CXCR3	658.8 ± 37.1	861 ± 111.3	0.21	840.9 ± 59.8	736.1 ± 96.9	0.60

CD69 (Cluster of Differentiation 69), HLA-DR (Human Leukocyte Antigen - antigen D Related), CD38 (cluster of differentiation 38), CD44 (homing cell adhesion molecule), VLA4 (Very Late Antigen-4), LFA1 (Lymphocyte Function-associated Antigen 1), CCR6 (C-C chemokine receptors 6), CXCR3 (C-X-C chemokine receptor 3).

In MS patients we observed a significant correlation between PM10 level in the previous30 days and expression on CD4+ T cells of CCR6, a chemokine receptor characteristic of Th17 cells, and LFA1, which is part of the family of leukocyte integrins essential for migration of T cells through the BBB (**Figure 3A,C**). Expression of CCR6 and LFA1 was significantly higher in MS patients which were exposed to PM10 levels over 35 $\mu g/m^3$ compared to those exposed at lower concentrations of PM10 (**Figure 3B,D**).

Figure 3.



*Scatterplots **[A,C]** and histograms **[B,D]** of expression of CCR6 on CD4+ T cells **[A,B]** and LFA1 on CD4+ T cells **[C,D]** in MS patients and PM10 exposure in the previous 30 days. CCR6: C-C chemokine receptor 6; LFA1: lymphocyte function-associated antigen 1. * p<0.05, *** p<0.001*

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In a linear regression model, the association between exposure to PM10 and the expression of CCR6 (β=9.1, 95%CI= 4.4-13.8, p<0.001) and LFA1 (β=28.7, 95%CI= 9.5-47.8, p=0.004) on CD4+ T cells was independent from age, sex, treatment and smoker status. A significant, although weaker, correlation was also observed between PM10 levels and expression of CCR6 on CD8+ T

cells, which was not confirmed in an age-, sex-, treatment and smoker status-adjusted regression model. These associations were at least partially specific of an effect of PM10 on AMs and CCRs because expression of T cell activation markers was not similarly altered upon PM10 exposure (**Table 3**).

Interestingly enough, in HC we did not observe any significant correlation between expression of the tested migratory or activation markers and PM10 (**Table 3**).

Table 3: Spearman's Rho between average PM10 levels 30 days before enrolment and expression level of the all tested molecules on CD4+ and CD8+ in MS (N=44) patients and HC (N=19).

Spearman's Rho	**MS (N=44)**		**HC (N=19)**	
	CD4	**CD8**	**CD4**	**CD8**
Activation molecules	***Rho, p***			
CD69	-0.03, 0.90	-0.11, 0.60	-0.21, 0.59	0.23, 0.56
HLA DR3	-0.25, 0.38	-0.28, 0.33	0.22, 0.61	0.35, 0.40
CD38	-0.17, 0.56	-0.14, 0.41	0.31, 0.45	0.36, 0.38
Adhesion molecules				
CD44	0.13, 0.41	0.10, 0.51	0.13, 0.59	0.42, 0.08
VLA4	0.08, 0.57	-0.04, 0.79	-0.05, 0.83	0.13, 0.59
LFA1	0.45, 0.004	-0.13, 0.39	0.02, 0.92	0.16, 0.49
Migration molecules				
CCR6	0.5, 0.0007	0.34, 0.03	-0.02, 0.93	-0.22, 0.37
CXCR3	0.13, 0.41	0.03, 0.83	-0.07, 0.78	0.13, 0.61

CD69 (Cluster of Differentiation 69), HLA-DR (Human Leukocyte Antigen - antigen D Related), CD38 (cluster of differentiation 38), CD44 (homing cell adhesion molecule), VLA4 (Very Late Antigen-4), LFA1 (Lymphocyte Function-associated Antigen 1), CCR6 (C-C chemokine receptors 6), CXCR3 (C-X-C chemokine receptor 3).

Patients were followed up after 3 months but none of them had a clinical relapse, thus preventing further analysis of the correlation between expression of AMs and CCRs, their changes upon PM10 exposure and clinical activity of the disease in our study population.

This notwithstanding, our results show in RR MS patients a positive association of ambient air pollution with expression of AMs and CCRs on circulating lymphocytes. In particular, the strong association between CCR6 expression on CD4+ T cells and PM10 may suggest a prominent effect of PM10 exposure on Th17 recruitment.

3. In vitro PM10 induces expression of migratory markers on PBMCs and enhance dendritic cells-dependent generation of IL17-producing T cells

We next tested in-vitro the effect of urban PM on expression of CCR6 and LFA1 as well as on cytokine production and T cell polarization.

PM10 increased the expression of CCR6 on CD4+ T cells and LFA1 on both CD4+ and CD8+ T cells (**Figure 4A**).

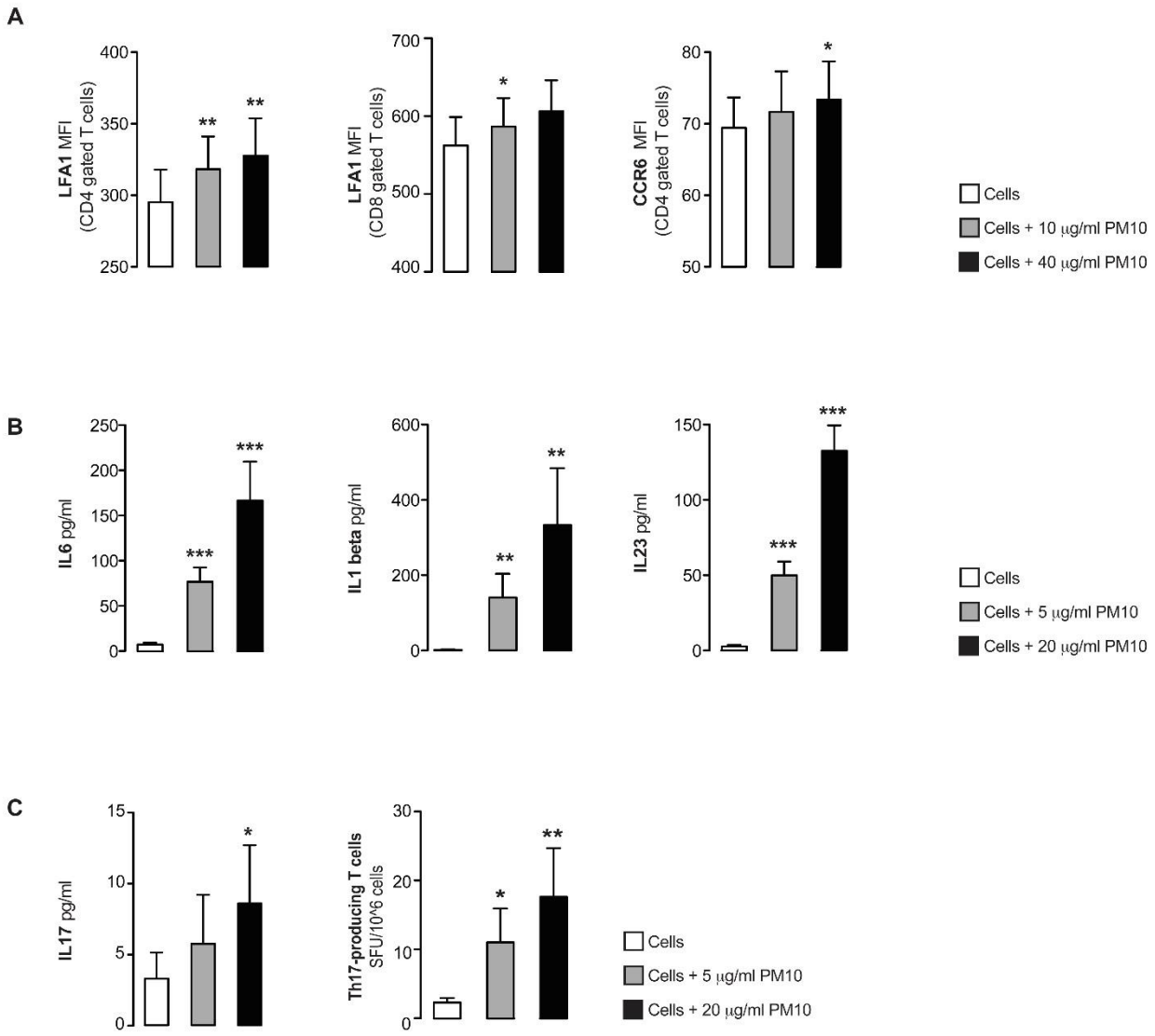
Based on the hypothesis that the effect of PM10 on amplification of CCR6+ Th17 cells may be mediated by resident innate immunity cells in the lung, we co-cultured PBMCs with PM-treated mdDC and analysed IL1 beta, IL6 and IL23 cytokine production by mdDC and assessed in mdDC/PBMCs mixed cultures the effect of PM treatment on Th17 cell polarization.

In vitro treatment of mdDC with PM, even at low concentration, induced a massive release of all Th17-polarizing cytokines tested, IL1 beta, IL6 and IL23 (**Figure 4B**). As well known, these cytokines are necessary for differentiation of Th17 into fully pathogenic cells (Langrish et al. 2005; McGeachy et al. 2007).

Co-culture of PBMCs with PM-treated mdDC significantly increased the number of IL17-producting T cells on ELISPOT-based assay (**Figure 4C**). Amplification of IL17 production T cells was paralleled by an increase of IL17 in cell culture medium (**Figure 4C)**.

Overall, these findings support the presence of a direct effect of PM10 on expression of AMs and CCRs on T cells, and a predominantly indirect effect of PM10 on Th17 polarization, by increasing production of Th17 polarizing cytokines by dendritic cells.

Figure 4.



In vitro treatment of PMBCs with PM induces LFA1 on CD4+ and CD8+ cells and CCR6 expression on CD4+ cells ***[A]****. In vitro treatment of monocyte derived dendritic cells (mdDC) with increasing doses of urban particulate matter (PM) induces secretion of IL1 beta, IL-6 and IL-23****[B]****. In mixed mdDC/PBMC co-cultures, in vitro treatment of mdDC with PM induces release of IL-17 and increases the percentage of IL-17 producing T cells* ***[C]*** ***.*** ** p<0.05, ** p<0.01, *** p<0.001*

DISCUSSION

Incidence of autoimmune disorders has steadily increased in the past century in industrialized countries, thus suggesting that a change in their environment may underlie this epidemiological observation.

Air pollution is increasingly recognized as a major public health concern and an increased concentration of airborne PM, one of the most studied component of air pollution, has been associated with both increased morbidity and mortality(Pope, Ezzati, and Dockery 2009; Dockery et al. 1993; Brunekreef et al. 2009; Hoek et al. 2002).

Particulate matter is a complex mixture of constituent chemicals. It is often classified by particle size, although this physical classification may oversimplify the molecular makeup, which may vary from urban to rural areas, including elemental and organic carbons, metals, sulphates, nitrates and microbial contaminants (Kelly and Fussell 2015).

In our study we found an association between MS inflammatory activity and concentration of PM10 in the month preceding MRI examination, with 10 $\mu g/m^3$ increase of PM10 corresponding to a 22% higher risk of disease activity, as shown by the presence of CE lesions on brain MRI.

Our results strengthen previous epidemiological evidences of an association between clinical MS exacerbations and levels of PM10 (Angelici et al. 2016; Oikonen et al. 2003; Gregory et al. 2008). In particular, a four-fold increase of monthly relapse rate in MS patients was reported in Sweden when air level of PM10 was in the higher quartile compared to the lowest (Oikonen et al. 2003). This clinical observation was confirmed by recent large retrospective study in our region which reported a 42% increase of hospital admission due to MS exacerbation when PM10 levels were in the highest quartile in the previous week (Angelici et al. 2016). Beside an effect on disease activity, increased concentration of airborne PM10 was also associated with an increased incidence of MS in female living in urban areas in Georgia, US (Gregory et al. 2008).

Low vitamin D levels are an established risk factor for MS incidence and exacerbation(Fitzgerald et al. 2015). Although we cannot exclude in our study a contribution of low vitamin D level to

enhanced disease activity in winter, vitamin D plasma concentration, which is high in summer-fall and low in winter-spring, does not seem to correlate with MS relapse rate during other times of the year at our latitudes. More recently, melatonin proved to be able to block differentiation of Th17 cells and boost the generation of Tr1 cells and, eventually, to exert a protective effect on MS activity(Farez et al. 2015). However, in our cohort disease activity was lower in summer, when melatonin levels are expected to be at lower levels. Finally, western dietary habits have also proved to play a role in increased incidence of autoimmune disorders, by altering the gut microbiome and through the pro-inflammatory effect of a sodium rich diet (Wu et al. 2013; Kleinewietfeld et al. 2013). However, dietary habits can hardly explain the seasonal changes in MS disease activity that we observed.

Our results suggest that air pollution may lead to MS exacerbation by two different mechanisms: on the one hand, PM10 may directly lead to up-regulation of AMs and CCRs on circulating lymphocytes, including CCR6 and LFA1, which may facilitate their entry to the CNS; on the other hand, PM10 could increase IL1 beta, IL6 and IL23 production by DC and enhance DC-dependent generation of IL17-producing T cells.

Migration of pathogenic self-reactive T cells to the CNS is an essential step for the development of EAE in immunized mice (Sallusto et al. 2012). Migration of lymphocytes trough the BBB is strictly controlled and requires the sequential interaction of AMs on T cells and BBB endothelial cells. Diverse studies have demonstrated how different subsets of encephalitogenic T helper cells use different molecular mechanisms to breach the BBB, probably at different sites within the CNS. In particular, CCR6 is known to mediate early recruitment of Th17 lymphocytes to CNS through its interaction with CCL20 on choroid plexus and mice lacking CCR6 were shown to be highly resistant to the induction of experimental autoimmune encephalomyelitis(Reboldi et al. 2009). LFA1 is an integrin heterodimer expressed on T cells that binds to its ligand ICAM-1 (InterCellular Adhesion Molecule-1) and mediates adhesion of lymphocytes to the vessel wall, including cerebral endothelium. In MS patients pharmacological blocking of lymphocyte migration through the BBB

by Natalizumab, a monoclonal antibody which target the alfa-integrin complex of which LFA1 is part, is considered one of the most effective treatments able to prevent formation of new demyelinating lesions(Miller et al. 2003).

Here we show in MS patients a significant correlation between respiratory exposure to PM10 and expression of CCR6 and LFA1 on circulating T cells. This observation was further supported by *in vitro* assays which showed a direct effect of urban PM on CCR6 and LFA1 expression.

Of note, increased level of airborne PM10 did not seem to lead in healthy subjects tested to an upregulation of AMs and CCRs, thus suggesting that chronic activation of the immune system may be a prerequisite for PM10 to exert its effects on the migratory state of lymphocytes.

Our results in MS patients parallel recent observations in EAE mice, which demonstrated a crucial role of the lung in the preclinical phase of EAE (Odoardi et al. 2012).

Moreover, increasing evidences support a crucial role of Th17 in the pathogenesis of EAE and as auto-aggressive cells in MS (Miossec, Korn, and Kuchroo 2009). In fact, frequency of Th17 cells is significantly higher in the cerebrospinal fluid of patients with RR MS during relapse compared to remission (Brucklacher-Waldert et al. 2009).

Our work suggests that PM can boost Th17 polarization by a DC-dependent IL1 beta, IL6 and IL23 release.

To this regard, IL17 was also significantly increased by CD4+ T cells isolated from the lung of rats after PM2.5 exposure (Becker et al. 1996). Moreover, in mice, ingestion of airborne particulate matter induced an inflammatory response in the intestine by enhancing the secretion of proinflammatory cytokines, including IL17, and altered gut microbiome and gut permeability(Kish et al. 2013).

PM10 is known to contain aryl hydrocarbon receptor (AHR) ligands and their interaction with the AHR, which has been shown to play a role in Th17 differentiation and IL17 secretion (Quintana et al. 2008), could represent a possible mechanism of its activity on the immune response. To this regard, previous studies showed that inhaled PM led to upregulation of genes related to polycyclic

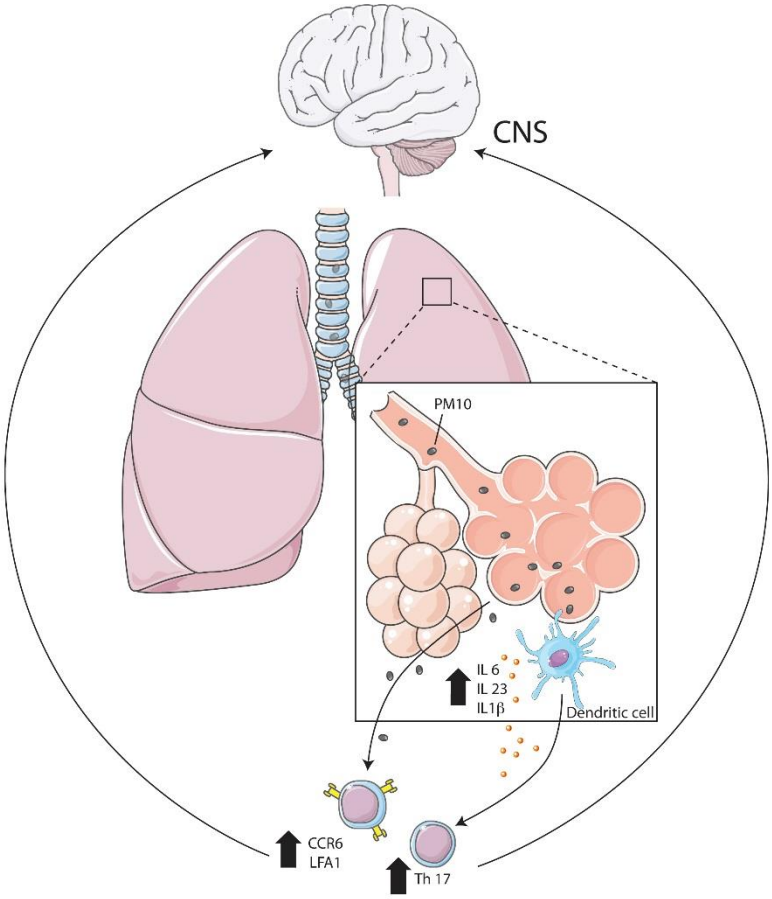
aromatic hydrocarbons (Sancini et al. 2014) and significantly increased polarization of Th17 in wild type mice but not AHR-/- mice (van Voorhis et al. 2013), suggesting a role of AHR as a mediator of PM10 proinflammatory properties. Moreover, changes in lipid plasma membrane composition and lipid peroxidation were also observed in different extra-pulmonary tissues, including brain, of PM-treated mice, and postulated to result from oxidative stress and inflammatory products following PM exposure (Rizzo et al. 2014)

Our results also imply a crucial role of DC in inflammatory response to PM10.

Of note, a previous study similarly found that in vitro urban PM enhanced DC maturation and IL6 production (Matthews et al. 2014). Enhanced secretion of IL6 and TNFα was also observed after in vitro treatment with airborne PM of human and rat alveolar macrophages (Becker et al. 1996). Importantly, diesel dust, which is over 90% elemental carbon, did not induce their release, thus suggesting that the cytokine –inducing activity of PM10 that we and others observed could be ascribed to different chemical composition of PM compared to diesel dust, including a higher content of extractable organic carbon and inorganic constituents such as sulphates, nitrates and metals, as well as microbial component. Interestingly enough, in a previous study, pre-treatment of UAP with polimixin B and heat shock inactivated its pro-inflammatory effect, thus pointing to a major role of microbial contaminants in proinflammatory effect of PM10 (Becker et al. 1996).

All together, these observations led us to hypothesize that lung resident DC may uptake PM10 in the lower respiratory tract, and, after migration to bronchial associated lymphoid tissue, induce a proinflammatory response with generation of Th17 cells and enhance their migratory properties. Although this response would be initially protective, in susceptible individuals, exposure to PM10 could therefore contribute to the activation of potentially auto-aggressive T cells and their transition to a migratory and pro-inflammatory Th17 mode, which, by enhanced accessing to the CNS, may lead to clinical exacerbation of the disease (**Figure 5**).

Figure 4.



Respiratory exposure to PM10 may lead to increase disease activity in MS patients by inducing a direct upregulation of adhesion molecules and chemokine receptors on circulating lymphocytes and by promoting lung-resident dendritic cells-mediated release of IL1 beta, IL6 and IL23 and subsequent enhanced generation of Th17-producing T cells

Of note, a crucial role of lung-resident CD103+ DC for the polarization of Th17 was recently demonstrated taking advantage of a mouse model of pulmonary aspergillosis, in which lung CD103 + DC control Th17 function by their finely tuned IL2-IL23 production (Zelante et al. 2015). Larger prospective studies are needed to confirm the effect of PM10 on Th17 cell polarization in MS patients, as well as to confirm the effect of PM10 on migratory properties of circulating lymphocyte and loss of BBB integrity. Future studies should also aim at understanding whether the pro-inflammatory effect of PM10 depends mainly on its physical properties or on chemical

properties of one or more of its components. Finally, molecular pathways underlying the cytokine-inducing activity of PM10 on DC are still largely unknown and need to be elucidated.

In conclusion, our study identified a role of air pollution as a novel contributor to MS disease activity. PM10 is able to increase expression of AMs and CCRs on circulating lymphocytes and enhance Th17 polarization by a prominent proinflammatory DC mediated cytokine release. We speculate that PM10 exposure in the lungs may lead to MS exacerbation by enhancing a DC-dependent production of autoreactive Th17 lymphocytes and by boosting their migratory capacity through the BBB.

4. INFECTIONS

COMBINED CENTRAL AND PERIPHERAL DEMYELINATION: CLINICAL FEATURES, DIAGNOSTIC FINDINGS, AND TREATMENT

INTRODUCTION

Acute disseminated encephalomyelitis (ADEM) is considered an inflammatory monophasic demyelinating disorder of the central nervous system (CNS), and has been first reported in children after infection or vaccination.

ADEM in children has typically a polysymptomatic presentation that includes encephalopathy, fever and meningeal signs. The latter can be dramatic and severe enough to warrant intensive care and mechanical ventilation. Despite the clinical severity, the final outcome is favorable with most cases recovering completely.

In adults, the clinical features of the disease are more heterogeneous, encompassing acute demyelinating syndromes with either polyfocal (encephalomyelitis) or monofocal (acute transverse myelitis, encephalitis, unilateral and bilateral optic neuritis) presentation. The encephalopathy is less frequent and the clinical features are usually dominated by the spinal cord involvement. The disease course can be relapsing or progressive in up to 20-30% of cases and, if looked at, involvement of the peripheral nervous system is not infrequent (Berzero et al. 2016).

In fact, we previously found that 36% of a selected cohort of patients with post-infectious inflammatory demyelinating disorders of the CNS showed either clinical or electroneurography (ENG) evidence of PNS involvement. Notably, these patients with CCPD showed worse prognosis and higher relapse rate compared to those with CNS-restricted variants (Marchioni et al. 2013). The contemporary occurrence of combined central and peripheral demyelination (CCPD) is rare and

data are limited to case reports or small case series (Thomas et al. 1987; Adamovic et al. 2008; Zéphir et al. 2008).

The aim of this study are to report the clinical and paraclinical features of a large cohort of patients with CCPD in order to gain information about clinical presentation, disease course, response to treatments and outcome

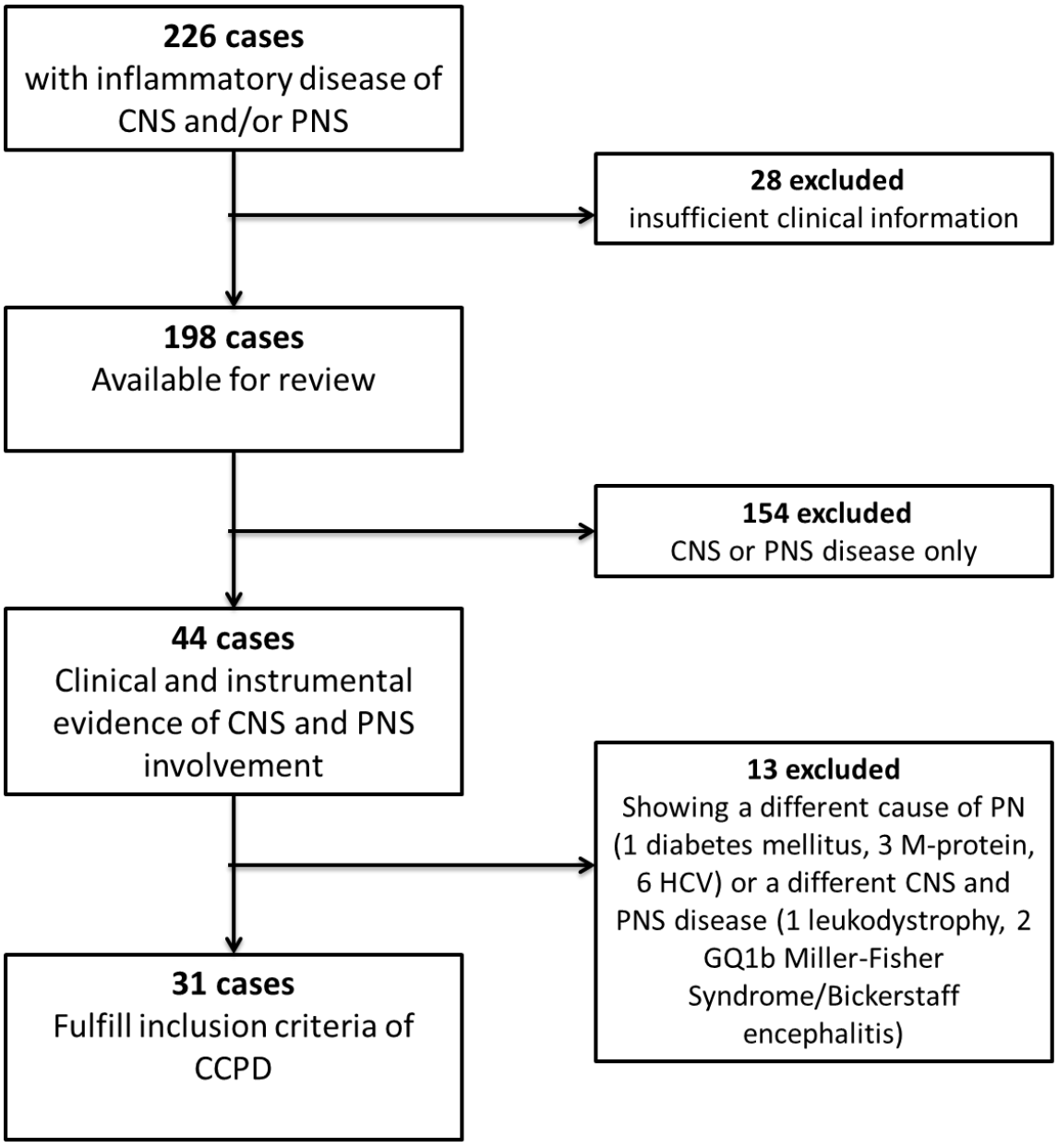
PATIENTS AND METHODS

This is a retrospective observational two-centre study. Inclusion criteria for CCPD patients' selection were:

A) presence of acute (within 1 month), subacute (between 1 month and 2 months) or chronic (over 2 months) onset of symptoms of CNS and/or PNS impairment; B) presence of CNS lesions suggestive of demyelination by brain and/or spine MRI; C) presence of peripheral neuropathy by nerve conduction studies (NCS); D) exclusion of other causes of combined CNS and PNS involvement, as detailed below.

Out of 276 cases with idiopathic inflammatory diseases of CNS or PNS who were identified from clinical databases and followed-up at the C. Mondino and C. Besta Neurological Institutes, 31 patients fulfilled all the inclusion criteria of CCPD and entered the study (**Figure 1**). The study was approved by C.Mondino Institutional Review Board and written informed consent was obtained from all the subjects.

Figure 1.



Flow-chart of CCPD cases enrolment

Clinical data were collected from in-ward and outpatient hospital charts. For descriptive purposes, disease course was classified into 3 categories: 1) monophasic, if characterized by one episode of acute demyelination but not followed by any further events; 2) relapsing, when the first event was followed, at least 3 months after the initial episode, by a clinical relapse defined by acute/subacute onset of new symptoms, or re-appearance of previously experienced symptoms; and 3) chronic progressive, defined by the presence of a steady or stepwise worsening of symptoms. Original MRI scans (1.5 Tesla), cerebrospinal fluid (CSF) examination, including agarose isoelectric focusing for oligoclonal IgG band (OCB) determination, and NCS exams were revised for technical and interpretation accuracy. We excluded from analyses of NCS: a) nerve roots and peripheral nerves stemming from metamers affected by inflammatory lesions, as detected by spine MRI; b) nerves showing alterations at entrapment points, possibly related to prolonged immobility.

The following investigations were performed in all patients in order to exclude other causes of combined CNS and PNS involvement, as well as distinct causes of neuropathy: fasting blood glucose, Hb1Ac, vitamin B12, folates, homocysteine, serum immunofixation, serology for HIV, HCV, Lyme disease, syphilis, ANA, ENA, ANCA, anti-cerebellum, anti AQP-4 antibodies (cell-based assay), anti-gangliosides, including anti GQ1b, and anti-sulfatides antibodies, serum ACE. In patients with chronic progressive disease course, metabolic testing for fitanic acid, galactocerebrosidase, arylsulfatase, very long chain fatty acids and gene tests for *PMP22* (CMT1A) and *GJB1* (CMTX1) were also performed.

After revision of clinical charts the following information was recorded for all patients: demographics, general medical history, risk factors for neuropathy, symptoms at presentation, previous infection or vaccination, outcome and response to treatment. Modified Rankin scale (mRS) was used to measure disability grade and outcome. Responders were defined as patients with an increase of at least 1 point on mRS after treatment. The first available neurological examination from disease onset was recorded. Muscle weakness was graded as mild (from 5- to 4 of Medical

Research Council (MRC) scale), moderate (from 4- to 3 of MRC scale) and severe/paralysis (less than 3).

Original NCS were evaluated for fulfilment of EFNS/PNS electrodiagnostic criteria for chronic inflammatory demyelination polyradiculoneuropathy (CIDP) (Joint Task Force of the EFNS and the PNS 2010). Original brain MRI were reviewed for fulfilment of 2010 McDonald criteria for multiple sclerosis (MS) (Polman et al. 2011).

Data were analysed with descriptive statistics methods. Continuous data were shown as mean or median (standard deviation, min-max)

RESULTS

Clinical presentation

Table 1 shows the demographic and clinical features at disease onset and at follow-up of 31 patients with CCPD. A summary of the disease history for each patient is also provided in **Table 2**. The majority of patients were male with a mean age at onset of 57 years, (range, 14 - 82). A previous infection or vaccination was reported in 20 subjects (65%), mostly infections of the upper respiratory tract, although a specific trigger was identified in 4 cases only (1 flu vaccine, 1 *S.pneumoniae* vaccine, 1 *C.jejuni* gastroenteritis and 1 *S.pyogenes* pharyngitis). The majority of patients presented with lower limb sensory-motor impairment and sphyncterial dysfunction, thus suggesting spinal cord lesions. However other symptoms, such as altered mental status and cranial nerve involvement, could characterize the onset of the disease. A primary PNS damage was present in 11 patients who presented with distal paraesthesia, and in 4 subjects with pseudo-GBS, namely with ascending motor-sensory impairment and abolished osteo-tendinous reflexes.

Table 1: Clinical features of CCPD patients at disease onset and in the chronic phase.

Demographic		
Age at onset (years; mean, range)	57 (17;14-82)	
Male	23 (74%)	
Disease course		
Monophasic	10 (32%)	
Relapsing	13 (42%)	
Chronic progressive	8 (26%)[§]	
	Disease onset	**Follow-up[#]**
Previous infection	20 (65%)	1 (5%)
Clinical features		
Lower limb motor and sensory	29 (94%)	19 (61%)
impairment	26 (84%)	2 (6%)
Urinary incontinence/retention	11 (35%)	4 (13%)
Distal paraesthesia	5 (16%)	-
Altered mental status	8 (26%)	7 (23%)
Upper limb motor and sensory	2 (6%)	-
impairment	4 (13%)	-
Headache	7 (23%)	4 (13%)
Ascending four limb sensory-motor		
impairment		
Other		
Response to treatment		
Steroids	17/23 (74%)	4/16 (25%)
IVIg	4/8 (50%)	4/11 (36%)
Other	1/1	1/4 (25%)
Overall response	19 (73%)	6 (19%)
Disability (mRS)	**N=25***	**N=31**
0	1 (4%)	2 (7%)
1	1 (4%)	2 (7%)
2	1 (4%)	1 (3%)
3	6 (24%)	4 (13%)
4	12 (48%)	4 (13%)
5	4 (16%)	18 (58%)

*All data are reported as number of cases (%), except age at onset, which is reported as median (min-max). # follow-up data for previous infection and clinical features refer only to cases with relapsing or chronic progressive disease course (N=21); § Including 6 cases with primary progressive disease course; * primary progressive CCPD cases are excluded. CCPD: combined central and peripheral demyelination; IVIg: Intravenous immunoglobulins; mRS: modified Rankin Scale.*

Table 2. Disease history in CCPD patients

#	Sex	Age at onset	prodroms	Symptoms at onset	compartment	Disease course	Time to relapse	Symptoms at relapse/progression	compartment	NCS	Final mRS	Neurologic syndrome at nadir*
1	F	51	gastroenteritis	lower limb weakness, sphincterial dysfunction, brainstem dysfunction	CNS	relapsing	118	upper limb weakness, facial emispasm	CNS & PNS	Definite CIDP	5	EMRN
2	M	59	flu-like syndrome	lower limb weakness, sphincterial dysfunction, acral paraesthesia	CNS & PNS	relapsing	8	upper limb paraesthesia	CNS & PNS	Possible CIDP	3	MRN
3	M	44	flu-like syndrome	altered mental status, ascending weakness with respiratory involvement	CNS & PNS	relapsing	25	worsening of lower limb weakness	CNS & PNS	Axonal PN	4	EMRN
4	M	41	flu-like syndrome	lower limb weakness, sphincterial dysfunction, headache	CNS & PNS	relapsing	6	worsening of lower limb weakness, Lehrmitte	CNS & PNS	Probable CIDP	0	EMRN
5	F	71	gastroenteritis	lower limb weakness, sphincterial dysfunction, weight loss	CNS & PNS	relapsing	6	worsening of lower limb weakness	CNS & PNS	Possible CIDP	5	MRN
6	M	82	none	lower limb weakness, sphincterial dysfunction	CNS & PNS	monophasic	NA	NA	NA	Definite CIDP	5	MRN
7	M	69	Gastroenteritis due to Campylobacter jejuni	lower limb weakness, sphincterial dysfunction, dysautonomia	CNS	relapsing	5	worsening of lower limb weakness	CNS & PNS	Possible CIDP	5	MRN
8	F	61	none	lower limb weakness, sphincterial dysfunction	CNS & PNS	monophasic	NA	NA	NA	Definite CIDP	3	MRN
9	F	73	gastroenteritis	ascending weakness	CNS & PNS	monophasic	NA	NA	NA	Axonal PN	5	MRN
10	M	74	pneumonia	altered mental status, lower limb weakness, sphincterial dysfunction	CNS & PNS	relapsing	1	worsening of lower limb weakness	CNS & PNS	Definite CIDP	5	MRN
11	M	75	streptococcal pharyngitis	lower limb weakness, sphincterial dysfunction	CNS & PNS	relapsing	3	upper limb weakness, respiratory involvement	CNS & PNS	Definite CIDP	5	EMRN

12	M	75	fever	altered mental status, lower limb weakness, sphincterial dysfunction	CNS, then PNS	monophasic	NA	NA	NA	Definite CIDP	5	EMRN
13	F	65	flu-like syndrome	altered mental status, lower limb weakness, sphincterial dysfunction	CNS & PNS	monophasic	NA	NA	NA	Axonal PN	5	MRN
14	M	77	Streptococcus pneumoniae vaccination	lower limb weakness, sphincterial dysfunction, distal paraesthesia	CNS & PNS	monophasic	NA	NA	NA	Probable CIDP	5	MRN
15	M	71	flu-like syndrome	lower limb weakness, sphincterial dysfunction, distal paraesthesia	CNS & PNS	relapsing	5	worsening of lower limb weakness	CNS & PNS	Definite CIDP	5	MRN
16	M	15	flu-like syndrome	lower limb weakness, sphincterial dysfunction, dystonia	CNS & PNS	relapsing	9	worsening of lower limb sensory ataxia	CNS & PNS	Definite CIDP	1	EMRN
17	M	51	none	lower limb weakness, sphincterial dysfunction	CNS	progressive	4	worsening of lower limb weakness	CNS & PNS	Axonal PN	5	MRN
18	F	63	none	ascending motor weakness	PNS	relapsing	58	upper limb weakness, dysphagia	CNS & PNS	Axonal PN	5	MRN
19	M	68	gastroenteritis, pneumonia	lower limb weakness, sphincterial dysfunction	CNS & PNS	monophasic	NA	NA	NA	Possible CIDP	0	EMRN
20	F	70	influenza vaccination	lower limb weakness, sphincterial dysfunction	CNS & PNS	monophasic	NA	NA	NA	Definite CIDP	5	MRN
22	F	46	none	lower limb weakness, distal paraesthesia	PNS	relapsing	12	upper limb weakness, worsening of lower limb weakness	CNS & PNS	Axonal PN	5	MRN
21	M	68	flu-like syndrome	lower limb weakness, sphincterial dysfunction, distal paraesthesia	CNS & PNS	monophasic	NA	NA	NA	Definite CIDP	4	MRN
23	M	58	fever	lower limb weakness, sphincterial dysfunction	CNS	progressive	130	worsening of lower limb weakness	CNS & PNS	Probable CIDP	5	MRN
24	M	38	none	lower limb weakness	CNS	progressive	44	worsening of lower limb sensory ataxia	CNS & PNS	Definite CIDP	2	EMRN

25	M	40	none	lower limb weakness, sphincterial dysfunction, distal paraesthesia	CNS	progressive	13	worsening of lower limb weakness	CNS & PNS	Possible CIDP	5	MRN
26	M	51	none	lower limb weakness, distal paraesthesia	CNS & PNS	progressive	108	worsening of lower limb weakness	CNS & PNS	Definite CIDP	1	MRN
27	M	35	none	distal paraesthesia	PNS	progressive	94	worsening of lower limb weakness	CNS & PNS	Definite CIDP	3	MRN
28	M	42	fever	left lower limb weakness, sphincterial dysfunction, cranial nerve involvement (diplopia and facial diplaegia)	CNS & PNS	progressive	163	right lower limb weakness	CNS & PNS	Possible CIDP	3	EMRN
29	M	23	none	lower limb weakness	CNS &PNS	progressive	56	worsening of lower limb weakness	CNS & PNS	Definite CIDP	4	MRN
30	M	59	none	lower limb weakness, sphincterial dysfunction	CNS & PNS	relapsing	12	upper limb weakness	CNS & PNS	Axonal PN	5	MRN
31	M	60	none	upper and lower limb weakness, cranial nerve involvement (diplopia, paraesthesia)	CNS & PNS	monophasic	NA	NA	NA	Axonal PN	4	EMRN

Neurologic examination

In 27 patients (87%) neurologic examination at disease nadir showed lower limb weakness, which was severe in 67% of them, with distal predominance in 5. Upper limbs weakness was found in 12 patients (39%). Tone was increased at four limbs in 11, decreased in 6 and mixed (increased at upper limbs and decreased at lower limbs) in 2 patients. Osteo-tendineous reflexes were more often increased at upper limbs (45% of the cases) and reduced or abolished at lower limbs (51%). Babinski sign was present in 58% of the cases. Dystonia was observed in one case **(Table 3).**

Disease course

Patients were followed-up for a mean of 84.3 months (43, 3 - 134). One third of the patients showed a monophasic disease course. Overall, in 21 cases (68%) we observed a progression of the disease: either a relapse, with subacute onset of new symptoms, in 13 cases (42%), or a steady chronic progression in other 8 patients (26%). Notably, six patients who presented with distal paraesthesia showed a progressive disease course from onset. In the relapsing subgroup, the mean delay between inflammatory events was 12 months (50, 3-126). In 5 cases, the relapse occurred in the first year after steroid withdrawal. Clinically, relapses and chronic progressions were characterized by worsening of lower-limb motor and sensory impairment, onset of upper limb paralysis or brainstem dysfunction, but not encephalopathy.

Table 3: Neurologic examination at disease nadir

Sign at neurological examination	**N (%)**
Rigor	0
Mental status	2 (6%)
Cranial nerves	2 (6%)
Motor impairment	
Upper limb	12 (39%)
mild weakness	6 (19%)
moderate weakness	5 (16%)
severe weakness/paralysis	1 (3%)
Distal worse	8 (25%)
Lower limb	27 (87%)
mild weakness	1 (3%)
moderate weakness	5 (16%)
severe weakness/paralysis	21 (67%)
Distal worse	5 (16%)
Tonus	
Upper limb	
Reduced	3 (9%)
Increased	7 (33%)
Lower limb	
Reduced	6 (22%)
Increased	11 (35%)
Osteo-tendineous reflexes	
Upper limb	
Reduced/Not elicitable	6 (19%)
Increased	15 (48%)
Lower limb	
Reduced/ Not elicitable	16 (51%)
Increased	10 (32%)
Babinski sign	18 (58%)
Sensory loss	
Upper limb	
Vibratory deep sensation	4 (12%)
Superficial sensation	2 (6%)
Lower limb	
Vibratory deep sensation	22 (70%)
Superficial sensation	22 (70%)
Sphincter dysfunction	18 (58%)
Dystonia	1 (3%)

Treatment and outcome

In the acute phase, 26 patients (84%) were treated with steroids, either 6-metil-prednisolone 500 to 1000 mg/day for 5 days followed by oral tapering over 3 months or chronic prednisone 1 mg/Kg/day, intravenous immunoglobulins (IVIg) or plasma exchange (PE) (**table 1**). An improvement of at least one mRS point was observed in 19/26 (73%). In 24 patients with relapses or chronic progression either steroids or IVIg were used later in the disease course but the response rate was lower (19%) than that at disease onset. Azathioprine and Methotrexate were used only in 2 cases with chronic progressive disease course without significant improvement.

Of interest, Natalizumab was used as an off-label drug in a young patient with unresponsiveness to steroids, and persistent inflammatory activity on brain MRI. The drug strikingly improved the brain MRI lesions. However, the clinical condition deteriorated due to a marked worsening of nerve conductions, thus leading to treatment discontinuation. The patient was subsequently started on rituximab with persistent clinical benefit (follow-up, 12 months).

Overall, outcome was poor (mRS≥4) in 22 cases (71%).

Cerebrospinal fluid examination

CSF analysis showed pleocytosis and elevated total proteins in 58% and 74% of patients, respectively **(table 4)**. Fourteen of 31 cases (45%) showed intrathecal IgG production, which was detected as either CSF-restricted oligoclonal IgG bands (OCB) (5 cases), or as OCB that were equal in serum and CSF in addition to CSF-restricted OCB (9 cases). CSF analysis was repeated in seven patients with chronic disease course. In the only two patients with CSF-restricted OCB at onset, the OCB pattern changed, as OCB disappeared in one case and formed a mirror pattern, namely absence of intrathecal IgG production, in the other one. In the remaining five CSF analysis did not show OCB at either disease onset or later in the disease course.

MRI

Multifocal demyelinating lesions of the brain and focal, multifocal and, less frequently, longitudinally extensive lesions involving the posterior-lateral white matter of the spinal cord characterized the CNS involvement in our cohort (**table 4, figure 2A-I**). Half of the patients had active lesions, mostly in the spinal cord, and 17 (56.7%) showed contrast enhancement of cauda and nerve roots, which tended to persist over time. Eleven patients (46%) fulfilled the Barkhoff-McDonald criteria for MS (*Polman et al*. 2011). On subsequent MRI scans, spatial dissemination of lesions occurred in 7 patients (22.6%) who had either relapsing or progressive disease course but not in patients with a monophasic disease course. New lesions occurred more frequently in the spinal cord.

Figure 2. MRI and nerve conduction study findings in CCPD.

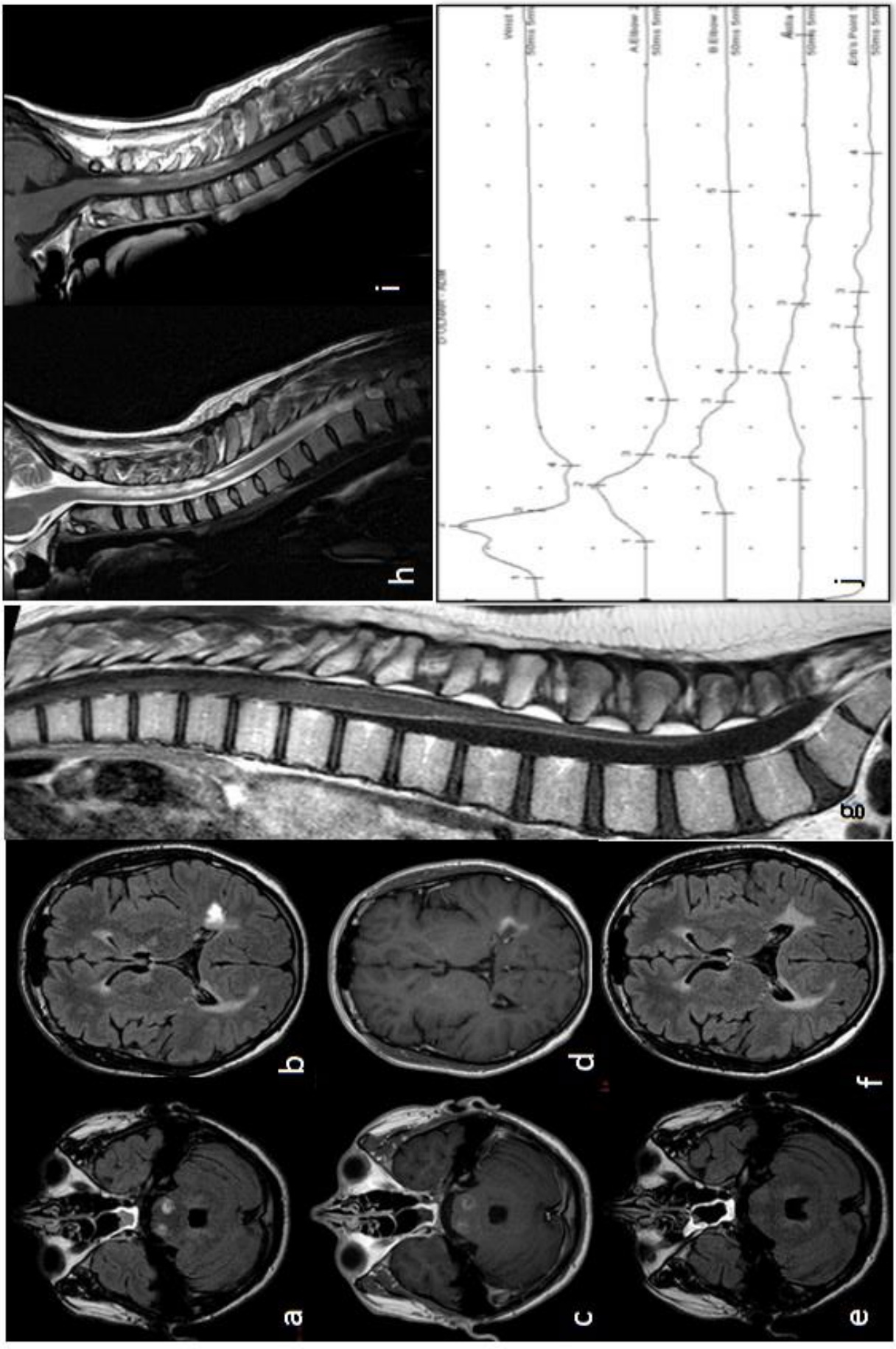


Figure 2 (continued). *Brain axial FLAIR images (**a, b, e, f**) and T1-w images post-gadolinium (Gd) administration (**c, d**) performed respectively pre- (**a, b, c, d**) and post- (**e, f**) immunosuppressive treatment with rituximab in a patient with CCPD. Pre-treatment spine MRI with T1-w image post-Gd (**g**). Pre-treatment brain MRI (**a,b,c,d**) evidences, in the context of diffuse mainly periventricular lesions, three round/ovoidal hyperintense signal alterations respectively at the level of the pons (**a**) and in left periventricular region (**b**) showing Gd enhancement (**c, d**). Post-treatment brain MRI shows marked reduction of the pons and left periventricular lesions (**e, f**). Pre–treatment spine MRI (**g**) shows marked enhancement both of caudal roots and conus lesions. Spine MRI with sagittal T1-w image (**h**) and post-Gd T1-w image (**i**) showing an extensive cervical spine lesion from C2 to D1 (**h**), hyperintense on T2 and iso-hypointense on T1 w.i., where a patchy vivid enhancement is detectable (i), mainly posteriorly. (**j**) Motor nerve conduction study of the ulnar nerve (recording from muscle abductor digiti minimi) showing temporal dispersion of the compound muscle action potential (CMAP) in proximal segments (roots and plexuses) in a patient with CCPD. Calibration: 5ms/div; 5 mV/div.*

Table 4: MRI and CSF findings in CCPD.

Brain MRI	**N=26**
T2 lesions	19/26 (73%)
Topography	
Periventricular	12/19 (63%)
Subcortical	12/19 (63%)
Infratentorial	10/19 (52.6%)
Deep grey matter	2/19 (10.5%)
Type of lesion	
MS-like	10/19 (53%)
ADEM-like	2/19 (10%)
Non specific	7/19 (37%)
T1 lesions with Gd-enhancement	3 (11%)
Spine MRI	**N=30**
T2 lesions	24/30 (80%)
Topography	
Postero-lateral white matter	16/24 (66.7%)
Anterior white matter	3/24 (12.5)
Central grey matter	3/24 (12.5)
White and grey matter	2/24 (8.3%)
Distribution	
Cervical	14/24 (58%)
Thoracic	16/24 (67%)
Lumbosacral (Conus-epiconus)	9/24 (37%)
Type of lesion	
Focal	9/24 (37%)
Multifocal	9/24 (37%)
LETM	5/24 (21%)
T1 lesions with Gd-enhancement	15/30 (50%)
T1 meningeal enhancement, nerve roots and conus/cauda Gd-enhancement	17/30 (56.7%)
Fulfilling the 2010 McDonald criteria for dissemination in space	12/26 (46%)
Temporal dissemination	7/31 (22.6%)
CSF examination	N=31
Pleocytosis	18 (58%), 54 (8-144)
Raised proteins	23 (74%), 97 (22-200)
OCB	
CSF-restricted OCB	5/31 (16%)
OCB equal in serum and CSF plus CSF-restricted OCB	9/31 (29%)

Table 4 (continued): *All data are reported as number of cases (%), except pleocytosis and raised CSF proteins, which are reported as number of cases (%), median (min-max). CSF pleocytosis is defined as more than 5 cells/μL; raised CSF proteins are defined as more than 40 mg/dL. ADEM: acute demyelinating encephalomyelitis, Gd: gadolinium, LETM: longitudinally extensive transverse myelitis. MS: multiple sclerosis, OCB: oligoclonal bands*.

Evoked potentials

Somatosensory evoked potentials (SEPs) from upper limbs were abnormal in 10/18 subjects (55%). SEPs from lower limbs were abnormal in all subjects, either slowed in 6 patients or not elicitable in 12, due to abnormal peripheral nerve conduction. Visual evoked potentials (VEPs) were altered in 9/14 patients (64%) and brainstem auditory evoked potentials (BAEPs) in 5/13 patients (38%).

Nerve conduction study

Twenty-three patients (74%) fulfilled the EFNS/PNS electrodiagnostic criteria for CIDP (*Joint Task Force of the EFNS and the PNS 2010)*, which were defined as definite in 14 (45%), probable in 3 (10%) and possible in 6 (19%), while 8 cases (26%) had a pure axonal neuropathy. Demyelination showed a proximal-distal gradient as documented by frequent F-response latency prolongation (6 cases, 19%) or absence (7 cases, 23%), and slow nerve conduction velocities (13 cases, 42%), compared to relatively preserved distal motor latencies (2 cases, 6%) (**figure 2-J**). Complete and partial conduction blocks were present in five (16%) and eight (26%) patients, respectively.
Peroneal and tibial nerves were the most frequently affected motor nerves, showing mixed demyelinating and axonal damage. Median and ulnar nerves often showed demyelinating involvement with relatively preserved motor amplitudes. Sensory nerve conduction was altered at sural nerves and, to a lesser extent, at median nerves (**table 5).**

Table 5. Nerve conduction study in patients with CCPD

	motor						sensory		
	Altered/ tested	**CMAP (mV)**	**MCV (m/s)**	**DML (ms)**	**F wave latency (ms)**		**Altered**	**SAP (µV)**	**SCV (m/s)**
ref		*>2*	*>40.6*	*<5.8*		*ref*		*>6*	*>42*
Peroneus R	17/23	0.9 (0.9)	36.1 (5.4)	4.3 (0.8)		***Suralis***	14/23	3.1 (2.3)	38 (1.9)
L	16/22	0.5 (0.5)	35.1 (4.4)	4.5 (1)			15/23	2.5 (1.6)	37.2 (2.1)
ref		*>5*	*>41*	*<5.5*					
Tibialis R	19/26	0.5 (0.5)	35.1 (4.4)	4.7 (0.8)	70 (9.8)				
L	17/26	2.2 (1.3)	33.3 (4.7)	4.6 (1)	67 (9.7)				
ref		*>5*	*>48*	*<3.3*		*ref*		*>6*	*>46*
Ulnar R	8/12	7.6 (2.9)	30.7 (8,3)	3 (0.6)	41.1 (10)	***Ulnar***	1/10	12 (12.5)	49.1 (6.1)
L	7/12	12.8 (14)	36.7 (8.1)	3 (0.6)	39.8 (7.5)		1/10	13 (10.2)	44.6 (10.8)
ref		*>5*	*>46.8*	*<4*		*ref*		*>8*	*>46.8*
Median R	11/15	8.5 (4.3)	37.7 (9.5)	3.7 (0.5)	35.7 (5.9)	***Median***	5/8	3.7 (2.2)	26 (4.6)
L	8/13	6.6 (6)	36.6 (5.6)	3.8 (0.9)	36.4 (6.2)		4/10	3.6 (4.6)	38 (2)

CMAP: compound motor action potential; MCV: motor conduction velocity; DML: distal motor latency; SAP: sensory action potential; SCV: sensory conduction velocity. Data are indicated as mean (standard deviation).

DISCUSSION

The examination of the clinical and paraclinical findings of the present cohort of patients, in keeping with previous reports (Thomas et al. 1987; Zéphir et al. 2008; Adamovic et al. 2008; Kawamura et al. 2013; Ogata, Matsuse, et al. 2015), highlights that CCPD is a rather heterogeneous disease.

A documented infection preceded CCPD onset in 65% of cases, but was unusual before relapses. Such percentage is higher than that reported recently in a Japanese CCPD cohort (10%) (Ogata, Matsuse, et al. 2015), but is consistent with a previous observation showing that the large majority (85%) of pediatric CCPD cases followed an infection (Adamovic et al. 2008). The high frequency of infections before CCPD onset pleads in favour of a role of infectious agents as triggers for subsequent self-sustained autoimmune processes.

PNS and CNS involvements were contemporary or rapidly subsequent in 22 cases (71%), CNS preceded PNS in 6 (19%) and PNS preceded CNS in 3 (10%). However, for some patients, the attribution of symptoms to either primary PNS or CNS involvement was difficult and subclinical alterations limited to one compartment could have been present before clinical onset. Relapsing symptoms occurred in 42% of the cases, and were mainly due to new CNS lesions, while PNS symptoms mainly showed an insidious onset and persisted over time. The preponderance of cases with contemporary PNS and CNS demyelination refutes the idea of a spreading of autoimmunity from one compartment to the other, following exposure of previously hidden epitopes. Conversely, it is conceivable that auto-reactive lymphocytes and/or antibodies against a common PNS and CNS epitope may cross the blood-brain- and blood-nerve-barriers and activate an inflammatory process concomitantly, as previously suggested (Kamm and Zettl 2012).

Regarding the PNS damage, NCS disclosed clear demyelinating features in 3/4 of the patients, with conduction blocks in 42% of them, while 26% of the patients had a pure axonal neuropathy. However the possibility that, in these latter cases, demyelinating changes were initially present and could not be detected in later disease stages due to severe axonal degeneration cannot be excluded.

Regarding the CNS damage, demyelinating lesions on MRI scans affected brain and spinal cord, particularly the dorsal and lumbosacral metamers, and were frequently associated with Gd-enhancement of cauda and nerve roots. Bilateral involvement of optic nerves was often asymptomatic but could be detected on VEPs in 64% of tested cases. Of note, six anti-aquaporin-4 antibody-seronegative cases had spinal cord lesions extending over 3 metamers, namely indistinguishable from those typically seen in neuromyelitis optica spectrum disorders, thus suggesting that CCPD should be considered in the differential diagnosis of such disorders. Unfortunately, these patients' serum samples are unavailable for anti-MOG antibody testing. Overall, 46% of the patients fulfilled the 2010 McDonald criteria for MS and 74% fulfilled PNS/EFNS electrodiagnostic criteria for CIDP, independently from their clinical presentation, disease course, response to treatment and outcome (data not shown). For this reason, beyond the fact that MS and CIDP can co-occur, definition of CCPD based on current diagnostic criteria for MS and CIDP may be too restrictive and arguably exclude several patients with a combined inflammatory disease of CNS and PNS, for whom extensive clinical investigation could not provide a better explanation. Analysis of larger international case series of patients with combined inflammatory disorders of CNS and PNS will be useful in order to define the spectrum of "typical" CCPD and its variants.

Of interest, 2/3 of patients entered a chronic phase of the disease, with either a relapsing or progressive disease course, analogously to what characterizes MS. However, the features that differentiate CCPD from MS in our series are: 1) the OCB patterns: persistent CSF-restricted OCB, which are typical of MS, were found in 2 patients only, and OCB that were equal in serum and CSF in addition to CSF-restricted OCB were more frequently detected. This latter abnormality suggests the presence of a mixed systemic plus intrathecal humoral immune activation. Nonetheless, we cannot exclude that patients whose CSF was not tested repeatedly, might have developed OCB later in the disease course; 2) the lack of asymptomatic spatial dissemination of lesions on MRI; and 3) the coexistence with PNS involvement itself. The CCPD cases here reported also differ from typical

ADEM because of their older age of onset, the rarity of large confluent and synchronous lesions on brain MRI affecting both white and grey matter, the relapsing and progressive courses of the majority of our cases, and the overall poor outcome (Krupp et al. 2013).

Efficacy of steroids and IVIg was only partial and mostly limited to the acute phase of the disease. Rituximab, whose efficacy has been observed in both MS and CIDP (Hauser et al. 2008; Benedetti et al. 2011), led to marked improvement in a CCPD patient with aggressive disease course and may thus represent an option for the treatment of steroids- and IVIg-resistant cases. Conversely, Natalizumab, although highly effective in dampening CNS inflammatory activity in one case with MS-like lesions on brain MRI, was detrimental on PNS symptoms, possibly due to drug induced inhibition of blood-brain barrier crossing by pathogenic T cells, which concentrate systemically. A previous study also reported the inefficacy of Natalizumab in CIDP patients (Wolf et al. 2010).

Clinically, the outcome was poor in 71% of patients, which is consistent with the previous observation of unfavourable outcome with EDSS ≥ 5 in 54% of children with CCPD (Adamovic et al. 2008), but it appears to be more severe than what was reported in a previous study showing that 26 out of 40 CCPD cases (65%) had no or mild disability (Hughes functional score ≤ 1) (Ogata, Matsuse, et al. 2015). The better outcome of the Japanese patients (Ogata, Matsuse, et al. 2015) could be due to differences in demographics, with a majority of young females in the Japanese series in comparison with the prevalence of older men in our study, and paraclinical features, with a higher frequency of patients with definite demyelination on NCS and cerebral lesions fulfilling the 2010 McDonald criteria for MS. In addition, the presence of diverse pathogenic mechanisms cannot be excluded.

Recently, antibodies directed to neurofascin-155 (NF155), a protein involved in axo-glial coupling at the paranodal regions which flank the node of Ranvier on both central and peripheral myelin, have been identified in five out of seven Japanese patients with CCPD (Kawamura et al., 2013) who showed a relapsing disease course and, differently from our patients, good response to IVIg. Also, antibodies to NF155 were identified in a low percentage of patients affected by CIDP who had

predominantly distal weakness, ataxia, disabling tremor and poor response to IVIg (Querol et al., 2014). A possible cerebellar origin of tremor was hypothesized, although there was no conclusive evidence of CNS involvement in these patients. However, their clinical phenotype differed from our patients who invariably showed symptomatic CNS damage, mainly long tracts impairment and altered mental status, but no tremor or cerebellar ataxia. Despite differences in clinical features, serological characterization for NF155 antibodies of larger cohorts of CCPD patients may thus help to define subtypes of patients with different prognosis and diverse response to treatment.

This study has some limitations. First, the retrospective design limits the definition of efficacy of the treatments. Second, the clinical evaluations were assessed at different time-points of the history of the disease, which may have resulted in non-homogeneous biological and instrumental findings.

In conclusion, our large cohort of CCPD patients was characterized by heterogeneous clinical manifestation, with either primary PNS or CNS involvement, frequent preceding infection and a monophasic or chronic disease course. The outcome was poor in the majority of patients and response to treatments, including steroids and IVIg, was partial and mostly limited to the acute phase of the disease. Definition of CCPD based on current diagnostic criteria for MS and CIDP may be too restrictive compared to the broad spectrum of combined inflammatory damage of CNS and PNS, whose pathogenesis still remains largely unknown.

ABSENCE OF ANTI NEUROFASCIN-155 ANTIBODIES IN COMBINED CENTRAL AND PERIPHERAL DEMYELINATION

INTRODUCTION

Combined central and peripheral demyelination (CCPD) encompasses a wide array of disorders ranging from myeloradiculoneuropathy and encephalomyeloradiculoneuropathy, which often occur after infection or vaccination, to co-occurrence of multiple sclerosis (MS) and chronic inflammatory demyelinating polyradiculoneuropathy (CIDP) (Kamm and Zettl 2012; Marchioni et al. 2013). Such phenotypic heterogeneity raises the question whether CCPD is a unique disease entity or a group of diseases with overlapping clinical phenotypes but different pathogenesis.

Antibodies directed to neurofascin-155 (NF155), a protein expressed on both central and peripheral nervous system myelin and involved in axo-glial coupling at the paranodal regions which flank the node of Ranvier, have been identified with high frequency in Japanese patients with CCPD (Kawamura et al. 2013; Ogata, Matsuse, et al. 2015). However, such data still await independent confirmation in different case series. On the contrary, antibodies to NF155 have been consistently reported, although with low frequency, in patients with CIDP (Ng et al. 2012; Querol et al. 2014; Devaux et al. 2016), thus questioning their specificity and pathogenicity in CCDP. Lastly, there is no current consensus on the gold standard technique for anti-NF155 antibody testing, and previous studies used rat or human NF155 based ELISA, cell-based assay (CBA), and immunohistochemistry (IHC) on mouse teased fibers.

The aims of this study were to confirm the association between anti-NF155 antibodies and CCPD in a Caucasian population, and to assess the diagnostic accuracy of the currently available techniques for anti-NF155 antibody testing.

METHODS

We examined sera from 16 CCPD patients (seven myeloradiculoneuropathy, six encephalomyeloradiculoneuropathy, three MS with CIDP). Clinical features of CCPD patients are summarized in **figure 1A**. As controls, 26 CIDP, 15 MS, 15 other peripheral neuropathy (PN) patients and 20 healthy controls (HC) were used. All the sera were collected for diagnostic purposes and stored at -80°C. A previously identified CIDP patient with antibody to NF155 was used as positive control. Anti-NF155 antibodies were detected by ELISA and CBA. Also, reactivity against the node of Ranvier was assessed by IHC on teased fibers from mouse sciatic nerve. Sera samples were tested in duplicate by two independent laboratories, which were blinded to patients' diagnoses. Briefly, for ELISA polystyrene microwells were coated with 4 µg/mL rat NF155-NS0 (R&D Systems), or 1 µg/mL human NF155 (OriGene), blocked with 5% nonfat milk in PBS, and, after washing, incubated with sera that were previously diluted at 1:200 (rat ELISA) or 1:100 (human ELISA) with 2% nonfat milk in PBS. Detection system was based on horseradish peroxidase/ tetramethyl benzidine reaction. Detailed methods for CBA and IHC are reported in section 6.*ANTIBODIES* (pp 68-70).

RESULTS

On rat NF155-based ELISA, anti-NF155 antibodies were found in 2/26 (7.6%) of CIDP, 1/15 (6.6%) of MS, 1/15 (6.6%) of other PN patients and in 2/20 (10%) of HC, but in none of the 16 CCPD patients **(Figure 1B)**. On human NF155-based ELISA, anti- NF155 antibodies were found in 1/26 (3.8%) of CIDP, but not in CCPD or healthy and disease controls **(Figure 1C)**.

Such isolated antibody reactivity, along with the positive control serum, was confirmed by CBA (**Figure 1D**) and showed paranodal staining by IHC on teased nerve fibers (**Figure 1E**).

Table 1.

Patient #	Origin	Gender, onset age	Diagnosis	Previous infection	EFNS criteria for demyelination	CNS and PNS	Disease course	Treatments	Response
1	Italy	F, 71	MRN	yes	Possible	contemporary	relapsing	steroids, IVIG	partial
2	Italy	M, 82	MRN	no	Definite	contemporary	monophasic	steroids, IVIG	no
3	Italy	F, 91	MRN	no	Definite	contemporary	monophasic	steroids	no
4	Italy	M, 74	EMRN	yes	Definite	contemporary	relapsing	steroids, IVIG	no
5	Italy	M, 75	EMRN	yes	Definite	contemporary	relapsing	steroids, IVIG	partial
6	Italy	M, 75	EMRN	yes	Definite	CNS, then PNS	monophasic	steroids	partial
7	Italy	M, 15	MS + CIDP	yes	Definite	contemporary	relapsing	steroids, IVIG, Natalizumab, Rituximab	good
8	Italy	M, 68	EMRN	yes	Possible	contemporary	monophasic	steroids	partial
9	Italy	M, 38	EMRN	no	Definite	CNS, then PNS	progressive	steroids, IVIG	partial
10	Italy	M, 40	MRN	no	Possible	CNS, then PNS	progressive	steroids, IVIG	no
11	Italy	M, 51	MRN	no	Definite	contemporary	progressive	steroids, IVIG	no
12	Italy	M, 42	EMRN	yes	Possible	contemporary	progressive	steroids, IVIG	partial
13	Italy	M, 23	MRN	no	Definite	contemporary	progressive	steroids, Azathioprine	no
14	France	M, 61	EMRN	yes	Definite	contemporary	monophasic	steroids, IVIG	partial
15	France	M, 6	MS + CIDP	no	Definite	CNS, then PNS	relapsing	steroids, IVIG, Methotrexate, Fingolimod	partial
16	France	F, 25	MS + CIDP	no	Definite	CNS, then PNS	relapsing then progressive	IVIG	no

Clinical features of the patients with combined central and peripheral demyelination (CCPD). MRN = myeloradiculoneuropathy, EMRN = encephalomyeloradiculoneuropathy, MS = multiple sclerosis, CIDP = chronic inflammatory demyelinating polyradiculoneuropathy.

Figure 1.

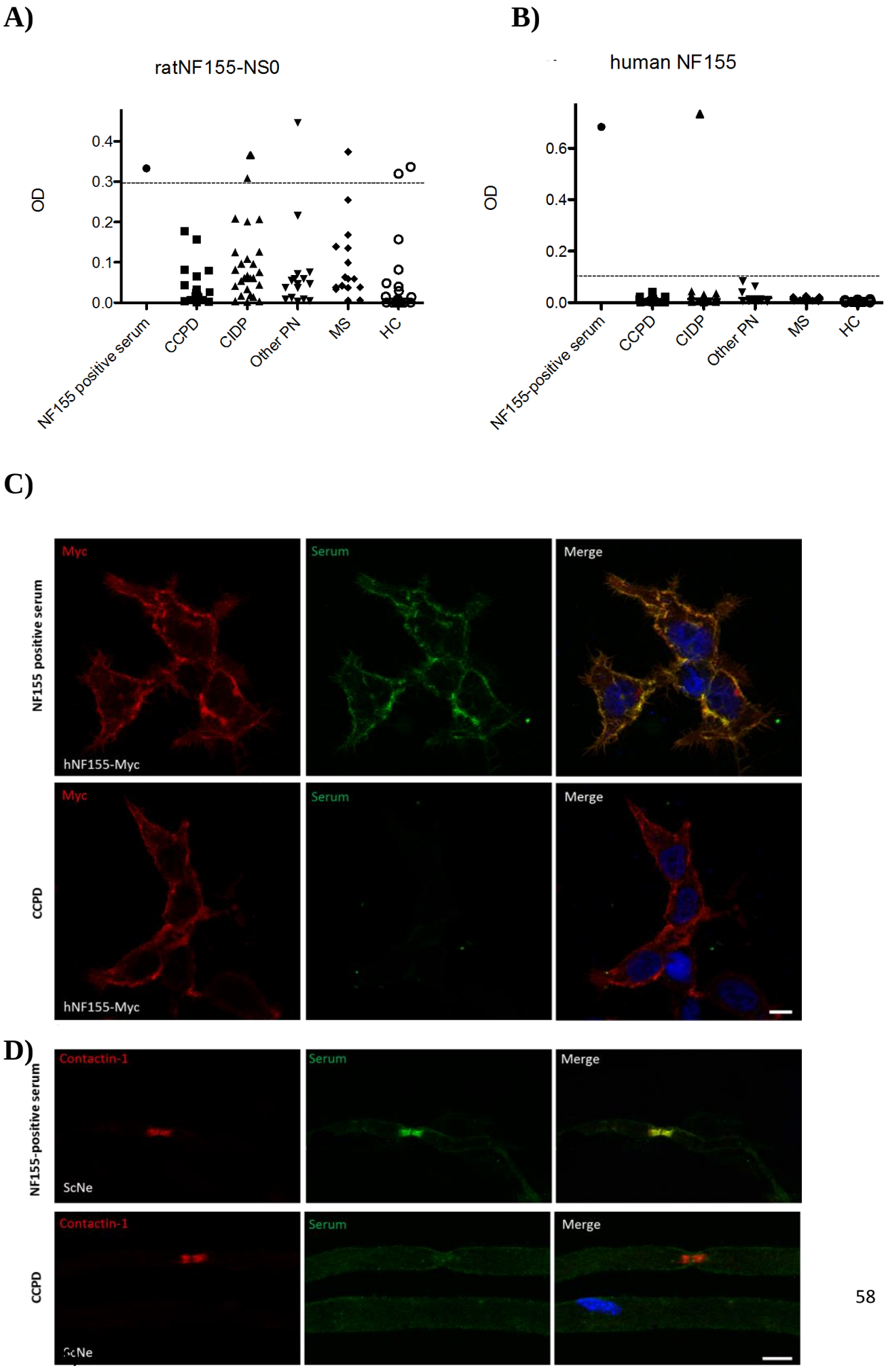

A)
ratNF155-NS0
OD
0.4
0.3
0.2
0.1
0.0
NF155 positive serum
CCPD
CIDP
Other PN
MS
HC
B)
human NF155
OD
0.6
0.4
0.2
0.0
NF155-positive serum
CCPD
CIDP
Other PN
MS
HC
C)
NF155 positive serum
Myc
Serum
Merge
hNF155-Myc
CCPD
Myc
Serum
Merge
hNF155-Myc
D)
NF155-positive serum
Contactin-1
Serum
Merge
ScNe
CCPD
Contactin-1
Serum
Merge
ScNe

Figure 1 (continued). A-D) *Serum samples from patients with CCPD (n=16), CIDP (n=26), other peripheral neuropathy (PN) (n=15), MS (n=15) and healthy control (HC) (n=20) were tested for autoantibodies to rat Neurofascin-155 (NF155)* ***(A)*** *and human NF155* ***(B)*** *by ELISA. Optical densities (OD) to NF155 are shown after subtraction of baseline OD reading to bovine serum albumin. The cut-off (dashed line) represents the mean of HC and other PN groups plus 3 standard deviations.* ***(C)*** *Serum from NF155-positive CIDP patients, but not CCPD patients, reacted against myc-tagged human NF155 in HEK293 cells.* ***(D)*** *NF155-positive CIDP serum, but not CCPD patients, colocalized with Contactin-1 at paranodes in mouse sciatic nerve fibers. Scale Bars: 10 µm.*

DISCUSSION

None of our patients with CCPD had serum antibody reactivity to NF155, differently from previous studies reporting their high prevalence (45.5-86%) (Kawamura et al. 2013; Ogata, Matsuse, et al. 2015). Differences in ethnicity (Caucasian *vs* Japanese), and in clinical features (our patients had later onset, more heterogeneous clinical presentations, and unsatisfactory response to IVIg) can at least partially account for the discrepancies. Therefore, we cannot exclude that anti-NF155 antibodies may be present in particular subgroups of patients with CCPD, as those with clear response to immunotherapies or of Japanese ethnicity.

We found anti-NF155 antibodies in 1/26 (3.8%) patients with CIDP. The patient had the "classical" clinical features of NF155-positive CIDP, including distally predominant weakness, ataxia, tremor and IVIg resistance, but no central demyelination. In a recent study, one of us also found that anti-NF155 antibodies were specifically associated with CIDP in a Japanese cohort (Devaux et al. 2016). A minority of those patients showed CNS demyelinating lesions (3/35) which suggested that the presence of antibodies to NF155 may increase the susceptible to develop CNS disorders. We thus believe that anti-NF155 antibodies in previously reported CCPD cases were mostly related to CIDP rather than a marker of combined central and peripheral inflammatory disease.

Regarding anti-NF155 antibody testing, ELISA using rat NF155 showed low analytical specificity, possibly due to unspecific reactivity to NS0 myeloma cell line derived- rat NF155, which is

abnormally glycosylated and potentially immunogenic (Ng et al. 2012). By contrast, human NF155-based ELISA and CBA allowed a clear distinction between anti-NF155 seropositive and seronegative cases. For this reason, both human NF155-based ELISAs and CBAs should be preferred for anti-NF155 antibody testing.

5. GENETICS

GENETICS AND ENVIRONMENT IN THE IMMUNE PATHOGENESIS OF ADEM AND OTHER ACUTE DEMYELINATING SYNDROMES IN ADULTS

INTRODUCTION

The cause of ADEM is still unclear. As with other autoimmune diseases, both environmental triggers and genetic susceptibility may contribute to breakdown of B and T cell self-tolerance and subsequent development of the disease. The fact that, in most cases, ADEM is preceded by an infection or vaccination suggests that environmental factors may play a primary role in its pathogenesis. Infectious microbes themselves may contain antigens that cross-react with, or mimic, self-antigens present in the CNS, and the immune response to microbes could result in reactions against the self-antigens, a phenomenon called molecular mimicry. Molecular sequencing has revealed various short stretches of homologies between numerous viral antigens and myelin proteins (Chastain and Miller 2012). Triggering of the autoimmune mechanism by neurotropic virus, leading to CNS tissue damage and systemic leakage of immunogenic CNS-confined autoantigens, has also been hypothesized (Menge et al. 2007). Moreover, infections of different tissues inducing local innate immune responses may, by activation of costimulatory pathways and cytokine release, also result in activation of T cells that are not specific for the infectious pathogen (a phenomenon called bystander activation) and, eventually, lead to breakdown of T cell tolerance. Various murine models of autoimmune encephalomyelitis are based on immunological mechanisms of molecular mimicry and bystander activation; however, conclusive evidence of their role in the development of ADEM in humans is still lacking (Menge et al. 2007).

Whatever the initial triggering event, autoreactive T and B cells are subsequently responsible for the immune-mediated damage to the CNS. A variety of chemokines and cytokines, including proinflammatory cytokines (TNFα, IL6), Th1-associated cytokines (IFNγ) and Th2-associated (IL4,

IL5), but not Th17 (IL17), are significantly elevated in the blood or CSF of ADEM patients (Franciotta et al. 2006; Ishizu et al. 2006). Upregulation, in the CSF, of chemokines active on neutrophils (G-CSF) and Th2 cells appears to be a distinctive feature of ADEM versus MS, and suggests that there is primary Th2- and Th1-mediated inflammation in ADEM, as opposed to the predominant Th1- and Th17-mediated damage that occurs in MS (Franciotta et al. 2006).

In recent years, several antibodies have been identified in patients with ADEM and acute CNS demyelination. Myelin oligodendrocyte glycoprotein (MOG) antibodies have recently been described in children with ADEM, and their presence in the acute phase of the disease, as tested by cell-based assay to full-length MOG, seems associated with a favourable clinical outcome, along with a decrease in antibody titres over time (Baumann et al. 2015; Di Pauli et al. 2011).

In addition, a range of CNS-directed autoantibodies, including AQP4, NMDAR, VGKC and GlyR-Ab, have been found in association with acute demyelination in children. Although these antibodies are clinically relevant when associated with specific neurological syndromes, their roles and clinical relevance in demyelinating diseases remains to be established (Hacohen et al. 2014).

Other protein targets, including myelin basic protein, proteolipid protein, myelin-associated oligodendrocyte basic glycoprotein and alpha-B-crystallin, have been identified in sera from ADEM patients by taking advantage of high-throughput protein array technology; however for the majority of them their diagnostic role still need to be confirmed by traditional antibody assays (Van Haren et al. 2013).

Antibodies directed to neurofascin-155 (NF155)-were identified in five out of seven Japanese patients with CCPD and confirmed by ELISA and cell-based assay (Kawamura et al. 2013). All the patients were diagnosed with chronic inflammatory demyelinating polyneuropathy (CIDP) according to the EFNS criteria and their MRI lesions fulfilled the Barkhoff criteria for a diagnosis of MS. Antibodies to NF155 have been consistently identified in subgroups of CIDP patients, some of whom had cerebellar tremor possibly secondary to CNS involvement (Querol et al. 2014). As previously reported (Cortese et al. 2016), we failed to identify reactivity to NF155 in patients with

ADEM and PNS involvement, which suggests that anti-NF155 may not be a relevant target in CCPD, but seems, rather, to be associated with chronic PNS demyelination with or without CNS involvement.

The role of lipids as targets of autoimmune damage has been extensively studied in inflammatory neuropathies, however their role in CNS demyelination is still largely unexplored. Transient anti-neutral glycolipid antibodies, including lactosylceramide and galactosylceramide, were found in four Japanese patients with acute encephalomyeloradiculoneuropathy (Shima et al. 2014). However, definite evidence of pathogenicity in ADEM for all the cited antibodies is still lacking.

As with most autoimmune diseases, genetic susceptibility to ADEM is probably inherited as a complex polygenic trait, in which affected individuals inherit multiple genetic polymorphisms that contribute to susceptibility to the disease. As an exception to this consideration, heterozygous mutations in the gene encoding RAN-binding protein 2 (RANBP2), a nuclear pore protein, have been reported in children with acute necrotizing leukoencephalitis (OMIM 601181) (Neilson et al. 2009), and represent the only example of single-gene mutation associated with ADEM.

In a large paediatric cohort with acquired demyelinating syndromes (ADSs), children harbouring one or more HLA-DRB1*15 alleles, known to be associated with susceptibility to adult-onset MS, were more likely to be confirmed to have MS compared to children with ADS who lacked HLADRB1*15 alleles, thus confirming the role of HLADRB1*15 as risk factor for paediatric-onset MS (Disanto et al. 2011) . To date, no specific HLA haplotype for susceptibility to ADEM has been confirmed.

Similarly, 57 non-HLA-related genetic risk loci recently identified in a large-scale genome-wide association study in adult patients with MS (International Multiple Sclerosis Genetics Consortium (IMSGC) et al. 2013), were also found to confer increased susceptibility to paediatric-onset MS in children with acute demyelinating syndrome, but not to monophasic ADSs, such as ADEM (van Pelt et al. 2013). The study also demonstrated that these single nucleotide polymorphisms do not appear to confer a general risk of CNS inflammation since they did not differ between monophasic

ADS children and controls. However no data on genetic susceptibility to adult-onset ADEM, as well as to other acute demyelinating syndromes of unknown aetiology, are available and it is not known whether MS-associated the HLA and non-HLA related genetic risk also confer increased susceptibility to ADEM and its variants.

The present study aims to investigate the impact of non-HLA and HLA MS-risk variants on the development and disease course of ADEM and other acute demyelinating syndromes (ADS) in adults.

METHODS

Patients

Adults older than 18 who presented with acute demyelinating syndrome between 1999 and 2014 and admitted to "C.Mondino" National Neurological Institute were enrolled. Initial phenotypes were characterized by clinical history and physical examination as clinically monofocal transverse myelitis, clinically monofocal optic neuritis (ON), acute disseminated encephalomyelitis (ADEM) or other clinically polyfocal disease.

All patients were followed up for at least 2 years and underwent at least a repeat MRI after that time in order to exclude MRI evidence of dissemination in time (Polman et al. 2011). Patients with a second clinical attack or progressive disease were included in the analysis, provided they did not fulfilled 2010 McDonald criteria of dissemination in space and time. A total of 1000 patients with adult-onset MS were already genotyped as part of a previous study and used as comparison (Sorosina et al. 2015). Institutional Review board approved the study and all patients gave written informed consent.

SNP selection and genotyping

DNA isolation and purification from whole blood samples was performed using Qiagen flexigene kit. Genotyping is currently being performed at the San Raffaele Hospital using the Illumina® OmniExpress arrays (San Diego, CA).

Weighted genetic risk scores will be calculated as described in (Sorosina et al. 2015)based on 106 non-HLA MS-associated risk variants and one HLA-DRB1*1501 marker.

We will determine the ability of the genetic risk scores to discriminate between adult with ADEM and adult with MS and its impact to distinguish patients with monophasic vs multiphasic disease course, as well as to distinguish patients with good *vs* bad outcome.

PRELIMINARY RESULTS

Hundred patient fulfilled the inclusion criteria and were enrolled. Clinical features are reported in **table 1.**

Table 1. Demographic and clinical features of patients with ADEM and other ADS enrolled

	N=100
Male	60 (60%)
Age of onset	49 ± 15 (18-74)
Type of onset	
Acute transverse myelitis	55 (55%)
ADEM	31 (31%)
Optic neuritis	9 (9%)
Other polyfocal disease	4 (4%)
PNS involvement	20 (20%)
Disease course	
Monophasic	69 (69%)
Multiphasic/progressive	31 (31%)
Poor outcome (mRANKIN ≥3)	42 (42%)
Follow-up (total, y)	3 ± 2 (2-17)

ADEM acute demyelinating encephalomyelitis ADS acute demyelinating syndrome

The majority were male with a mean age at disease onset of 49 years. Acute transverse myelitis was the most common presentation, followed by ADEM, optic neuritis and other polyfocal disease including one patient with rhomboencehalitis, two with limbic encephalitis and one cerebellitis. Twenty % of the patient had concurrent evidence of peripheral neuropathy. The disease was monophasic in 2/3 of them and 58% had a good functional outcome without residual disability. Array-based genotyping of enrolled cases is currently ongoing in collaboration with Dr Filippo Martinelli-Boneschi (Ospedale San Raffaele, Milan).

6. ANTIBODIES

PREVALENCE AND CLINICAL IMPACT OF ANTIBODIES TO NEUROFASCIN-155, CONTACTIN-1 AND CONTACTIN ASSOCIATED PROTEIN-1 IN PATIENTS WITH CHRONIC INFLAMMATORY DEMYELINATING POLYRADICULONEUROPATHY

INTRODUCTION

Chronic inflammatory demyelinating polyneuropathy (CIDP) is the most common acquired inflammatory neuropathy worldwide and is clinically heterogeneous. Proven treatments for CIDP include corticosteroids, plasma exchange, and IV immunoglobulin (IVIg). However, the response rates to treatments are highly heterogeneous between patients and there is great need of biomarkers to identify CIDP subgroups and guide specific immunotherapeutic options.

Several evidences suggest a role of humoral immunity in CIDP pathogenesis: the efficacy of plasma exchange (PE) as a treatment of CIDP (Mehndiratta, Hughes, and Pritchard 2015); the presence of Ig and complement deposition on the outer surface of Schwann cells in patients' sural nerve biopsies (Dalakas and Engel 1980); the ability of serum collected from plasma exchange-responsive to induce demyelination if injected intraneurally in rats (Yan et al. 2000), as well as the fact that sensitization of rats to purified myelin proteins P0, P2 and PMP-22 induces an experimental autoimmune neuritis (Soliven 2012).

However, numerous studies failed to demonstrate a marker of the immune attack to the peripheral nervous system.

More recently, antibodies to single components of the neurofascin 155 (NF155)- Contactin -1 (CNTN1)- Contactin-associated protein (Caspr1) paranodal complex have been identified in in 3-18% of Japanese or Caucasian patients with chronic inflammatory demyelinating polyradiculoneuropathy (CIDP), and IgG4 has been demonstrated to be the predominant isotype of

these antibodies in most cases (Lonigro and Devaux 2009; Devaux, Odaka, and Yuki 2012; Querol et al. 2013; Querol et al. 2014; Doppler, Werner, and Sommer 2013; Ogata, Yamasaki, et al. 2015; Miura et al. 2015; Doppler et al. 2016). Seropositive patients reportedly show specific clinical features, including earlier age of onset, sensory ataxia and tremor in NF155-positive cases, older age of onset and either predominant sensory or motor impairment in CNTN1-positive subjects and neuropathic pain in Caspr1-positive patients. Moreover the presence of these antibodies predicts a poor response to IVIg, as well as to other first-lines treatments.

However, their prevalence in Italian CIDP patients is unknown and current knowledge on the clinical impact of these novel antibodies is limited by the low numbers of seropositive patients so far reported. Moreover, there is no consensus on the gold standard technique for testing of these antibodies, among ELISA, cell-based assay (CBA), and immunohistochemistry (IHC) on mouse or rat teased fibers. Finally, evidence of the pathogenicity of these antibodies, although increasing, is still limited.

In this multicentre study we aimed 1) to assess the prevalence and isotype of antibodies anti-NF155, anti-CNTN1 and anti-Caspr1 in patients affected by CIDP and to report on their associated clinical features 2) to compare diagnostic accuracy of the currently available techniques for antibody testing; 3) To gather information on antibodies pathogenicity by assessing morphological changes of node of Ranvier in skin biopsies from seropositive patients.

METHODS

Patients and sera

Sera from 200 patients fulfilling the diagnostic criteria for CIDP (Joint Task Force of the EFNS and the PNS 2010) were collected in 10 centres with specific expertise in neuromuscular disorders from six different Italian regions (Lombardia, Veneto, Liguria, Toscana, Lazio, Sicilia) and were tested by two independent laboratories ("C Mondino" National Neurological Institute and CRN2M, Aix-Marseille Université, Dr Jerome Devaux). Samples from 50 GBS, 50 MS, 30 inherited neuropathies,

10 multifocal motor neuropathy and 50 healthy volunteers were used as controls for ELISA testing. Clinical information were collected from the CIDP patients that presented IgG reaction against NF155, CNTN1, or Caspr1, using a standard assessment questionnaire sent to the treating physicians. This questionnaire included data about demographics (current age and gender), disease onset (age at disease onset, comorbidities, triggering events, mode of onset and main symptoms at onset), serum collection (date of collection and temporal relationship with previous therapies), clinical features (presence of proximal and distal limb weakness, pain, impairment of pinprick and proprioception, cerebellar signs, sensory ataxia, tremor, cranial nerves involvement, respiratory failure, dysphagia, need for admission to an intensive care unit (ICU), CNS demyelination and other associated clinical features), disease course. Disease severity was scored using Overall Neuropathy Limitation Scale and modified RANKIN (mRANKIN) Score (Nobile-Orazio et al. 2011). Information was also collected about response to treatments (IVIg, steroids, PE, other treatment), paraclinical features (nerve conduction study, neuroimaging, CSF protein and cell count, presence of other antibodies or M-component, nerve biopsy, barin and spine MRI).

Data collected in seropositive patients were compared with available data from 30 seronegative CIDP patients followed-up at "C Mondino" National Neurological Institute, using appropriate statistical methods. All analyses were performed using STATA.

Constructs

Human Caspr1 (NM_003632.2) was amplified by PCR from a human brain cDNA library and sub-cloned into pcDNA3.1 (ThermoFisher scientific) at XbaI and EcoRI sites. Procedures for Myc-tagged human NF155 (NM_001160331.1) and Human CNTN1 (NM_001843.3) have been described in previous studies (Miura & Devaux, 2015) (Devaux J, 2016).

Cell binding assay

Human embryonic kidney (HEK) cells were plated at a density of 50.000 cells per wells. We used 24-well plates containing a 12 mm glass cover slip, which was coated with poly-L-lysine (0.1 mg/ml). Cells were kept for 24 hours at 37°C in culture milieu composed of Dulbecco's modified Eagles medium, 1 mM sodium pyruvate, penicillin/streptomycin, and 10 % fetal calf serum. Then HEK cells were transfected with Contactin-1, NF155, Caspr-1 or Caspr1/Contactin-1 using JetPEI (Polyplus-transfection. The day after, cells were incubated with serum free Opti-MEM medium (Life technologies) for 24 hours. On the third day we incubated cells with patients' sera (20 minutes at 37°C, diluted 1/50 in Opti-MEM). Then we washed cover slips thrice in PBS, fixed them with PAF2% and blocked with blocking solution (5% fish skin gelatine containing, 0.1% Triton X-100 in PBS). This step is aimed to saturate the free epitopes and to prevent non-specific binding of the primary and secondary antibodies. In some experiments, transfected cells were fixed and permeabilized prior to incubation with patients' sera (see results and discussion). HEK cells were then incubated with primary antibodies (Rabbit anti Caspr1, Goat anti Contactin-1; 1/2000) for 1 hour, then with the appropriate Alexa conjugated secondary antibodies (Donkey anti Human, anti-Rabbit, anti-Goat IgG; 1/500) or FITC conjugated mouse anti-human IgG1, 2, 3, or 4 (1/500) for 30 minutes. Coverslips were washed three times in PBS, stained with DAPI, and mounted with Mowiol.

Immunohistochemistry on mouse teased nerve fibres

Procedures were in line with the European Community's guiding principles on the care and use of animals (86/609/CEE). Adult C57BL/6J mice (8-12 weeks old) were euthanized and the sciatic nerves were quickly dissected out and fixed by immersion in 2% paraformaldehyde in PBS for 1 hour at 4°C, then rinsed in PBS. Sciatic nerve axons were gently teased, dried on glass slides and stored at -20°C. Teased fibers were permeabilized by immersion in −20°C acetone for 10 min and washed in PBS for 10 min. Slides were incubated for 1 hour in blocking solution and then incubated with sera diluted at 1/200 and antisera against nerve proteins (1/2000) overnight at 4°C. We used antisera against MBP, Nav channels and CNTN1 to localize myelin, nodes and paranodes respectively. Slides

were then washed thrice in PBS and incubated for 1 hour at room temperature with the appropriate Alexa conjugated secondary antibodies (1/500). Slides were washed three times in PBS, stained with DAPI and mounted with Mowiol. Staining were examined using an ApoTome fluorescence microscope (ApoTome, AxioObserver and AxioCam MRm, Carl Zeiss MicroImaging GmbH). Digital images were manipulated into figures with CorelDraw Home & Student X7.

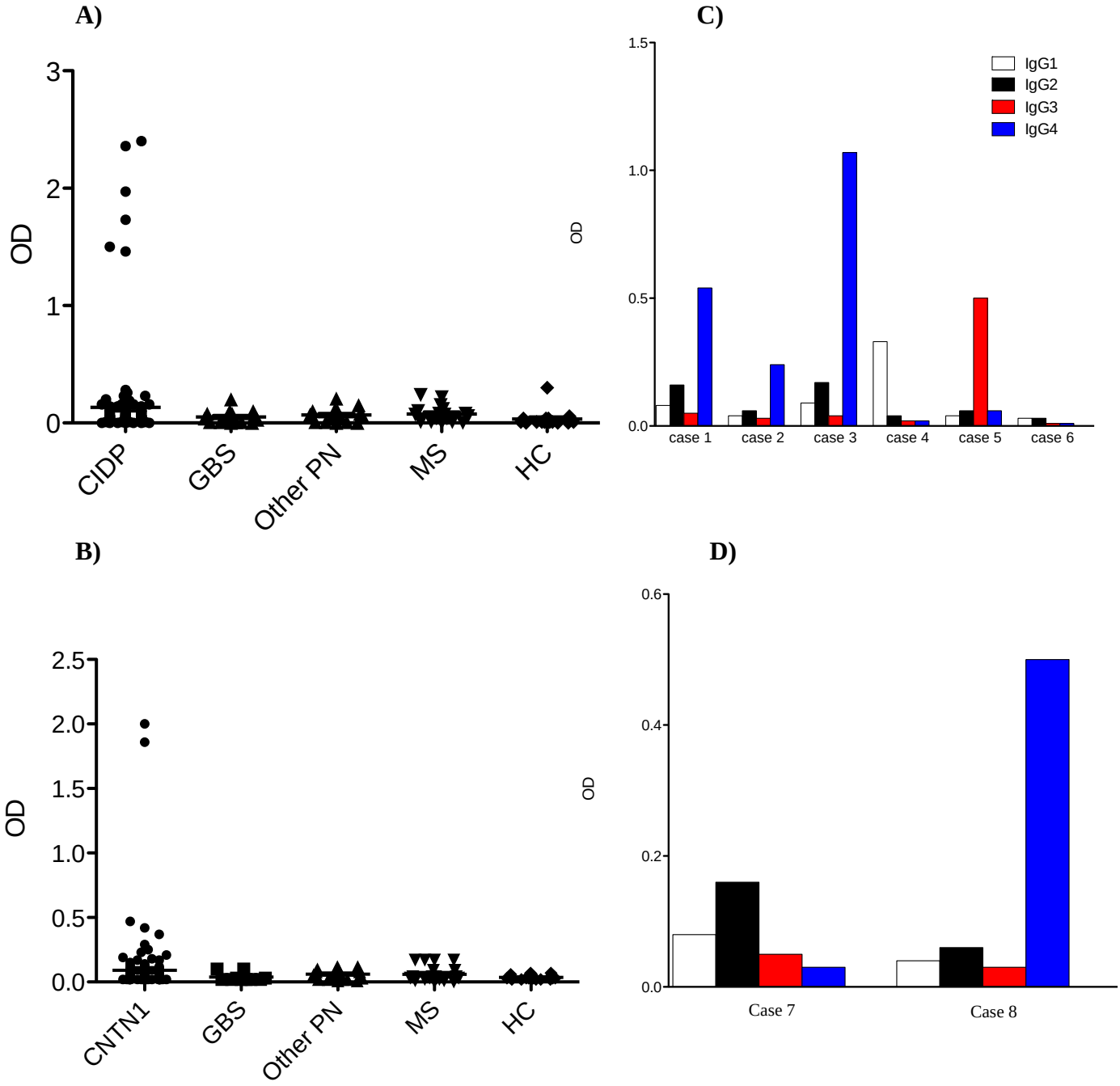
ELISA

Polystyrene microwells were coated with 1µg/mL human recombinant NF155 (OriGene) or 0.5µg/mL Contactin-1 (Sino Biological Inc) at 4°C overnight. Then the plates were blocked with 5% nonfat milk in PBS at room temperature (RT) for 1 hour and incubated with sera diluted 1:100 in 2% nonfat milk in PBS, at RT for 1 hour. Horseradish peroxidase-conjugated goat antibody to human IgG (1:6000; DAKO) was added, and plates were developed with tetramethylbenzidine (Sigma-Aldritch). Plates were washed between each incubation with PBS, and subsequently read at 450 nm (ELx800, Biotek). Positive cases were defined as those showing optical density (OD) values above the mean OD values of HC plus 3 standard deviations.

RESULTS

On ELISA, sera of six patients with CIDP showed reactivity against NF155 and 2 patients against CNTN1 **(Figure 1 A,B).** None of the sera from healthy and disease controls had antibodies to either NF155 or CNTN1. IgG isotype was IgG4 in 3 NF155 seropositive patient and 1 patient with anti CNTN1 antibodies. Other isotype were IgG1 in one NF155 seropositive case, IgG3 in another NF155 seropositive case and non-determinable in one anti-NF155 and one anti-CNTN1 positive cases **(Figure 1 C,D).** Of note, in the latter, sera was collected during the recovery phase after succesful immunosuppressant treatment.

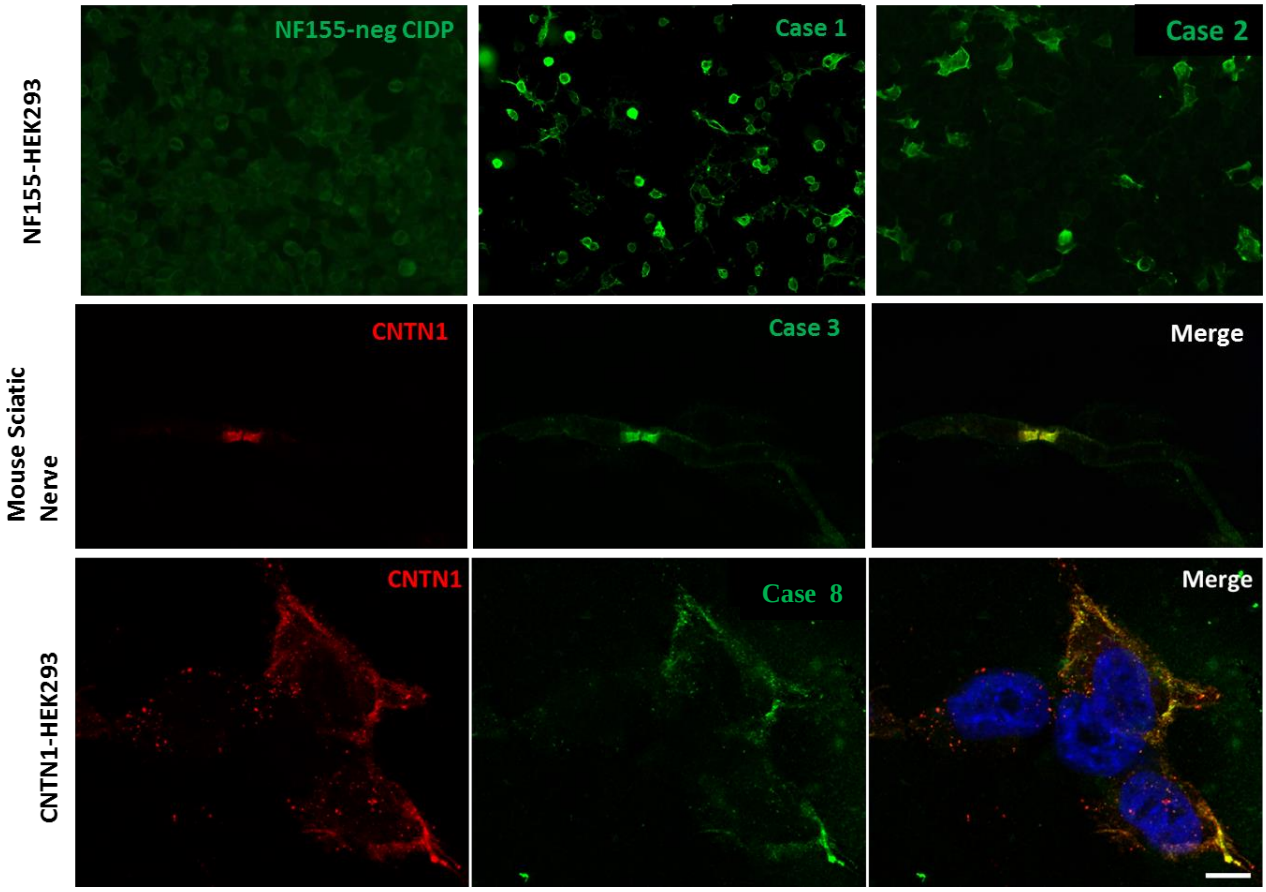
Figure 1. Reactivity to NF155 and CNTN1 by ELISA.



*Six CIDP patients reacted to NF155 (**A**) and two to CNTN1 (**B**). None of sera tested from patients with GBS, other peripheral neuropathy, multiple sclerosis and from healthy controls showed reactivity to NF155 or CNTN1. IgG4 was the predominant isotype in 3/6 NF155-positive patients (**C**) and 1/2 CNTN1-positive cases **(D).***

All sera from CIDP patients were also tested on live-cell CBA with NF155 or CNTN1 transiently transfected HEK cells. We could confirm reactivity to NF155 and CNTN1 in all patients positive on ELISA testing and no additional positive sera for NF155 or CNTN1 were identified (**figure 2).**

Figure 2: reactivity to NF155 and CNTN1 on CBA and IHC on murine sciatic nerve teased fibers

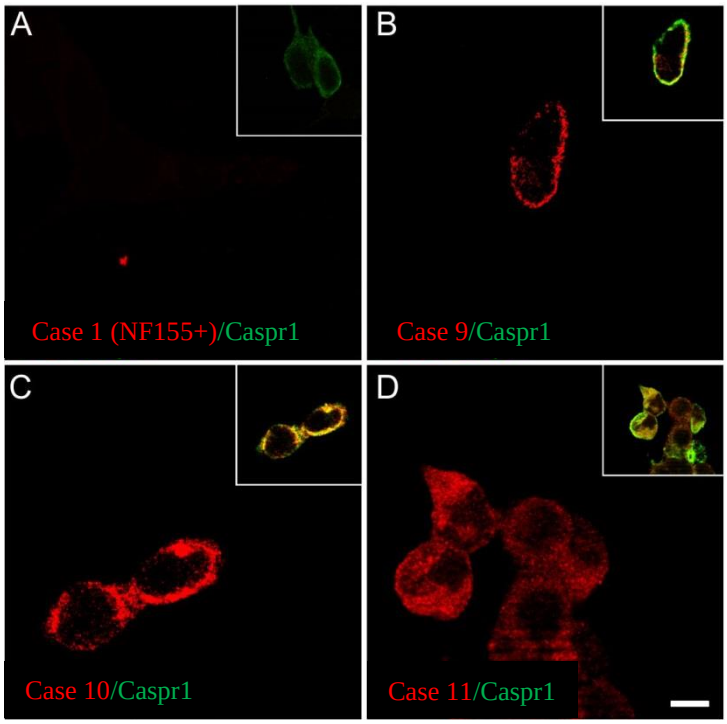


Upper and lower rows showed reactivity of sera from patients 1,2 and 8 to NF155 or CNTN1 transfected HEK cells, respectively. Binding of seropositive sera to paranodal regions (marked by the presence of CNTN1 staining) was confirmed on murine sciatic nerve teased fibers (as shown for NF155-positive case 3 in the middle row) Scale Bar: 10 µm

Moreover, in collaboration with Dr Jerome Devaux (CRN2M, Aix-Marseille Université), we identified three patients who were reactive to intracellular Caspr1, on fixed and permeabilized cell CBA (**Figure 3).** It is necessary to underline here that, differently from NF155 and CNTN1, Caspr1

transfected alone is retained in the endoplasmic reticulum (ER) and is poorly expressed at the cell surface, making difficult to evaluate the binding of antibodies to surface antigens on standard live-cell CBA. Taking advantage of co-transfected HEK cells with both Caspr1 and CNTN1, which enable Caspr1 incorporation in the lipid rafts and delivery to the cell surface, we further confirmed that anti Caspr1 antibodies recognize native extracellular domains of Caspr1 on live-cell CBA (**Figure 3**) .

Figure 3: CIDP IgG recognize Caspr1 in HEK cells.



HEK cells were transfected with Caspr1 alone, then were fixed and permeabilized, then immune-stained with sera (red), and antibodies to Caspr1 (green). Red channel are shown as main panels. Merge panels are shown in the inserts. Case 9 ***(B),*** *Case 10 (**C**) and Case 11 (**D**) recognized Caspr1. As control, we tested the serum from the NF155 reactive patient (Case 1). This latter did not react against Caspr1. Scale Bar: 10 µm* ***(A).***

Finally we confirmed that these antibodies were specific to Caspr1 as none of the Caspr1-positive sera reacted against CNTN1 alone (data not shown). CBA was preferred to ELISA for evaluation of reactivity to Caspr1 because the human recombinant Caspr1 is not commercially available yet.

All patients with IgG4 Ab to NF155 and seropositive patients to CNTN1 or Caspr1 also showed

reactivity against paranode when tested on teased fibers from murine sciatic nerve, but not the NF155 patients with other predominant IgG isotype or no detectable isotype.

Overall 11/200 (5.5%) patients had antibodies to one of the components of the paranodal NF155-Caspr1-CNTN1 complex. Eight of them (4%) showed consistent results on all available techniques, including binding of sera to the paranodal regions on teased fibers preparations (**table 1**).

Table 1. Comparison of currently available techniques for antibodies to NF155, CNTN1 and Caspr1

	ELISA (N=200)	**IgG4 isotype**	**CBA (living cells) (N=200)**	**IHC Paranodal staining on sciatic nerve fibers**
NF155 Abs	6 (3%)	3/6	6 (3%)	3/6 (3/3 IgG4)
CNTN1 Abs	2 (1%)	1/2	2 (1%)	2/2
Caspr1 Abs	NA	TBD	3 (1.5%)	3/3
Overall	8 (4%)	4/8	11 (5.5%)	8/11 (8/200, 4%)

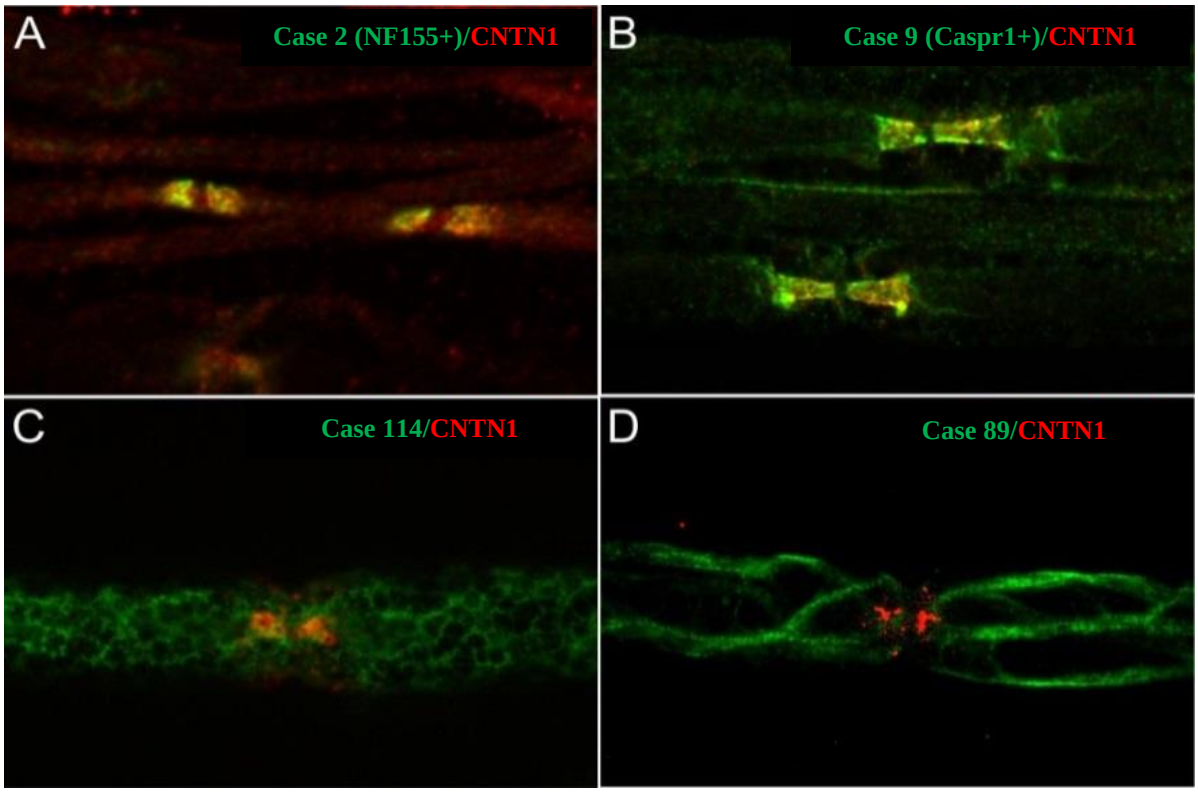
Testing of antibodies against NF155, CNTN1 and Caspr1 by ELISA, CBA and IHC on murine sciatic nerve. NA not available, TBD to be determined

Reactivity against nerve fibers in seronegative patients for NF155, CNTN1 or Caspr1 antibodies

We found that 21 patients (10,5%) showed IgG reactivity to the node, 35 patients (17,5%) to paranodes **(Figure 4A-B)** and, overall, 44 patients (22%) had reactivity against node and/or paranodes when sera were tested on murine teased fibers. This is in keeping with the results from a previous study (Devaux, Odaka, and Yuki 2012). In addition, we found other interesting staining patterns in our cohort: for instance, 11 patients (5.5%) presumably stained the Cajal bands (**Figure 3D**), as the deposition of IgG localized in a spiral shape along the fiber surface. This finding is interesting and novel, as this is the first time that human antibodies are shown to target this structure. Cajal bands are cytoplasmic channels flanked by regions of tight interaction between the

outer surface of compact peripheral myelin and the Schwann cell plasma membrane, involved in myelin formation and maintenance and mRNA delivery to the distal sites of the myelin. However, no transmembrane proteins have been described in this region so far, and the only known proteins enriched in this region, periaxin and dystrophin-related protein 2 (DRP2), are localized in the intracellular surface. In addition, 23 sera (11.5%) reacted against myelin (**Figure 3C**). Altogether, up to 40% of patients reacted against nerve fibers on IHC.

Figure 4: Patterns of immunoreactivity of CIDP sera



*These are teased mouse sciatic nerve fibers immuno-stained for IgG antibodies (green) from four patients with CIDP and with antibodies to CNTN1 (red) in order to localize the paranodes. Some CIDP IgG colocalize with CNTN1 at paranodes (**A-B**), whereas IgG from other CIDP patients show a different pattern of staining (**C-D**). In C, IgG stain in a diffuse manner the myelin sheath. In **D**, IgG bind the nerve fiber surface in a spiral way, presumably the Cajal bands.*

Clinical features of NF155-seropositive patients

Patients with IgG4 NF155 were predominantly male, with an average age of onset of 42 years. Onset of the neuropathy was subacute in two, and one of them was initially diagnosed with GBS. All patients had symmetric four limb muscle weakness, which was mild to moderate in proximal muscles and moderate to severe distal muscle groups. Pinprick and position sense of hallux was abolished in all of them. Sensory ataxia and tremor were also invariably present, although this latter was mild in two. One patient reported neuropathic pain at onset.

The neuropathy was moderately to severely disabling with a mean mRANKIN score at onset of 4 and a mean ONLS of 7. Lower limbs were always more affected than upper limbs and one patient was wheel-chair bound one month after disease onset. None of them had clinical signs of CNS involvement.

Although patients frequently reported a subjective improvement after IVIG or steroids, this was transitory and was not followed by any change in their disability grade, except for a 1 point change of RANKIN scale in one case treated with steroids.

Patients with NF155 Ab of IgG1 or IgG3 isotype or undetectable isotype had a less severe neuropathy, with only mild distal weakness, reduced sensation at hallux, and only mild disability. None had sensory ataxia, tremor or neuropathic pain. All 3 patients showed good response to IVIg leading to either normalization of significant improvement of neurological examination, without residual disability.

Clinical features of CNTN1-seropositive patients

Patients with CNTN1 Ab were two males with onset at 59 and 63 years, respectively, of a severe sensory and motor neuropathy. Onset was subacute in one of them. Strength was impaired in both proximal and distal muscles at upper and lower limbs, ranging from mild weakness to complete limb paresis. Pinprick sensation and proprioception were either reduced or abolished and both of them had sensory ataxia. One of them reported neuropathic pain at onset.

One patient had a contemporary onset of CIDP and membranous glomerulonephritis. At routine blood test his serum albumin was reduced and proteinuria was 10 gr/24 hours, thus leading to the diagnosis of nephrotic syndrome. Kidney biopsy showed sub epithelial deposits of immune complexes and complement deposition. Treatment with IVIg and corticosteroids did not improve neurologic function. A six-month course of cyclophosphamide was thus started. After that renal function and muscle strength normalized, while sensory ataxia showed only partial improvement. Anti-CNTN1 antibodies could not be detected in his serum one year after treatment. The patient did not require any further treatment and his condition has remained stable for the last 10 years. The second patient was treated with IVIg and steroids, which were ineffective. The patient died 6 months after disease onset of likely unrelated cardiac disease.

Clinical features of Caspr1-seropositive patients

Caspr1 positive subjects were two males, aged 24 and 67 at onset, and a young female, with onset of the disease at the age of 9 years. Mode of onset was chronic in two and subacute in one, while disease course was progressive in all of them. Disease severity was generally lower than CNTN1 and NF155 seropositive patients, with a mRANKIN Score of 3 or lower in all of them. All patients had lower limb-predominant weakness, grading from mild to severe. Strength in proximal muscle groups of upper and lower limbs was usually normal. Two patients had sensory ataxia and one had concomitant tremor. None of them reported neuropathic pain.

One patient was treated with IVIg, steroids and IFN-alfa, but did not show clear response to any of these treatment. Another patient showed no response to steroids and only a partial response to IVIg, while the third patient was only very recently diagnosed with CIDP and no therapy has been started so far.

Clinical features of seropositive vs seronegative patients

Next, we compared the clinical features of 9 seropositive patients (3 patients with antibodies against NF155 of IgG4 predominant isotype, 2 CNTN1 patients and 3 Caspr1 patients) vs 30 CIDP patients without antibodies to NF155, CNTN1 or Caspr1 and whose sera did not stain teased fibers when tested on murine sciatic nerve.

Overall, seropositive patients with antibodies against NF155, CNTN1 or Caspr1 had more frequently other autoimmune diseases in their past medical history (2 patients with Basedow disease and 1 patient with psoriasis). A subacute onset was observed more frequently in seropositive patients, although this did not reach statistical significance. Clinical examination showed more frequently sensory ataxia and tremor, together with abolished position sense at hallux in seropositive patients. Weakness was in general more severe in patients with positive antibodies and particularly in distal muscle of lower limbs. Seropositive patients tended to have a higher disability and showed worst response to IVIg compared to seronegative IVIg patients. Regarding the paraclinical investigations, seropositive patients had more frequently increased distal motor latencies and temporal dispersions on nerve conduction study and had a higher protein level in CSF. Patients with Caspr1 had the higher increase in CSF proteins up to 5 gr in 2 of them along with a mild increase of CSF cells (**Table 2**). Individual features of seropositive patients are available in **Table 3.**

Morphological changes of node of Ranvier in skin biopsy of seropositive patients

In collaboration with Dr Giuseppe Lauria (C. Besta Neurological Institute, Milan) we evaluated the skin biopsies from three patients with IgG4 NF155 antibodies, one patient with anti CNTN1 antibodies and one patients with antibodies anti NF155 antibodies of IgG1 predominant isotype. Analysis of myelinated fibers from IgG4 NF155 patients (case 1 and case 2) showed marked elongation of nodes of Ranvier and loss of paranodal NF155 staining. Moderate elongation of node of Ranvier and loss of NF155 paranodal staining were also observed in myelinated fibers of a

CNTN1 positive patient, despite the skin biopsy being performed during disease remission, 10 years after successful treatment with cyclophosphamide. Contrarily, we did not observed similar changes in in one patient with anti NF155 IgG1 antibodies and another seronegative CIDP patient (**Figure 5**).

Table 2. Clinical features of seropositive vs seronegative patients

	Ab negative (N=30)	Ab positive (N=8)	p
Age of Onset	53 ± 3 (14-80)	45 ± 9 (7-82)	0.31
Male gender	22 (73%)	6 (75%)	0.92
Other autoimmune disease	1 (3%)	3 (37%)	**0.005**
M-protein	7 (23%)	0 (0%)	0.13
Triggering infection/vaccination	4 (13%)	1 (12%)	0.95
Subacute onset	8 (26%)	4 (50%)	0.20
Disease course			
relapsing	15 (50%)	4 (50%)	
progressive	15 (50%)	4 (50%)	1
Clinical phenotype			
Typical	24 (80%)	8 (100%)	
DADS	1 (3%)		
MADSAM	3 (10%)		
Pure motor	2 (7%)		0.59
Weakness			
Upper limb proximal	0.3 ± 0.1	0.5 ± 0.2	0.63
Upper limb distal	0.8 ± 0.2	1.3 ± 0.4	0.21
Lower limb proximal	0.7 ± 0.2	1.1 ± 0.4	0.35
Lower limb distal	0.7 ± 0.2	2.3 ± 0.3	**0.006**
Distal predominant weakness	9 (30%	6 (75%)	**0.02**
Pinprick sensation hallux			
Reduced	20 (69%)	6 (75%)	
Abolished	2 (7%)	1 (12%)	0.7
Position sensation hallux			
Reduced	12 (46%)	1 (12%)	
Abolished	3 (11%)	5 (62%)	**0.01**
Sensory ataxia	14 (46%)	7 (87%)	**0.04**
Tremor	5 (16%)	4 (50%)	**0.049**
Pain	11 (36%)	3 (37%)	0.9
ONLS	3.9 ± 0.6	6 ± 1	**0.09**
EDx features			
Prolonged distal motor latency	6 (23%)	6 (75%)	**0.007**
Reduced conduction velocity	19 (73%)	8 (100%)	0.1
Prolonged F wave	11 (52%)	5 (71%)	0.17

Conduction blocks	10 (38%)	5 (71%)	0.12
Temporal dispersion	4 (15%)	7 (87%)	**0.00**
CSF albumin	69 ± 11	183 ± 49	**0.001**
CSF cells	1.7 ± 0.7	4.1 ± 1.9	0.14
Response to IVIg			
No	3 (15%)	2 (28%)	
Partial/transitory	7 (35%)	5 (72%)	
Good	10 (50%)	0 (0%)	**0.06**
Response to steroids			
No	10 (41%)	2 (28%)	
Partial/transitory	6 (25%)	4 (57%)	
Good	8 (33%)	1 (14%)	0.26
Response to first line Treatment			
No	4 (14%	1 (14%)	
Partial/transitory	5 (18%)	5 (72%)	
Good	19 (68%)	1 (14%)	**0.01**

Table 2 (continued). *Weakness was graded as follows: 0=normal, 1=mild (MRC=4), 2=moderate (MRC=3), 3=severe (MRC<3*)

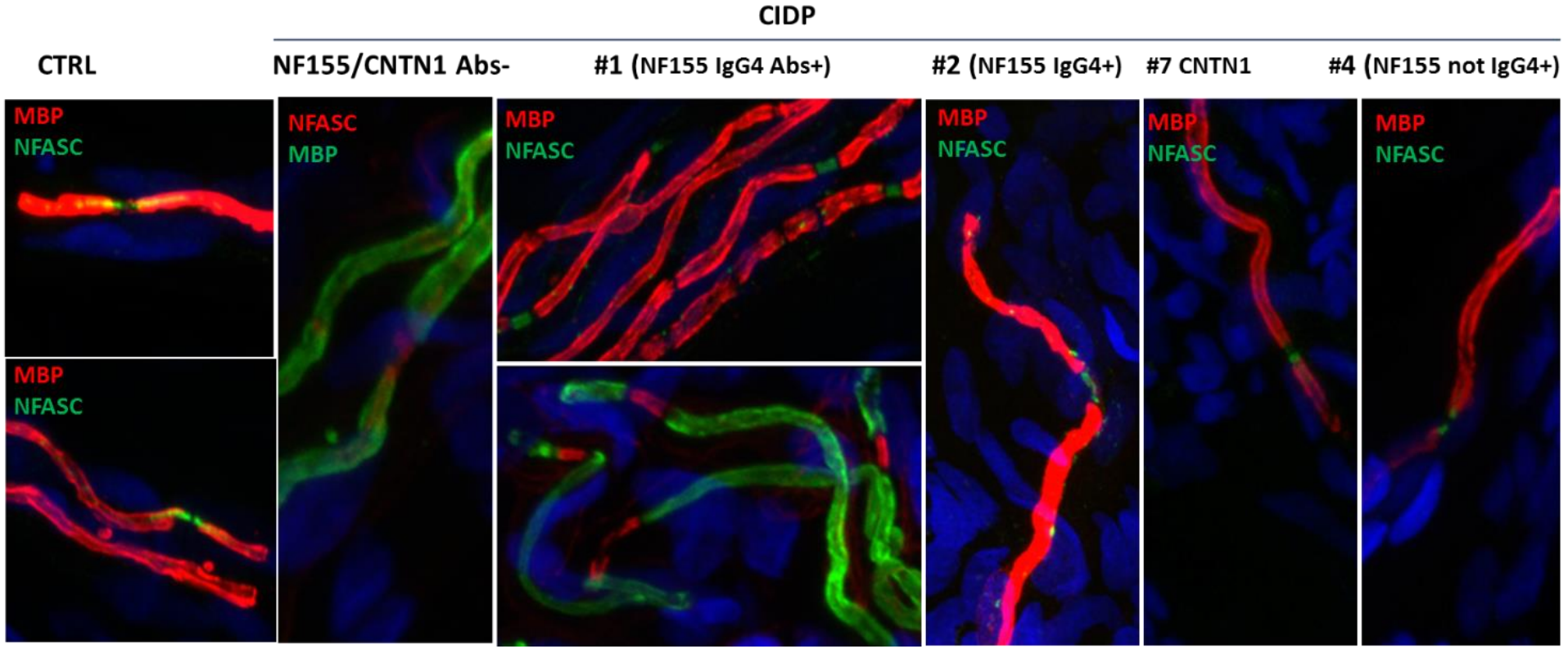
Table 3. Clinical features of patients with antibodies to NF155 (IgG4 only), CNTN1 and Caspr1

Patient	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7	Case 8	Case 9	Case 10	Case 11
Ab	NF155	NF155	NF155	NF155	NF155	NF155	CNTN1	CNTN1	Caspr1	Caspr1	Caspr1
Positive test	ELISA, CBA, IHC (paranode)	ELISA, CBA, IHC (paranode)	ELISA, CBA, IHC (paranode)	ELISA, CBA	ELISA, CBA	ELISA, CBA	ELISA, CBA, IHC (paranode)	ELISA, CBA	CBA, IHC (paranode)	CBA, IHC (paranode)	CBA, IHC (paranode)
IgG isotype	IgG4	IgG4	IgG4	IgG1	IgG3	ND	ND	IgG4	NP	NP	NP
Other Abs	none	none	IgM antiGM1 (low titre)	none	none	none	none	none	none	none	Anti-MAG (low titre)
Demographic	18, male	63, female	45, male	2, male	53, female	73, male	58, male	82, male	24, male	7, female	67, male
Comorbidities	-	NHL, Basedow disease, diabetes	Diabetes	-	Hypercolesterolemia	Hyperthensio	Membranous glomerulonephritis, Basedow disease, acquired cervical and lumbar stenosis	COPD, hypertension, ischemic cardiac disease	-	early pubarche	Hypertension, alcohol abuse, psoriasis
Mode of onset	Chronic	Acute	Subacute	Chronic	Chronic	Chronic	Subacute	Chronic	Chronic	Subacute	Chronic
Chief complain	Distal numbness, walking difficulties, unsteadiness	Walking difficulties and unsteadiness	Walking difficulties and pins-and-needles sensation at hands	Difficulties climbing stairs	Distal paraesthesia	Paraesthesia, unbalance	Paraesthesia at four limbs, paraparesis, sensory ataxia	Upper and lower limb paralysis	Paraesthesia, weakness	Proximal and distal weakness, postural and intentional tremor , mild ataxia	Gait ataxia
Weakness	++	++	++	+	+	+	+++	+++	+	++	+
Sensory ataxia	++	+	+	+/-	+	+	++	++	-	+	+
Tremor	+	++	+	-	-	-	-	-	-	+	-

mRANKIN at acme	3	4	4	2	2	2	5	5	3	2	3
ONLS upper limbs	2	3	3	0	3	2	2	4	2	1	0
ONLS lower limbs	3	3	6	5	2	2	6	6	3	2	2
ONLS total	5	6	9	5	5	4	8	10	5	3	2
EFNS/PNS criteria	Definite CIDP	Definite CIDP	Definite CIDP	Definite CIDP	Definite CIDP	Definite CIDP	Definite CIDP	Definite CIDP	Definite CIDP	Definite CIDP	Definite CIDP
Response to IVIg	Partial, not definite	Partial, not definite	Partial, transitory	yes	yes	yes	no	no	Partial, transitory	Partial, transitory	NP
Response to steroids	Yes, transitory)	Yes, transitory)	Partial, transitory	NP	yes	NP	yes	no	Partial, transitory	no	NP
Other treatment	NP	NP	NP	NP	NP	NP	Yes, to cyclophosphamide	Not performed	partial to Interferon-alfa,	NP	NP
Disease course and outcome	Relapsing-remitting	Relapsing-remitting, improved	progressive	Relapsing-remitting, improved	Relapsing-remitting, improved	Relapsing-remitting, improved	Relapsing, improved	Monophasic, died 6 months after onset	progressive	progressive	progressive

Table 3 (continued): *NHL non-Hodgkin lymphoma, COPD chronic obstructive pulmonary disease, ND not determinable, NP not performed*

Figure 5. Analysis of myelinated fibers in patients skin biopsies.



Skin biopsies NF155 IgG4 positive case 1 (#1) and 2 (#2), CNTN1 positive Case 7 (#7) and NF155 IgG1 case 4 (#4) and a seronegative CIDP patient were analysed

DISCUSSION

The diagnosis of CIDP can be very challenging, considering the heterogeneity of the clinical presentation, the presence of mimicking disorders and the absence of specific biomarkers. Moreover, the response to therapies is unpredictable. One third of the patients has no benefits from IVIg but can respond to other treatments (Kuitwaard et al. 2015; Nobile-Orazio and Gallia 2015; Cocito et al. 2010). The pathogenesis is considered to be autoimmune but early attempts failed to identify the responsible autoantibodies. A previous study showed that up to 30% of CIDP patients have reactivity against the node of Ranvier or adjacent structures when investigated by immunohistochemistry on teased fibers from murine sciatic nerve (Devaux, Odaka, and Yuki 2012), and further studies have identified NF155, CNTN1 or Caspr1 as target of this response in 3-18% of patients (Querol et al. 2013; Querol et al. 2014; Doppler et al. 2016). Although they account for a minority of patients, these findings are of clinical relevance because these antibodies are associated to distinct clinical phenotypes and predict an unsatisfactory response to IVIg.

In our study we confirmed the low prevalence (5.5%) of these antibodies in Italian CIDP patients. Frequency of NF155 and CNTN1 antibodies was similar to the prevalence of these antibodies in Caucasian patients reported by previous studies but lower compared to their prevalence in Japanese patients. The discrepancy may be attributable to differences in inclusion criteria and ethnicity. Frequency of the more recently identified Caspr1 antibodies was equal to that of antibodies to NF155, confirming that Caspr1 may also represent a relevant target of the immune-response in CIDP patients.

Table 4. Comparison of frequencies of antibodies to NF155, CNTN1 and Caspr1 between previous and the present study

Neurofascin -155	**N (%)**	**isotype**	**Method for Abs testing**	**ref**
	5/117 (4%)	IgG4, IgG3, IGM, IgA	ELISA/CBA	Ng, 2012
	4/61 (6%)	IgG4	ELISA/CBA	Querol, 2014
	6/7 (CCPD)	Total IgG	ELISA	Kawamura, 2013
	0/16 (CCPD)	-	ELISA/CBA	Cortese, 2015
	9/50 (18%)	IgG4	CBA/flow cytometry	Ogata, 2015
	38/533 (5%)	IgG4	ELISA/CBA	Devaux, 2016
	6/200 (3%)	IgG4, IgG1, IgG3	ELISA/CBA	Present study
Contactin-1				
	3/46 (6%)	Total IgG	CBA	Querol, 2013)
	4/53 (7.5%)	IgG4, IgG3	ELISA, CBA, WB	Doppler, 2015
	16/533 (3%)	IgG4	CBA	Devaux, 2015
	2/200 (1%)	IgG4	ELISA/CBA (Present study)	Present study
Caspr1				
	1/35 (3%) CIDP 1/10 GBS	IgG4 IgG3	CBA/Flow cytometry	Doppler, 2016
	3/200 (1.5%)	TBD	ELISA/CBA (Present study)	Present study

CCPD combined central and peripheral demyelination.

Clinical features of our patients were in keeping with previously reported antibody/phenotype relationships, thus confirming the existence of specific clinical subtypes associated with different antibodies.

In particular, patients with antibodies to NF155 of predominant IgG4 isotype showed earlier onset, predominantly distal neuropathy, gait disturbance, and poor response to IVIg. Tremor was also present, although only in one case this was disabling. In contrast with a previous study (Kawamura et al. 2013), none of our NF155 reactive patients had clinical evidence of CNS involvement.

Patients with anti-CNTN1 antibodies presented at older age with subacute or rapidly progressive onset of severe sensory and motor neuropathy. Previous studies have found that CNTN1 antibodies

were associated with either predominant motor or sensory impairment(Querol et al. 2013; Miura et al. 2015; Doppler et al. 2016). In our patients both sensory and motor fibers were equally affected, leading patients to be wheelchair–dependent few months after disease onset. Contrarily, patients with Caspr1 antibodies the neuropathy was milder compared to NF155 and CNTN1 positive cases. Differently from the first report of two patients with Caspr1-associated inflammatory neuropathy(Doppler et al. 2016), pain does not seem to be a clinical feature associated to the presence of Caspr1 antibodies in our series.

Antibodies were of predominant IgG4 isotype in 3/6 NF155 cases and 1/2 CNTN1 cases, while determination of isotype is still in progress in seropositive sera for Caspr1. Predominant IgG4 antibodies have been described in several other neurological disorders, as MuSK-associated myasthenia gravis (McConville et al. 2004), limbic encephalitis and Morvan syndrome (Irani et al. 2014), and also in non-neurological disorders, such as pemphigus vulgaris (Rock et al. 1989), kidney inflammatory diseases (Beck et al. 2009) and thrombotic thrombocytopenic purpura (Ferrari et al. 2009). IgG4 show very peculiar features: for example, they have low affinity for Fcγ receptors and no ability to bind C1q, the first component of the complement cascade, and therefore do not show properties of antibody-mediated cell cytotoxicity or complement activation (Huijbers et al. 2015). This observations partially explain the poor response of patients affected by IgG4-mediated disorders to complement-inhibiting IVIg treatment. The pathogenic mechanism of IgG4 antibodies in all these diseases seems to be rather the disruption of cell adhesion protein complexes by inhibiting the protein-protein interaction (Manso et al. 2016)and therapies aiming at clearing the sera from the pathogenic antibodies, such as rituximab, showed some evidence of efficacy in NF155 and Caspr1 associated CIDP, as well as in other IgG4-related diseases, probably due to the depletion of IgG4-producing B cells (Querol et al. 2015; Díaz-Manera et al. 2012; Irani et al. 2014).

Of note, three NF155-positive cases had antibodies of different or undetectable isotype. These patients had lower diability grade, showed a good response to IVIg and analysis of myelinated fibres from the skin biopsy in one of them demonstrated milder changes and better preservation of node of Ranvier morphology compared to IgG4 NF155 cases. Altogether these data point to a different mechanism of action of non-IgG4 anti-NF155 antibodies, and highlight the need for isotype testing in NF155 seropositive cases.

Although we were unable to determine the isotype in one of the two CNTN1 seropositive cases, we think that the clinical features of the patient, who had subacute onset of severe IVIg-resistant motor and sensory neuropathy, are in keeping with previous description of IgG4 CNTN1 associated CIDP. The first sera available for testing was collected during improvement of the disease after cycophamide treatment, which limits the interpretation of the result, and subsequent sera during remission resulted negative for anti-CNTN1 antibodies. Of note, this patient showed the contemporary occurrence of membranous glomerulonephritis. This association had been reported in another case with positive anti-CNTN1 antibodies(Doppler, Werner, and Sommer 2013). Contactin-1 is expressed at low levels in the kidney and a direct damage by anti-CNTN1 antibodies could be hypothesized. Alternatively, renal damage might follow unspecific immune complexes deposition. Notwithstanding the single-case observation, our report suggests that also cyclophosphamide may be considered an effective therapy in anti-CNTN1 antibody-associated CIDP, leading to persistent clinical remission and clearance of the antibodies.

Regarding the testing of this antibodies, recombinant human NF155 and CNTN1 based ELISA, proved reproducible, showed high specificity, and could be used for the screening of anti-NF155 and anti-CNTN1 antibody in routine diagnostics, but should be always followed by isotype determination in seropositive sera. Nevertheless, confirmatory tests by CBA and IHC on teased fibers from murine sciatic nerve in specialized laboratory are still useful to demonstrate the

specificity of the binding of antibodies to the native NF155 and CNTN1 antigens and, so far, represent the only available tool for anti-Caspr1 antibody testing.

Finally, analysis of myelinated fibers in the skin biopsy of IgG4 NF155-seropositive patients showed morphological changes of nodes of Ranvier, including elongation of the node and loss of neurofascin staining at paranodes, which were absent in non-IgG4 seropositive and seronegative CIDP cases, and provide further evidence of the pathogenicity of these antibodies.

Another finding of the study is the observation that a relevant number of CIDP sera, up to 40%, react against other sites than the paranode, including myelin and Cajal bands, if tested on murine teased fibers. The nature of these targets remains to be demonstrated. In several previous studies, antibodies have been shown to target myelin components (P0, PMP22, P2, and Cx32). Several criticisms can be raised against these previous studies: 1) the results were controversial, as they have not been replicated; 2) the immune reaction lacked specificity and some patients affected by genetic neuropathies were found to be reactive against these myelin proteins; and 3) some studies used inappropriate techniques, which required denaturation of the proteins (predominantly western blot). Here, we have not examined the reactivity of the sera against these targets. However, we think that it could be interesting to re-address this question and study the reactivity to myelin components using standardized and multi-approach protocols. To this regard, the evaluation of IgG isotypes could increase the specificity of the findings and help defining the isotype/phenotypes relationship as it was clearly shown for the anti-paranodal proteins in CIDP. Also, evaluation of the binding of the sera against the NF155-CNTN1-Caspr1 complex may identify additional reactivities. In fact, there may be patients reacting against the paranodal NF155-CNTN1-Caspr1 complex, but not to its single components, and similarly to what has been previously demonstrated for reactivity to ganglioside complexes (Nobile-Orazio, Giannotta, and Briani 2010). Also, no studies have investigated the reactivity of sera to proteins situated at Cajal bands, such as the periaxin-DRP2-

dystroglycan complex, which may represent interesting possible targets of the humoral response in CIDP patients to be evaluated in future studies.

7. DIAGNOSTIC CHALLENGES AND MISDIAGNOSIS

DIAGNOSTIC CHALLENGES IN HEREDITARY TRANSTHYRETIN AMYLOIDOSIS WITH POLYNEUROPATHY: AVOIDING MISDIAGNOSIS OF A TREATABLE HEREDITARY NEUROPATHY

INTRODUCTION

Hereditary transthyretin (ATTR) amyloidosis is a systemic disorder caused by mutations of transthyretin *(TTR)* gene leading to extracellular deposition of amyloid fibrils composed of misfolded TTR protein. Peripheral and autonomic nerves and heart are the most frequently affected organs, but also eye, leptomeninges and kidneys can be involved (Violaine Planté-Bordeneuve and Said 2011a). ATTR amyloidosis typically presents in endemic areas for the common Val30Met mutation, such as Portugal, Sweden and endemic foci in Japan, as a small-fibre peripheral neuropathy causing loss of nociception and thermal sensation and autonomic dysfunction. There is usually clear dominant family history, onset of the neuropathy is before the age of 40 and cardiomyopathy often follows(Sekijima 2015).

Conversely, in Italy, ATTR amyloidosis shows broad genetic and phenotypic variability. In some cases, cardiomyopathy can be the unique clinical feature, such as in patients carrying Ile68Leu mutation. In other cases, peripheral nerve damage can be isolated and ATTR amyloidosis thus presents as a sporadic late-onset progressive large fibre sensory and motor peripheral neuropathy, in the absence of cardiac and autonomic involvement. Such presentation makes it often difficult to distinguish ATTR amyloidosis-related peripheral neuropathy from other acquired peripheral neuropathies of adulthood (Cappellari et al. 2011; Russo et al. 2012; Mariani et al. 2015; Mazzeo, Russo, and Di Bella in press).

Nowadays, avoiding misdiagnosis of ATTR amyloidosis is of vital importance because diverse treatment options are available, including liver transplantation and anti-amyloidogenic therapies with Tafamidis or Diflunisal, which all appear to be particularly effective in early disease stages(Obici et al. 2012; Coelho et al. 2012a; Berk et al. 2013; Obici and Merlini 2014).

Aim of this study was firstly to assess frequency, type and causes of misdiagnosis of ATTR amyloidosis in Italy. Secondly, given the high rate of ATTR cases misdiagnosed as CIDP, to further investigate common and distinguishing electrodiagnostic features of ATTR from CIDP.

METHODS

Clinical data collection

We reviewed the medical records of 150 ATTR patients diagnosed at the Amyloid Research and Treatment Centre between 1999 and 2013.

Hundred-four (73%) were male with an average age of onset of 61 years (31-86). Most frequent mutations were Val30Met (39; 26%), Glu89Gln (28; 19%), Phe64Leu (20; 13%), Ile68Leu (14; 9%), Thr49Ala (8; 5%).

We collected the following information for all patients: age of symptom onset, TTR gene mutation, age at diagnosis, previous alternative diagnosis, diagnostic delay, known or possible family history of ATTR amyloidosis, presence of cardiac symptoms, autonomic dysfunction and tissue biopsy findings. When available the following information was collected: Electrodiagnostic (EDx) study reports at disease onset, protein level at cerebro-spinal fluid (CSF) examination.

A different cause of neuropathy, including diabetes, was ruled out in all patients.

Ethic approval was not required for the present study. All patients gave their written consent permitting the use of their anonymized data for research purposes.

Electrodiagnostic studies

We reviewed original EDx studies of 49 patients with ATTR amyloidosis and 33 patients with CIDP, fulfilling European Federation of Neurological Societies/Peripheral Nerve Society (EFNS/PNS) criteria for definite CIDP. All EDx studies had been performed at the time of diagnosis at C. Mondino National Neurological Institute. All EDx studies were performed using a Medelec Synergy SINC5-C (Viasys Healthcare, Manor Way, Old Woking, Surrey, UK) electromyograph and conventional procedures. We collected the following information: compound muscle action potential (CMAP) amplitude, distal motor latency (DML), distal and proximal CMAP duration and motor nerve conduction velocities measured in the unilateral median, ulnar, peroneal and tibial nerves; unilateral F wave latency in the ulnar and tibial nerves; antidromic sensory action potential (SAP) amplitude and sensory nerve conduction velocities measured in the unilateral median and sural nerves.

We further assessed in ATTR amyloidosis patients fulfilment of EFNS/PNS electrodiagnostic criteria for demyelination(Joint Task Force of the EFNS and the PNS 2010) For this purpose, we excluded DML prolongation and distal CMAP duration in the median nerve because of frequent median neuropathy at the wrist from carpal tunnel syndrome and partial motor conduction block in the tibial nerve because of possible technical sub-maximal stimulation at the knee.

Statistical analysis

Differences between groups were determined with 2-tailed t test for quantitative variables and with $\chi 2$ test for categorical variables, as appropriate. Univariate logistic regression was performed with misdiagnosis as outcome variable and late onset after 55 years, type of mutation (Val30Met *vs* non-Met30 mutations), symptomatic cardiac involvement (NYHA≥2), autonomic dysfunction and absence of amyloid deposits on tissue biopsy as independent variables. The correlation between distal latency, distal CMAP amplitude, conduction velocity and distal CMAP duration and between distal CMAP amplitude and disease duration was analysed by computing the Spearman's correlation coefficient. All analyses were performed using STATA statistical software, version 14.

RESULTS

Clinical features

ATTR amyloidosis had been misdiagnosed in 49/150 (32%) cases. Most frequently considered alternative diagnoses were CIDP, lumbar and sacral radiculopathy and lumbar canal stenosis, paraproteinaemic peripheral neuropathy, AL amyloidosis and other causes of acquired neuropathy **(Table 1)**.

Table 1. Alternative diagnosis for patients with ATTR amyloidosis and variables associated with misdiagnosis of ATTR amyloidosis

Misdiagnoses			**N=49**	
Chronic inflammatory demyelinating polyneuropathy (CIDP)			30 (61%)	
Lumbar and sacral radiculopathy and lumbar canal stenosis			11 (22%)	
Paraproteinaemic peripheral neuropathy			3 (6%)	
AL amyloidosis			3 (6%)	
Senile amyloidosis			1 (2%)	
Toxic peripheral neuropathy			4 (8%)	
Vasculitic peripheral neuropathy			1 (2%)	
Motor neuron disease			1 (2%)	
Fibromyalgia			2 (4%)	
Other diagnosis			2 (4%)	
Multiple misdiagnosis			9 (18%)	
Variables Associated with misdiagnosis of ATTR amyloidosis	**Misdiagnosed patients (N=49)**	**Not misdiagnosed patients (N=101)**	**OR [95%CI]**	**P val**
Late onset (after 55 yo)	39 (80%)	58 (58%)	2.89 [1.30-6.42]	0.006
Absence of family history	28 (58%)	36 (36%)	2.4 [1.19-4.83]	0.01
Absence of symptomatic heart involvement (NYHA<2)	31 (63%)	46 (46%)	2.05 [1.02-4.14]	0.04
Negative tissue biopsy	14/36 (39%)	8/40 (20%)	2.5 [0.9-7]	0.07

CI: confidence interval, NYHA: New York Heart Association, OR: Odds Ratio,

Motor neuron disease was suspected in a patient with spinal and bulbar muscle atrophy and weakness. Two patients with a painful sensory and motor neuropathy were misdiagnosed as fibromyalgia while myotonic dystrophy type 2 was hypothesized in one patient with myalgia associated with proximal and distal lower limbs muscle weakness.

Thirty (61%) of patients received immune-therapy, including intravenous immunoglobulins (22 patients, 45%), steroids (25 patients, 51%), and immune-suppressors (6, 12%) or a combination of them (22 cases, 45%) without clinical improvement. Moreover, 11 patients (22%) previously diagnosed with lumbar spinal stenosis and radiculopathy secondary to degenerative spine disorder underwent spine surgery with no or only transient clinical improvement of symptoms.

Delay to correct diagnosis was significantly longer in misdiagnosed patients compared to those not misdiagnosed (46.4 months ± 25.4 *vs* 34.7 months ± 26; p=0.01).

Late onset after 55 years, absence of family history and absence of symptomatic heart involvement were significantly associated with misdiagnosis **(Table 1)**. Seventy-six patients underwent a tissue biopsy before being referred to our Centre (35 patients underwent nerve biopsy, 32 fat pad biopsy, 10 heart biopsy, 16 biopsy in other sites and 18 had multiple biopsies). The tissue biopsy failed to show amyloid deposit in 9/35 (25%) nerve biopsies, 15/32 (47%) fat pad biopsies, 7/16 (43%) biopsies performed in other sites, but in none of heart biopsies. A negative tissue biopsy was more frequent in misdiagnosed vs non-misdiagnosed cases (40% vs 20%), although this difference did not reach statistical significance. In nine patients with a previously negative result, fat pad biopsy was repeated in our Centre and showed the presence of amyloid deposits.

Presence of autonomic dysfunction and mutation type (Val30Met vs non-Val30Met) did not appear to significantly impact on correct diagnosis of ATTR amyloidosis (data not shown).

Lumbar puncture was performed in 7/30 patients diagnosed with CIDP and showed cyto-albuminologic dissociation with mild elevation of proteins in 5 cases (70±21.5 mg/dL, range: 49-96). EDx study at onset was available for review in 19 cases and showed a demyelinating neuropathy in 9 of them (47%).

The contemporary presence of M-protein was misleading in 6 cases. In particular, 3 cases showing contemporary κ or λ chains deposition on biopsy were diagnosed as AL amyloidosis, while 2 patients with IgM M-protein and low-titre anti-MAG reactivity and 1 patient with IgG M-protein were diagnosed with paraproteinaemic peripheral neuropathy.

EDx studies

In order to ascertain the prevalence and specificity of demyelinating changes in ATTR amyloidosis we reviewed original EDx studies at diagnosis of 49 consecutive ATTR amyloidosis patients, including 15 cases formerly misdiagnosed, and compared them with 33 patients with definite CIPD.

Each demyelinating feature as defined by EFNS/PNS electrodiagnostic criteria for CIDP was observed in at least 1 patient with ATTR amyloidosis.

The most frequently observed demyelinating features were: increased distal CMAP duration (10/46, 21.7%), especially at ulnar nerve, reduced conduction velocity (9/46, 19.6%), particularly at median and peroneal nerve and ≥30% amplitude reduction of the proximal negative peak CMAP relative to distal (8/46, 17.4%). Motor conduction block (≥50% amplitude reduction) was observed at median nerve in 2 patients and temporal dispersion of CMAP in 3 patients, 2 at median and 1 at ulnar nerve. Prolonged DML of tibial nerve was present in 2 cases while 29/40 (72.5%) had prolonged DML of median nerve, most likely due to long-standing carpal tunnel syndrome, and because of that not

considered for further analysis. F wave were tested in only 5 patients and resulted altered in 4 of them, either prolonged in 2 or absent in 2 (**Table 2**). **Figure 1** shows representative examples of demyelinating changes on EDx observed in patients with ATTR amyloidosis.

Table 2. Prevalence of demyelinating features according to EFNS/PNS criteria (2010) in patients with ATTR amyloidosis at upper and lower limb nerves

	Overall (%)	**Median nerve**	**Ulnar nerve**	**Peroneal nerve**	**Tibial nerve**
Prolonged distal motor latency	2/46 (4.3%)	NA	0/21 (0%)	0/27 (0%)	2/16 (12.5%)
Reduced conduction velocity	9/46 (19.6%)	5/39(12.8%)	0/21 (0%)	4/29 (13.8%)	0/16 (0%)
Conduction block 50%	2/46 (4.3%)	2/32 (6.2%)	0/21 (0%)	0/24 (0%)	NA
Conduction block 30%	8/46 (17.4%)	1/32 (3.1%)	3/21 (14.39%)	2/24 (8.3%)	2/9 (22.2%)
Temporal dispersion	3/46 (3.5%)	2/33 (6.1%)	1/17 (5.9%)	0/21 (0%)	0/8 (0%)
Increased distal CMAP duration	10/46 (21.7%)	NA	9/17 (52.9%)	1/23 (4.3%)	NA
F waves prolongation	2/5	2/5	0/5	0/5	0/5
F waves absence	2/5	1/5	1/5	0/5	0/5

NA: not applicable; CMAP: compound motor action potential

Figure 1. Electrodiagnostic pitfalls in ATTR amyloidosis.

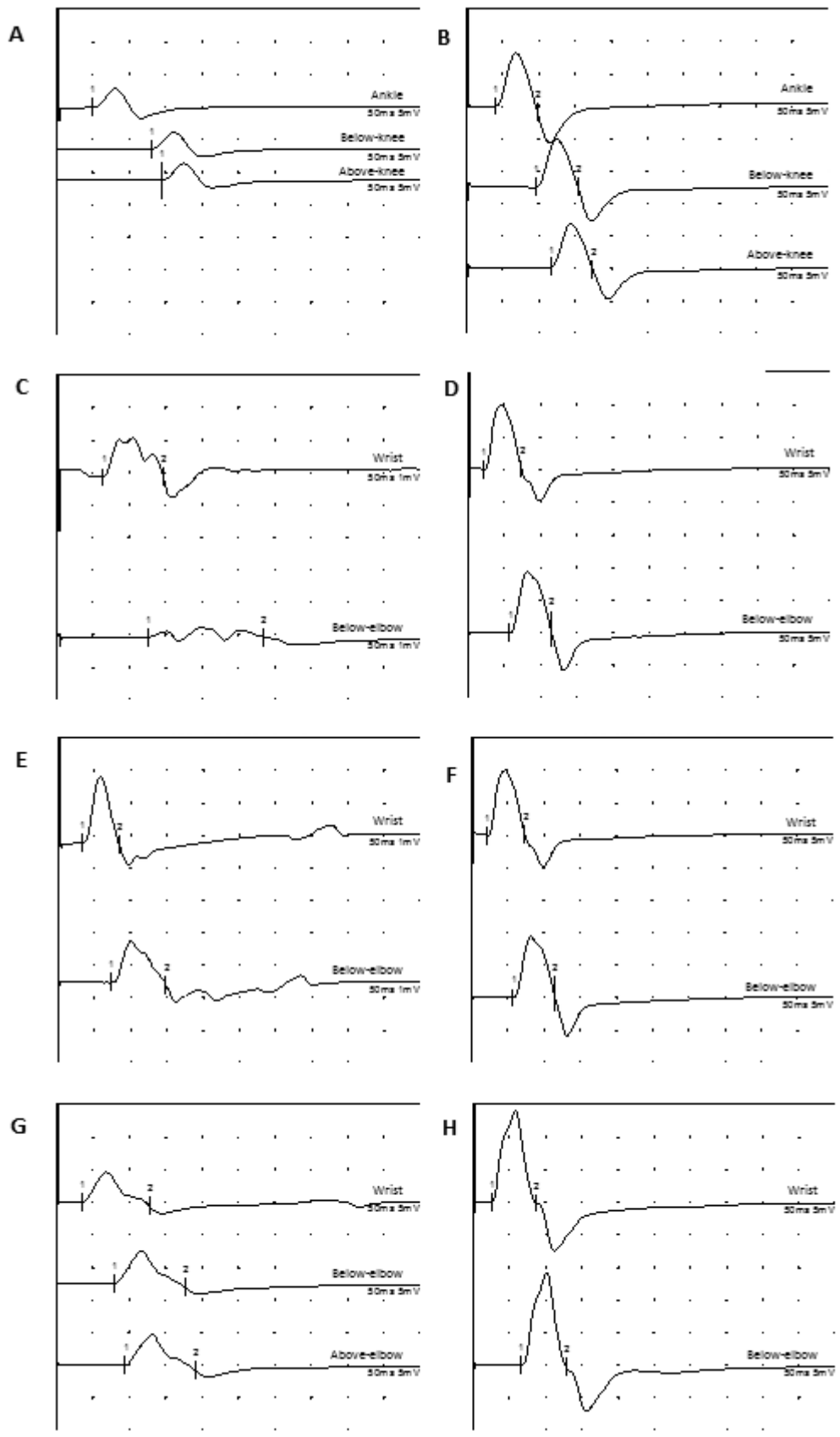


***Figure 1 (continued)**. Reduction of CV (32 m/s, normal > 38 m/s) at left peroneal nerve in a 53 years old patient carrying Glu54Gln mutation (**A**). Conduction Block at right median nerve in a 75 years old patient carrying Phe64Leu mutation and history of long-standing bilateral carpal tunnel syndrome (**C**). Temporal dispersion of CMAP at left median nerve in a 73 years old patient carrying Glu89Gln mutation (**E**). Increased distal CMAP duration (8.6 ms, normal< 6.7 ms) at ulnar nerve in a 71 year old patient carrying Tyr78Phe mutation **(G).** Representative examples of normal EDx studies in healthy controls at peroneal (**B**), median (**D, F**) and ulnar nerve (**H**).*

Overall 10/49 (20%) showed definite demyelination according to EFNS/PNS criteria on EDx study review, however this percentage raised to 27/49 (55%) considering less stringent criteria, including probable or possible demyelination **(Figure 2)**.

Figure 2. Electrophysiological features of peripheral neuropathy in ATTR amyloidosis

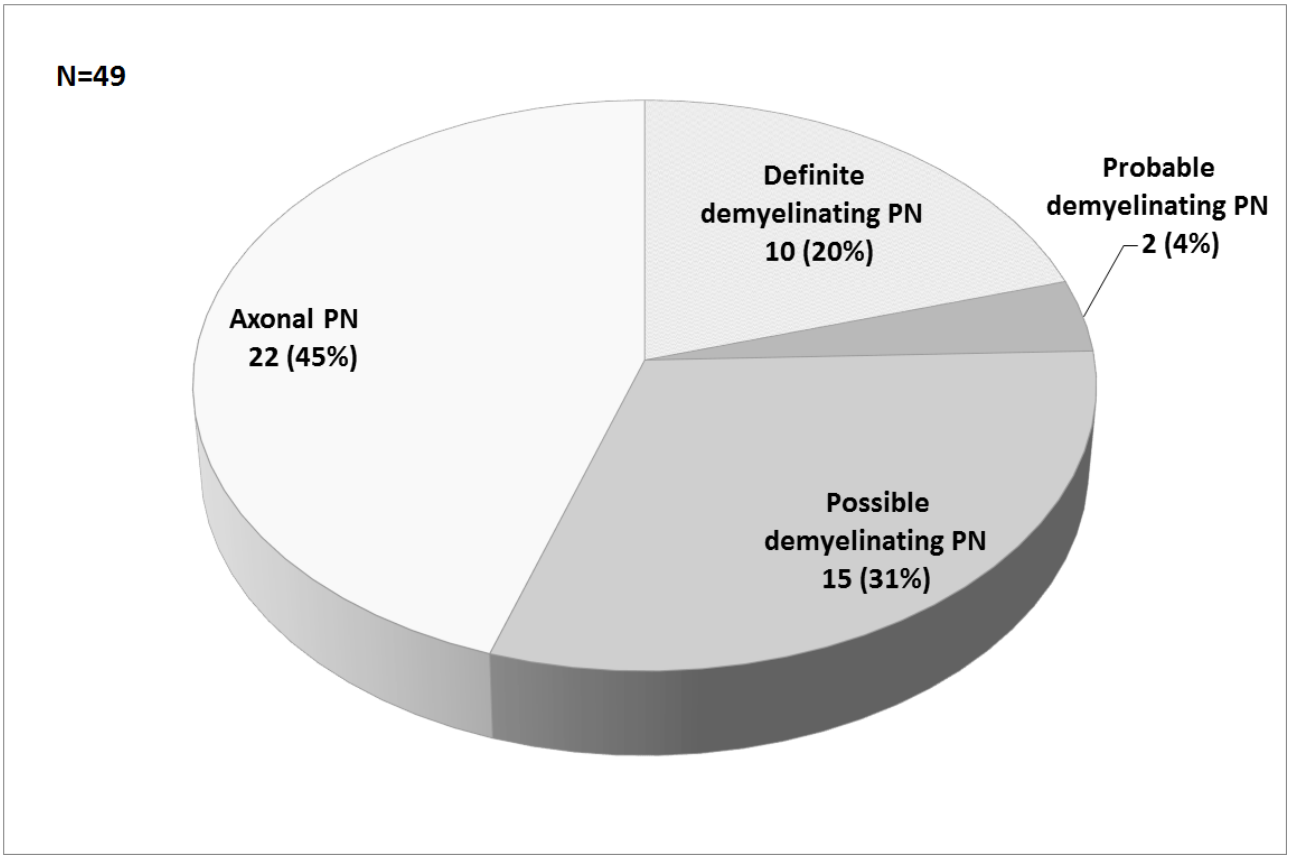


Figure 2 (continued). *Pie-charts showing the distribution of ATTR amyloidosis patients according to European Federation of Neurological Societies/Peripheral Nerve Society electrodiagnostic criteria for demyelinating polyneuropathy. PN: peripheral* neuropathy

Demyelinating changes were observed across different mutations, including Val30Met, Phe64Leu, Glu89Gln and Tyr78Phe. However, definite demyelinating features were particularly frequent in patients with Tyr78Phe mutation (3/7, 43%).

EDx features in ATTR amyloidosis and CIDP

These data confirm that definite demyelinating features are possible in a minority of cases affected by ATTR amyloidosis. However it is not fully understood whether such changes represent primary demyelination or depend on loss of fast conduction large diameter fibers.

In order to address this question we compared EDx studies of patients with ATTR amyloidosis with 33 patients with CIDP. A detailed summary of EDx findings in ATTR amyloidosis and CIDP is available in **Table 2**.

Table 2. Comparison of EDx study findings in ATTR amyloidosis and CIDP patients.

	TTR (N=49)	**CIDP (N=33)**	**P-value (0.05)**
Motor nerves			
Ulnar			
Distal latency (*ms*)	3.1 (0.6)	4.7 (3.5)	0.05
dCMAP duration (*ms*)	6.8 (1.5)	8.4 (3.5)	0.08
Conduction velocity (*m/s*)	51.5 (6.8)	37.3 (14.7)	0.0002
Amplitude (*mV*)	5.2 (3.5)	6.0 (3.5)	0.4
Median			
Distal latency (*ms*)	5.2 (2.0)	7.0 (6.6)	0.1
dCMAP duration (*ms*)	7.1 (2.4)	9.2 (4.9)	0.04
Conduction velocity (*m/s*)	45.3 (11.5)	35.9 (13.0)	0.05
Amplitude (*mV*)	3.6 (3.0)	5.9 (6.4)	0.02
Peroneal			
Distal latency (*ms*)	4.2 (1.2)	5.5 (2.1)	0.006
dCMAP duration (*ms*)	5.5 (1.4)	7.3 (3.2)	0.02
Conduction velocity (*m/s*)	41.4 (6.8)	33.1 (10.4)	0.00
Amplitude (*mV*)	2.4 (2.2)	2.8 (3.1)	0.6
Tibial			
Distal latency (*ms*)	5.1 (1.6)	6.9 (4.7)	0.15
dCMAP duration (*ms*)	5.0 (0.6)	10.4 (6.8)	0.02
Conduction velocity (*m/s*)	42.7 (7.2)	33.1 (10.4)	0.002
Amplitude (*mV*)	4.0 (5.5)	4.8 (6.1)	0.7
Sensory nerves			
Median			
Conduction velocity (*m/s*)	32.6 (12.1)	32.9 (13.6)	0.9
Amplitude (*mV*)	6.2 (9.7)	6.6 (7.9)	0.8
Sural			
Conduction velocity (*m/s*)	38.9 (7.9)	35.6 (9.8)	0.2
Amplitude (*mV*)	4.4 (4.8)	4.2 (4.7)	0.9

dCMAP distal compound muscle action potential, ATTR Amyloidosis TTR, CIDP chronic inflammatory demyelinating polyradiculoneuropathy

In ATTR amyloidosis we observed a high correlation between conduction velocity and distal CMAP amplitude in any examined nerve (**Figure 3 A, D**). Reduction of conduction velocity below the cut-off of 35 m/s, which represents the lower limit of normality of the slowest conducting fibers, was possible but invariably associated to a severe axonal damage. Contrarily, above 50% of CIDP patients presented slow conduction velocity below 35 m/s but relatively preserved CMAP amplitude. As expected, in CIDP reduction of conduction velocity and reduction of distal CMAP amplitude were paralleled by increased duration of CMAP. Contrarily, we did not observe any correlation between

CMAP duration and conduction velocity (**Figure 3 B,E)** or CMAP duration and CMAP amplitude in ATTR amyloidosis patients (**Figure 3 C,F) (Table 3)**

Table 3. Correlation analysis of nerve conduction parameters in ATTR and CIDP.

Pearson's correlation coefficients (r, p)	**ATTR**			**CIDP**		
	dCMAP Amplitude	Conduction velocity	dCMAP duration	dCMAP Amplitude	Conduction velocity	dCMAP duration
Ulnar nerve						
dCMAP Amplitude	1	-	-	1	-	-
Conduction velocity	0.76; <0.001	1	-	0.62. <0.001	1	-
dCMAP duration	-0.37; 0.1	-0.17; 0.5	1	-0.53; 0.02	-0.67; 0.002	1
Median nerve						
dCMAP Amplitude	1	-	-	1	-	-
Conduction velocity	0.69. <0.001	1	-	0.58. 0.006	1	-
dCMAP duration	-0.17. 0.3	-0.31. 0.06	1	-0.57; 0.03	-0.67; 0.007	1
Tibial nerve						
dCMAP Amplitude	1	-	-	1	-	-
Conduction velocity	0.77;<0.001	1	-	0.55;0.002	1	-
dCMAP duration	0.35;0.31	0.08; 0.8	1	-0.28;0.26	-0.67;0.002	1
Peroneal nerve						
dCMAP Amplitude	1	-	-	1	-	-
Conduction velocity	0.63; <0.001	1	-	0.45. 0.01	1	-
dCMAP duration	-0.04; 0.8	-0.40; 0.05	1	-0.30; 0.20	-0.51. 0.05	1

CMAP compound muscle action potential, dCMAP distal CMAP, ATTR Amyloidosis TTR, CIDP chronic inflammatory demyelinating polyradiculoneuropathy

Moreover, in ATTR amyloidosis but not in CIDP there was a correlation between disease duration and CMAP amplitude at peroneal (-0.6, p=0.0004), tibial (-0.50, p=0.049) and median nerve (-0.32, p=0.039). No correlation was found between ulnar CMAP, sural and median SAP amplitude and disease duration in either CIDP or TTR patients.

Finally, asymmetry of CMAP amplitudes, as defined by > 50% difference between sides, was slightly more common in CIDP vs TTR (34% vs 21%), although this did not reach a statistical significance.

Together these observations demonstrate that in ATTR amyloidosis conduction slowing is mainly secondary to time-dependent loss of fast conducting fibers, although true demyelinating changes are possible, particularly in severely affected nerve trunks.

Figure 3. Demyelinating changes and fiber loss in ATTR and CIDP.

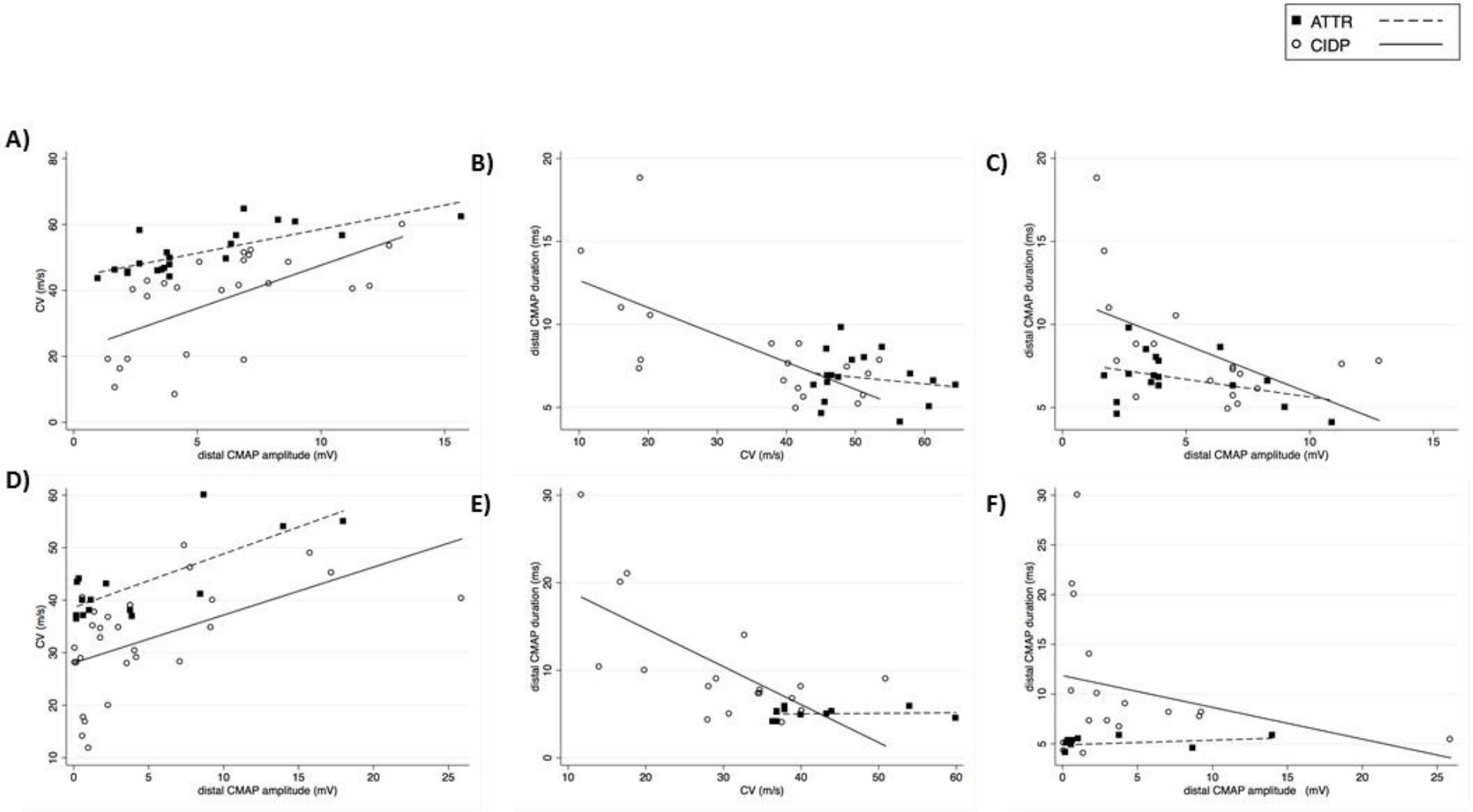


Figure 3 (continued). Demyelinating changes and fiber loss in ATTR and CIDP. *Scatter-plots showing, in ATTR (dotted line) and CIDP (continuous line), respectively, the relation between conduction velocity and distal CMAP amplitude at ulnar (**A**) (r=0.76 p<0.001 vs 0.62 p<0.001) and tibial (**D**) nerves (r=0.77 p<0.001 vs 0.55 p=0.002), between distal CMAP duration and conduction velocity at ulnar (**B**) (r= -0.31 p=0.06 vs -0.67 p=0.002) and tibial (**E**) (r= -0.08 p=0.8 vs -0.67 p=0.002) nerves, between distal CMAP duration and distal CMAP amplitude at ulnar (**C**) (r= -0.37 p=0.1 vs -0.53 p=0.02) and tibial (**F**) (r= 0.35 p=0.31 vs -0.28 p=0.26) nerves. ATTR transthyretin amyloidosis; CIDP chronic inflammatory demyelinating polyradiculoneuropathy; CMAP compound motor action potential; CV conduction velocity; PN peripheral neuropathy*

DISCUSSION

ATTR amyloidosis is a debilitating highly penetrant autosomal dominant disease leading to motor disability within 5 years and generally fatal within a decade without treatment (Ando et al. 2013).

Peripheral nerves are almost invariably involved, although differences in disease onset, clinical phenotype and disease progression exist across diverse mutations and in different geographic regions. In fact, a more aggressive disease course is reported in late-onset Val30Met patients and subjects carrying non-Val30Met mutations(David Adams et al. 2015). Such patients usually come from non-endemic areas for ATTR amyloidosis and often do not show a clear family history of the disease. It is therefore of vital importance for neurologists to suspect ATTR amyloidosis also in cases of sporadic relentless sensory-motor neuropathy in order to reach a timely diagnosis and promptly start anti-amyloidogenic treatment.

Our study show that ATTR amyloidosis is still misdiagnosed in a high proportion of cases, with significant increase, up to 1.5 fold and 4 years, in diagnostic delay.

Lack of family history, late onset of the neuropathy and absence of cardiac involvement were significantly more frequent in misdiagnosed patient and often misled practitioners into suspecting a different cause of acquired neuropathy. Even when the diagnosis of amyloid neuropathy was suspected and a tissue biopsy performed, the absence of amyloid deposits drove clinicians to reject the diagnosis of amyloid neuropathy in 40% of them and do not perform further genetic testing. To this regard it is well known that diagnostic sensitivity of biopsy varies greatly across different tissues and various stages of the disease(Fernández de Larrea et al. 2015) and negative biopsy result does not rule out the disease, particularly in patients with typical signs and symptoms (Ando et al. 2013). The immune-histochemical characterization of amyloid deposit was also confounding in 3 cases in which amyloid deposits were labeled by both anti-TTR antibodies and anti-light chain antibodies.

Such pathologic finding is known to possibly occur in patients with a concomitant monoclonal component and should prompt clinicians to perform *TTR* gene testing also in doubtful cases of AL amyloidosis (Lachmann et al. 2002; V. Planté-Bordeneuve et al. 2007).

We observed that CIDP was the most frequent misdiagnosis of ATTR amyloidosis in Italy and demyelinating feature on nerve conduction study were found to be a relevant factor leading to disease misdiagnosis.

Previous studies reported a low prevalence of demyelinating changes in ATTR amyloidosis ,[16–18]. However, such observations were mainly based on early onset Val30Met cases from endemic areas, for which loss of unmyelinated and small myelinated fibers is the predominant pathologic feature, at early stage s(Misu et al. 1999a; Haruki Koike et al. 2012).

In fact, a French study, which included late-onset Val30Met cases from non-endemic areas and patients with non-Val30Met mutations, also found a high rate (18/90, 20%) of misdiagnoses as CIDP, which were supported by increased CSF protein content in seven and marked decrease of conduction velocities in eight of them (V. Planté-Bordeneuve et al. 2007). A slowing of conduction velocities and a prolongation of distal latencies, which suggests demyelination, were also conspicuous in some late-onset Val30 Met sporadic patients from non-endemic area from Japan (Haruki Koike et al. 2008). A later report from a large French case series identified demyelinating changes on EDx study in 48/84 (56%) of non-Val30met and late-onset Val30Met cases. Seventeen of them (20%) fulfilled electrodiagnostic criteria for definite demyelination (Mariani et al. 2015), thus further confirming that CIDP-like presentation of ATTR amyloidosis is not uncommon (Mathis et al. 2012).

Our study provided similar results, with 55% of cases showing demyelinating features on EDx study and 20% having a definite demyelinating neuropathy according to EFNS/PNS criteria. Of note, demyelinating features were particularly frequent in patients with Tyr78Phe mutation. Moreover, we showed that all hallmarks of demyelination, which usually support a diagnosis of CIDP, can also be

observed in ATTR amyloidosis. In particular, reduction of conduction velocity was frequent at peroneal and median nerve. In the latter, conduction blocks and temporal dispersion were also possible. These changes could represent true demyelination due to ATTR deposition, although we cannot exclude that, at median nerve, conduction blocks and reduced conduction velocity may be secondary to long-standing carpal tunnel syndrome causing Wallerian degeneration with preferential loss of fast conducting fibers.

Distal CMAP duration increase was particularly frequent at ulnar nerve, in the absence of focal ulnar neuropathy at the elbow. F waves were also frequently altered, although these were tested in only few cases. Interestingly, recent studies suggested in ATTR amyloidosis the presence of a proximal-distal gradient of pathologic changes at MRI-neurography with more affection of proximal tracts of sciatic nerves compared to the distal segments, and which parallel pathologic demonstration of greater deposition of amyloid at this level (Misu et al. 1999a; Kollmer et al. 2015).

Previous pathologic studies have shown that severe loss of myelinated and, to various extent, unmyelinated fibers characterize nerve biopsies of ATTR amyloidosis patients. Although less frequently, segmental demyelination, remyelination as well as distortion of myelin sheets in contact with amyloid are also possible (Misu et al. 1999a; V. Planté-Bordeneuve et al. 2007) and could explain EDx findings suggestive of predominant demyelinating neuropathy observed in some of our patients.

This notwithstanding, we provided evidence that slow conduction velocities, which are often observed in ATTR amyloidosis, are mostly related to loss of fast conducting fibers. In fact, despite reduced conduction velocity <35 m/s, so below the limit of the slowest conducting fibers, points to a primary demyelinating damage, marked conduction slowing was invariably associated to severe axonal loss.

In fact, differently from CIDP, in ATTR amyloidosis conduction slowing and reduction of CMAP

amplitude were not paralleled by increase in distal CMAP duration, thus not giving evidence of the temporal dispersion of motor potentials which is typically seen in segmental demyelination (Joint Task Force of the EFNS and the PNS 2010; Tankisi et al. 2012). Moreover, in ATTR, but not in CIDP, we observed in multiple motor nerves a significant correlation between CMAP amplitude and disease duration, expression of the relentless loss of large fast conducting fibers in ATTR patients. However, we cannot exclude that "phase cancellation" effect due to desynchronized distal CMAP, which was previously hypothesized in CIDP patients in order to explain the occurrence of low amplitude potentials in a primary demyelinating disorder (Tankisi et al. 2007), may also partly account for the reduction of CMAP amplitude in ATTR.

Regarding CSF examination, high protein content, although never above 1 gr/dL, was found in 5 out of 7 patients. and previous studies also reported the presence of cyto-albuminologic dissociation in 29-70% of ATTR amyloidosis cases (Mariani et al. 2015; Misu et al. 1999a; D. Adams et al. 2000) There is no clear explanation for this increase in CSF proteins. We speculate that a disruption of integrity of blood-nerve barrier and blood-brain barrier at the level nerve roots may occur, possibly due to amyloid deposition at this level, and similarly to what observed in the context of diabetes mellitus (Prasad et al. 2014).

Finally, spondilogenic radiculopathies and lumbar canal stenosis were also frequently suspected before the diagnosis of ATTR amyloidosis and a not negligible proportion of these patients underwent spine surgery with partial or no benefit. Of note, lumbar canal stenosis was also found to be a misdiagnosis of ATTR amyloidosis in 12% of 60 patients from the French reference centre for familial amyloid polyneuropathy (David Adams et al. 2012). Moreover, a positive history of bilateral carpal tunnel syndrome, often requiring surgical release, was present in the majority of patients and EDx study showed, at diagnosis, persistent alteration of median nerve conduction in keeping with carpal tunnel syndrome in ¾ of them. Together these data should encourage to raise awareness in

neurosurgeons and orthopaedic surgeons about the possibility of ATTR amyloidosis in patients with sensory disturbances and progressive motor deficit at lower limbs, particularly in association with bilateral carpal tunnel syndrome.

In conclusion, ATTR amyloidosis is still a frequently misdiagnosed disease, particularly in sporadic cases of isolated late-onset peripheral neuropathy. CIDP is the most common alternative diagnosis, which is supported by EDx finding of a demyelinating neuropathy in half of them, together with a frequent mild raise in CSF proteins. Such demyelinating changes, which are particularly frequent at median nerve in the context of long-standing carpal tunnel syndrome and in other severely affected motor nerves, appear to be mostly related to loss of large fast conducting fibers. Therefore, ATTR amyloidosis should be timely considered in the differential diagnosis of late-onset sporadic progressive axonal and axonal-demyelinating polyneuropathies in order to avoid detrimental diagnostic delay. Patients with idiopathic progressive polyneuropathies, as well as patients with suspected CIDP refractory to immune therapies, frequently undergo many repeat EDx studies, which often add little information but have considerable costs. *TTR* gene comprises 4 exons coding for 147 amino acids, and the cost of its full sequencing is under 350 US$. Therefore, *TTR* gene testing could be advised at early stages of the diagnostic work-up in cases of suspected CIDP with poor response to immune-therapies and other idiopathic late-onset progressive axonal and axonal-demyelinating polyneuropathies.

8. IMMUNITY AND DEGENERATION

ALTERED TDP43-DEPENDENT SPLICING IN INCLUSION BODY MYOSITIS

INTRODUCTION

Inclusion body myositis (IBM) is the most common acquired muscle disease in adults over the age of 50. The muscle pathology is characterized by the presence of both inflammatory (CD8+ lymphocytes invading non necrotic muscle fibres, MHC class I up-regulation) and degenerative changes (cytoplasmic inclusions containing amyloid Beta and other Alzheimer type proteins, rimmed vacuoles), as well as loss of mitochondrial cytochrome c oxidase (COX) activity on muscle fibres. Unlike other inflammatory myopathies, IBM is unresponsive to immune-suppressive treatments and shows a relentless clinical course with progressive weakening of muscles (Cortese et al. 2013). At present, the exact pathogenesis of the disease is still poorly understood and both a primary inflammatory and primary degenerative cause have been hypothesised.

TDP-43 is 414-amino-acid RNA binding protein, which localizes in the nucleus and is involved in RNA processing including mRNA splicing, transport and stabilization. Of particular interest is the observation that TDP-43 is a major component of inclusions that characterize fronto-temporal lobe dementia (FTLD) and amyotrophic lateral sclerosis (ALS) pathology and mutation in TDP-43 cause FTLD and ALS (Neumann et al. 2006; Buratti and Baralle 2012). Recent studies have shown the presence of cytoplasmic TDP-43 inclusions in muscle fibres of patients with IBM, as well as in other myopathies with protein aggregates including myofibriallr myopathies and oculopharyngeal muscular dystrophy, thereby placing IBM nearer to a unique category of neuro-degenerative diseases termed TDP-43 proteinopathies, pathologically characterized by mislocalization of the TDP-43 to the cytoplasm and the loss of its normal nuclear localization (Weihl et al. 2008; Salajegheh et al. 2009; Olivé et al. 2009a).

However it is not known yet whether TDP43 aggregation in muscle is paralleled by impaired TDP43 function, of possible pathogenic relevance to muscle fiber degeneration.

To this date, several experimental cellular and mice models have provided evidence that depletion of TDP-43 is associated with alteration of the splicing regulation of its target transcripts (Polymenidou et al. 2011; Tollervey et al. 2011). Some of these changes were also observed in brains of FTLD patients suggesting that alteration of RNA metabolism could be a primary mechanism by which TDP-43 causes neuro-degeneration (Shiga et al. 2012). Widespread alteration of the expression of genes involed in RNA metabolism were also observed in muscle tissue of IBM patirents by us in a previous study, suggesting that impaired RNA metabolism could also play an important role in IBM muscle degeneration (Cortese et al. 2014).

To better address this issue, in this study we have taken advantage of the excellent preservation of frozen muscle biopsies from several patients with IBM and other protein aggregate myopathies (PAM) to investigate TDP43 function, by looking at the splicing profile of previously validated TDP43 target exons.

METHODS

Patients' samples

Muscle biopsies were obtained from 5 IBM patients and 3 genetically-confirmed OPMD patients and 1 HSPB8-related myofibrillar myopathy. Muscle biopsies from 4 patients with non-specific symptoms and normal histology were used as controls. Biopsies were processed for routine histology and immunohistochemistry. Moreover, we assessed by immunohistochemistry the presence of TDP43 aggregates (Abnova, Taipei City, Taiwan; 1:800). IBM patients fulfilled Griggs

criteria for IBM (Griggs et al. 1995). Institutional board reviewed the study and ethical approval was obtained.

RNA purification and RT-PCR analysis and quantitative real time PCR analysis

One µg of tissue from muscle biopsy were homogenized in 1 mL of Trizol at 4 °C. Total RNA was isolated using RNeasy mini kit (Quiagen). Total RNA from sample II-1 was purified using Trizol reagent (Invitrogen) according to manufacturer's instructions. The cDNA synthesis was performed using 1 µg of each RNA sample and Moloney murine leukemia virus reverse transcriptase (Invitrogen) and random hexameric primers. PCR analysis was performed using the following primers: SKAR/POLDIP3 Fw 5′-gcttaatgccagaccgggagttg-3′; SKAR/POLDIP3 Rv 5′-tcatcttcatccaggtcatataaatt-3′; FNIP1 Fw 5′-gctacaagatagtcttgaattcatc-3′; FNIP1 Rv 5′-cagaccgtgctatgccactgtctct-3′; TNIK Fw 5′caattcttggccacgcagttgatta-3′; TNIK Rv 5′-ctcagagagaaagtggaggaggttc-3′.

In all experiments exon inclusion/exclusion was quantified using Image J according to the methodology described in (Schneider, Rasband, and Eliceiri 2012).

The quantification of TDP43 gene expression levels was performed by real-time PCR using SYBR green technology. Specific primers for TDP43, RPL13a and GAPDH genes were designed using Beacon designer software (Bio-Rad) and are as follows:

The housekeeping genes GAPDH and RPL13a were used to normalize the results. All amplifications were performed on a CFX96 real-time PCR detection system (Bio-Rad). The relative expression levels were calculated according to Livak method (using the equation ΔCT = CT(target) – CT(normalizer) for Ct normalization; and the difference between ΔCT test ($HSPB^{8K141E}$) and ΔCT calibrator (normal muscle) to calculate the expression ratio and compare the expression levels of TDP43. Statistical significance was calculated using student's t-test

SYSH-SY5 cell line siRNA

Human neuroblastoma SH-SY-5Y cells were cultured in DMEM–Glutamax-I (Gibco-BRL, Life Technologies Inc., Frederick, MD, USA) supplemented with 10% fetal bovine serum (Gibco-BRL, Life Technologies Inc., Frederick, MD, USA) and Antibiotic-Antimycotic-stabilized suspension (SigmaAldrich, St. Louis, MO, USA) at 37°C incubator with humidified atmosphere of 5% CO2. The siRNA sense sequences used for silencing the different target proteins were the following: luciferase (control): uaaggcuaugaagagauac, TDP43: gcaaagccaagaugagccu.

RESULTS

Firstly we confirmed the presence of TDP43 aggregates in all sIBM patients and OPMD patients, but not in HC muscle (**Figure 1**). Due to limited availability of muscle tissue we could not assess the presence of TDP43 aggregates in the biopsy from the HSPB8 case (**appendix 2**). Nonetheless, TDP43 inclusion were previously reported in another case of HSPB8-related myofibrillar myopathy carrying the same mutation.

In parallel, as described in the Material and Methods section, total RNA from each of the patient samples was extracted and analyzed using RT-qPCR for TDP-43 expression. Interestingly, we observed, that the relative expression of TDP-43 mRNA was significantly reduced in sIBM muscle compared to HC (**Figure 2A**). There was also a trend of lower expression of TDP43 in OPMD, although this did not reach statistical significance. These results are of particular interest because they suggest that the autoregulatory mechanism which should keep TDP-43 mRNA concentration constant is impaired in the muscle tissue of IBM patients.

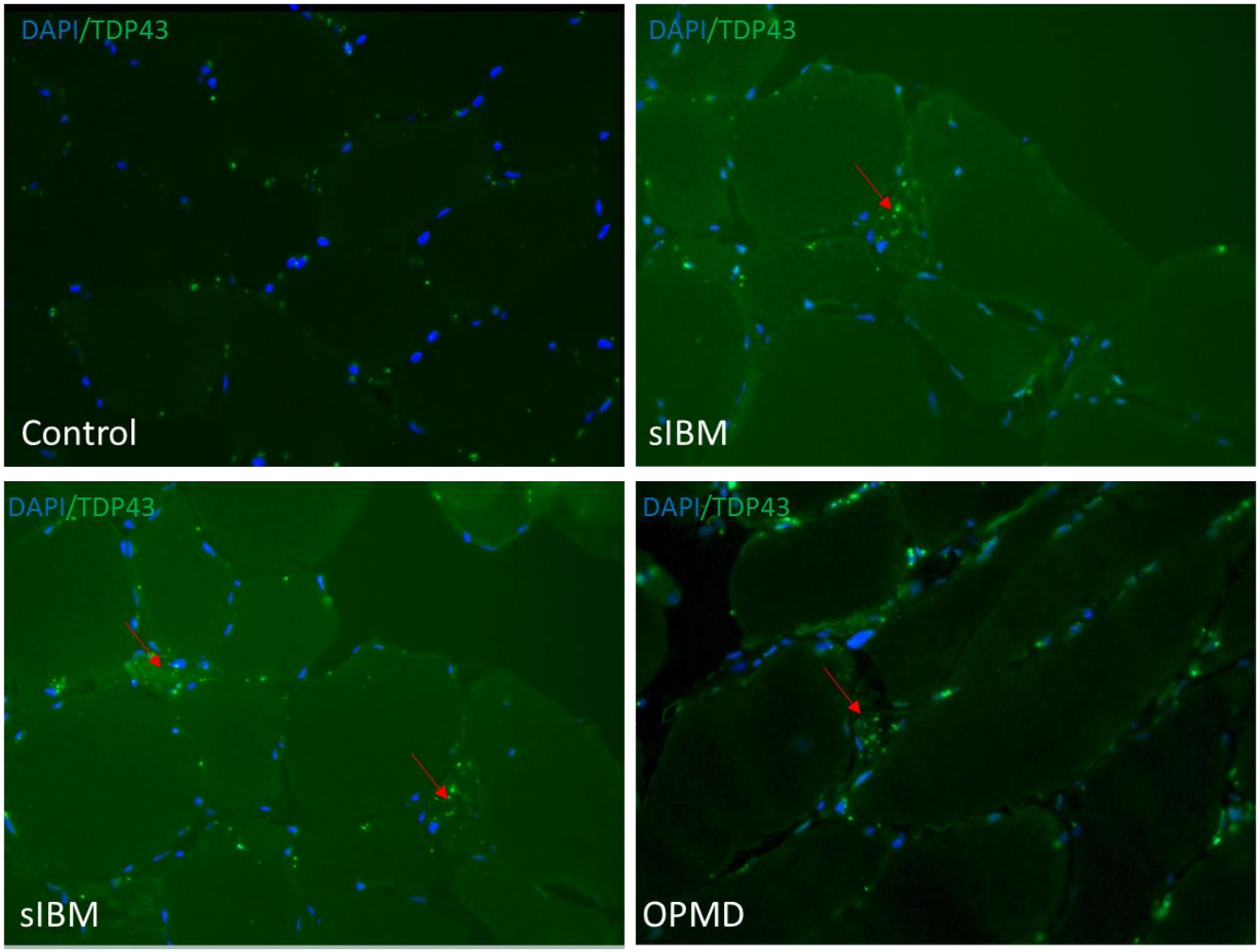
It was then of interest to see whether this drop in TDP-43 expression was reflected in the splicing profile of genes that have been previously described to be regulated by this protein (De Conti L, 2015; Shiga et al., 2011). As control for these amplifications we have amplified in parallel RNA obtained from a human neuroblastoma cell line SH-SY-5Y treated with siLuciferase and siTDP-43. This experiment was performed to compare the inclusion/skipping levels detected in the tissue of the patients with a well-validated experimental system of TDP-43 loss-ofo-function.

The results of RT-PCR analysis using specific primers showed that splicing of POLDIP3 was altered in 3/5 sIBM patients and 2/3 OPMD cases, but not in HC. Splicing of FNIP was altered in all 3/5 sIBM cases and 1/3 OPMD cases. Assessment of the splicing of Exon 15 of TNIK was uninformative, because this exon is already completely included in normal muscle tissue and thus prevented evaluation of an increased inclusion, as could be expected based on results on TDP-43 depleted cell lines **(figure 2B**).

In the case with HSPB8-related myofibrillar myopathy/distal hereditary motor neuropathy we were able to demonstrate alteration of TDP43 dependent splicing in both POLDIP3, FNIP and additional TDP43-dependenent target (BRD8) (**appendix 2**).

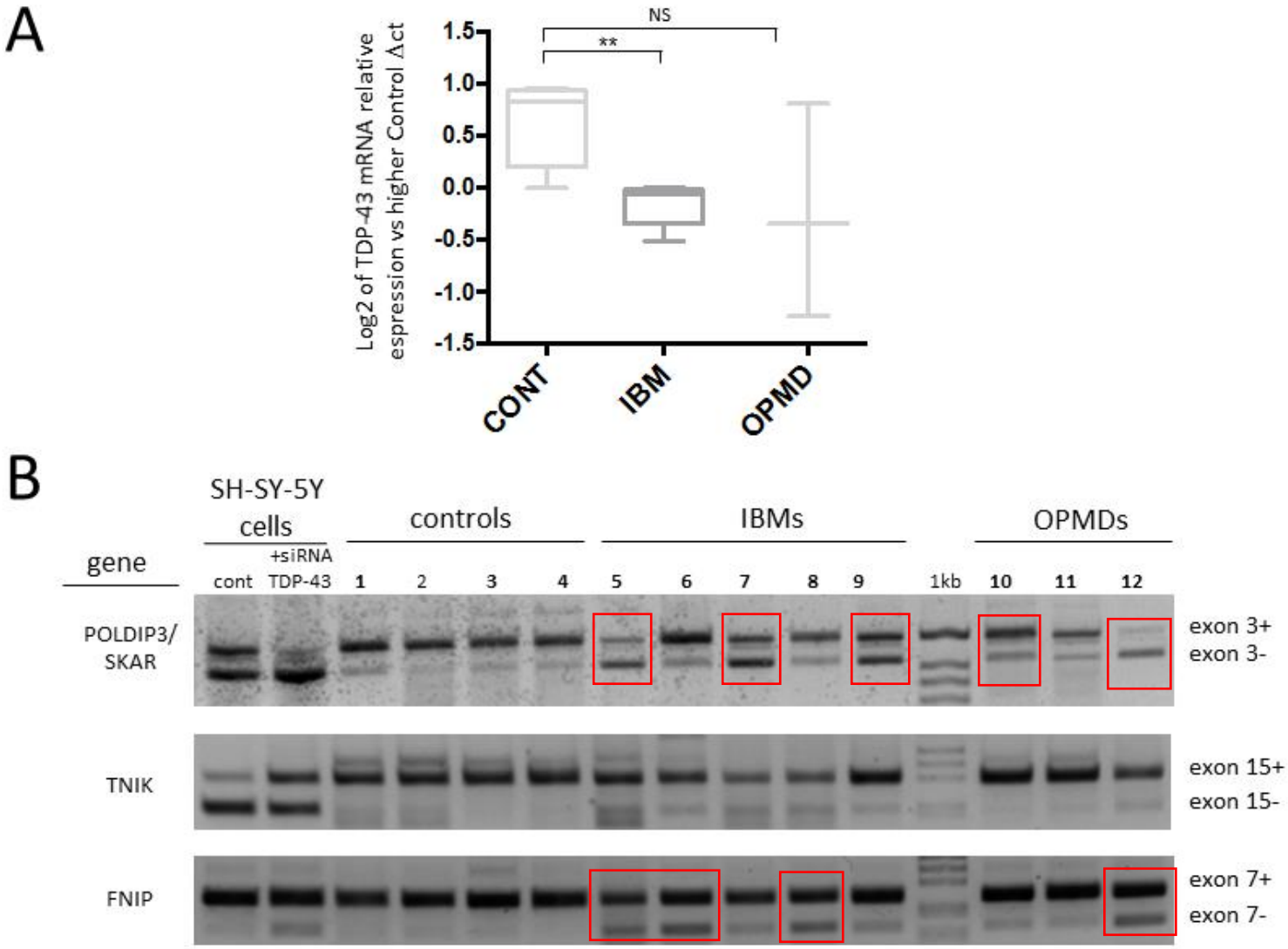
Overall, all IBM samples tested and in 2 out of 3 OPMD cases, but none of healthy controls, showed evidence of alteration of the splicing profile of at least one TDP43 target transcript.

Figure 1. TDP43 aggregates are found in muscle fibres from patients with PAMs



The red arrows indicate TDP43 sarcoplasmic aggregates in myofibres from patients with IBM and OPMD. No TDP43 aggregates or pathologic TDP43 mislocalization was observed in muscle from healthy controls

Figure 2. Altered TDP43 expression and TDP43-dependent splicing in PAMs



(A) *qPCR of TDP43 expression* ***(B)*** *Splicing analysis of 3 previously validated TDP43-dependent target exons. Red-highlighted are transcript with an altered splicing profile, similar to that observed in the TDP43 depleted cell lines (Left)*

DISCUSSION

TDP-43 is a RNA binding protein involved in different steps of RNA metabolism including RNA maturation and transport. TDP-43 mislocalization from the nucleus to cytoplasmic inclusions has a primary role in neurodegenerative disease of CNS, notably ALS and FTD and both loss of nuclear function and gain of toxic function in the cytoplasm have been hypothesised as disease causing-mechanism. TDP-43 mislocalization and sarcoplasmic aggregation was also identified by several studies as a pathologic feature of sIBM, along with the presence of inflammatory infiltrates. Of great interest, TDP43 aggregates, along with *tau* inclusions, were also recently found in the neurons of patients affected by IgLON5-mediate autoimmune encephalitis, further strengthening the link between autoimmunity, TDP43 and neurodegeneration (Gelpi et al. 2016).

In this study, we provide evidence for the first time that aggregation and mislocalization of TDP43 in sIBM is paralleled by loss of TDP-43 function in muscle, as demonstrated by alteration of the splicing profile of TDP-43 dependent transcripts. These transcripts were chosen because they represent robust, previously validated TDP43 target genes and reliable marker of TDP43 function. An alteration at their level, however, demonstrates that loss of TDP-43 activity is present in most muscles of IBM patients and this may lead to altered splicing and processing of genes involved in muscle fiber survival.

Interestingly, we found similar alteration of the splicing profile in other PAMs, including OPMD and HSPB8-related myofibrillar myopathy (**APPENDIX 2**), highlighting that TDP43 aggregation and loss of function can be common pathologic mechanisms underlying different genetic and acquired disorders affecting the skeletal muscle.

Whether the inflammatory response which characterizes the muscle pathology of sIBM is causing or rather the consequence of TDP43 aggregation is so far still unknown. Future studies should aim to clarify the cross-talk between the impairment of RNA metabolism and the inflammatory response in sIBM, as well as to investigate the effect of TDP43 aggregation and loss of function on the splicing of transcript of possible pathogenic relevance to muscle degeneration.

9. CONCLUSIONS

Inflammatory disorders of the nervous system have a broad clinical expressivity, affecting the CNS, the PNS or both compartments, and variable disease course, from self-limited monophasic disorders to chronic relapsing and progressive variants. Both genetic susceptibility and environmental factors play a role in their onset and progression. However, as for most autoimmune disorders, their ultimate cause is still unknown.

We found that air pollution can influence the inflammatory activity of MS and, by a combined *in vivo* and *in vitro* approach, we identified two candidate mechanisms, including the upregulation of adhesion molecules on circulating lymphocytes and the increased generation of T helper 17 cells. In recent years there has been a great interest in the relationship between the gut and the central nervous system and tremendous progress has been made in characterizing their bidirectional interactions (Mayer, Tillisch, and Gupta 2015). Based on studies using rodents raised in a germ-free environment, the gut microbiota appears to influence the development experimental autoimmune encephalitis and there are increasing evidences that changes of the gut microbiota, associated with western life-style, are can impact on the onset and progression of MS (Berer et al. 2011; J. Chen et al. 2016) . In our study we suggest that the lung, another organ where our immune system is constantly challenged by external infectious and non-infectious agents, may contribute to the inflammatory activity of MS. Future studies should aim to clarify the mechanisms of action of air pollution, and particulate matter in particular, on lung-resident dendritic cells, which seem the primary mediator of the proinflammatory properties of particulate matter.

Of note, respiratory infections and flu-like syndrome are also the most common triggers identified in patients with ADEM and other ADS of adulthood. By reviewing the clinical data of adult patients with acute demyelinating syndrome (ADS) admitted in the last 15 years in our Institute we

demonstrated that, contrarily to the typical monophasic and polyfocal presentation of ADS in children, ADEM in adults is much more heterogeneous, encephalopathy is less frequent while involvement of the PNS is not rare and has negative prognostic impact. Although the contemporary expression of neurofascin-155 at axo-glial junctions on both CNS and PNS makes it an excellent candidate as a target of the immune-mediated damage in combined demyelination of central and peripheral nervous system, we were unable to confirm the association of CCPD with antibodies against neurofascin-155. Whether ADEM and its variants represent a unique disease with variable expressivity or are rather a group of disorders with different pathogenesis remain still unknown. We believe that the unbiased assessment of the genetic susceptibility profile of patients with ADEM, as compared to that of patients with MS and healthy individuals, could provide useful information about the disease-causing mechanisms which are common or distinctive of these inflammatory demyelinating disorders.

As opposed to the negative result of the search for a serological marker of CCPD, antibodies against Neurofascin-155 and other components of the axo-glial junctions at paranodes, including Contactin-1 and Caspr1 were found, although at a low frequency, in subgroups of patients with chronic inflammatory demyelinating polyneuropathy, the most common inflammatory disorders affecting the PNS. Of note these antibodies are of predominant IgG4 isotype and therefore their pathogenicity, if any, is independent from complement activation and Fc receptor-mediated inflammation. Future studies should aim at better understanding the pathogenic mechanisms underlying the antibody-mediated damage of the nerve and the node of Ranvier in particular. Alternatively, these antibodies could be a simply a biomarker of an autoimmune, and possibly T-cell mediated, disorder affecting the peripheral nerves. This should also be addressed by future works.

In fact, the impact of CIDP misdiagnosis in terms of cost (and risk) of unnecessary IVIg treatment is enormous and, despite their low prevalence, the identification of these novel antibodies as reliable hallmark of the immune-mediated process of CIDP is highly valuable. In fact, the diagnosis of this inflammatory neuropathy is challenging and even in highly specialized centres half of the patients diagnosed as having this condition are misdiagnosed and their neuropathy could be better explained by a different cause(Allen and Lewis 2015). In particular, hereditary amyloidosis due to mutation in transthyretin gene (ATTR amyloidosis) can show similar clinical and neurophysiological features to CIDP and we observed that up to 20% of a large series of patients with ATTR amyloidosis were previously given a diagnosis of CIDP, with significant delay in appropriate treatment onset. Although recent evidences point to a possible role of the immune response in influencing the course of this inherited degenerative disease (Kurian et al. 2016), patients with ATTR amyloidosis fail to respond to immune-active treatments, while different therapies targeting the production or the stability of the mutated protein are now available and appear to be particularly effective in early disease stages, hence the need for a timely diagnosis of this otherwise progressively degenerative condition.

In recent years great progresses have been made in the management of acquired demyelinating disorders of the nervous system and we now have a wide array of immune-active treatments to tackle the inflammatory component of these diseases. However, treatment options are much less limited when patients enter a progressive phase of the disease, and current effort are aimed at understanding the underlying processes linking inflammatrion to neurodegeneration and relentless axonal loss. To this regard, inclusion body myositis represent a unique benchmark to study this interaction as inflammatory and degenerative changes are the pathologic hallmark of the disease since the earliest disease stages. We demonstrated that impaired RNA metabolism due to loss of TDP43 function is also part of the molecular signature of the disease and could contribute to the decreased survival of skeletal muscle fibers in sIBM, as well as in other myopathies with TDP43

protein aggregates. Of interest, the altered processing of RNA due to mutation in genes involved in RNA metabolism is known to underlie the pathogenesis of Aicardi-Goutières Syndrome (Rice et al. 2007), an inherited auto-inflammatory and degenerative disorder. It is tempting to speculate that impaired RNA metabolism in sIBM could sustain the inflammatory response against muscle fibers, which is refractory to immunotherapies and immune suppressive treatments, and future work would need to confirm or reject this hypothesis.

10. APPENDIX 1

MONITORING EFFECTIVENESS AND SAFETY OF TAFAMIDIS IN TRANSTHYRETIN AMYLOIDOSIS IN ITALY: *A LONGITUDINAL MULTICENTER STUDY IN A NON-ENDEMIC AREA*

INTRODUCTION

Inherited transthyretin amyloidosis (ATTR) is an autosomal dominant disorder due to mutations of the transthyretin (*TTR*) gene. TTR is synthetized mainly by the liver and released in plasma as a tetrameric transport protein. Mutations in *TTR*, of which Val30Met (p.Val50Met) is the most common, cause transthyretin tetramer dissociation, monomer misfolding, and aggregation into insoluble fibrillar proteins in different tissues. Peripheral nerves and heart are the most frequently affected organs, but also eye, leptomeninges and kidneys can be involved (Violaine Planté-Bordeneuve and Said 2011b; Haruki Koike et al. 2012).

Orthotopic liver transplantation (OLT), by removing the main site of mutated TTR production, proved able to halt or slow neurological progression and is, at present, the standard-of-care treatment in patients aged <50 with Val30Met mutation. However, mortality rate following OLT is not negligible (around 10%) (Herlenius et al. 2004; Okamoto et al. 2009). Moreover, OLT is often not curative of ATTR, since cardiac disease tends to progress after OLT, possibly due to continued accumulation of wild type TTR(Suhr, Friman, and Ericzon 2005; Yamashita et al. 2012; D. Adams et al. 2000). Altogether, these observations warranted the search for new treatment options in ATTR.

Tafamidis meglumine (Vyndaqel®) is a small molecule, which kinetically stabilizes the TTR tetramer and prevents its dissociation into amyloidogenic monomers. It has been approved by EMA for the treatment of transthyretin amyloidosis in adult patients with stage 1 symptomatic polyneuropathy to delay peripheral neurologic impairment.

There have been a few encouraging studies on safety and long-term efficacy of Tafamidis in early-onset Val30Met ATTR patients (Coelho et al. 2012b; Coelho et al. 2013; Lozeron et al. 2013)]. However, less is known about its efficacy in later stages of the disease and in non-Val30Met mutations, which represent a significant proportion of ATTR genotype spectrum in Italy, as well as in other countries where ATTR is non-endemic (Merlini et al. 2013; Mariani et al. 2015)]. Moreover, diverse studies reported different rate of response to Tafamidis treatment, possibly based on genetic and demographic heterogeneity of treated patients [8–10]. In fact, prognostic factors affecting the response to treatment are still largely unknown.

We therefore designed a protocol to evaluate patients who have just been prescribed Tafamidis meglumine (and who were going to start treatment) in Italy. Study entry occurred after the decision to prescribe the drug but before its administration. *Sinsu strictu* this is a longitudinal observational study on a series of patients on treatment with Tafamidis. Aims of this multicentre study were: 1) to prospectively collect data about safety and exploratory efficacy on nerve and heart function of Tafamidis in ATTR patients, including Val30Met and non-Val30Met mutations, in early and late stages of the disease (as it was possible to prescribe Tafamidis according to Italian regulation in patients in different disease stages at the beginning of this study); 2) to identify factors associated with response to treatment and better preservation of neurological function; 3) to test the usefulness of a reproducible, easy-to-administer clinical scale: the Charcot-Marie-Tooth (CMT) Neuropathy Score (CMTNS), to monitor disease progression in ATTR.

METHODS

Patients and study protocol

Patients were enrolled between October 2011 and December 2014 in eight Centres across Italy. Inclusion criteria were: 1) Women and men aged ≥18 affected by symptomatic ATTR-related neuropathy, defined by the presence of a pathogenic *TTR* mutation and symptomatic sensory, sensory-motor and/or autonomic peripheral neuropathy; 2) Patients starting treatment with

Tafamidis meglumine (20 mg once daily); 3) Written informed consent to participate in the study. Exclusion criteria were: 1) previous orthotopic liver transplantation; 2) pregnancy or breastfeeding; 3) other current anti-amyloidogenic treatment such as Diflunisal, doxycycline, tauroursodeoxycholic acid.

Patients were assessed at treatment start and every six months thereafter up to three years according to a standardized protocol which included the following assessments: Neuropathy Impairment Score (NIS) and NIS-Lower Limb subscale (NIS-LL)(Dyck et al. 1997; Coelho et al. 2012b)]; Kumamoto Scale (Tashima et al. 1997) CMTNS and its clinical component CMT Examination Score (CMTES), version 2 (Murphy et al. 2011)]; Familial amyloid polyneuropathy (FAP) Stage ("Coutinho P, Martins Da Silva A, Lopes Lima J, Resende Barbosa A. Forty Years of Experience with Type I Amyloid Neuropathy. Review of 483 cases.In: Glenner GG, Pinho E Costa P, Falcao de Freitas A, Editors. Amyloid and Amyloidosis. Amsterdam: Excerpta Medica; 1980. P. 88–98.," n.d.)] and polyneuropathy disability (PND) score (Ando et al. 2013); Modified Body Mass Index (mBMI) as defined by BMI (Kg/m^2) multiplied by serum albumin (g/L), in order to compensate for possible oedema (Suhr et al. 2014)]; blood routine testing including N-terminal pro-hormone brain natriuretic peptide (NT-proBNP) (Lehrke et al. 2009); echocardiography with measurement of inter-ventricular septum (IVS) thickness and electrocardiogram. Adverse Events (AEs) were monitored throughout the study. Ethics committees of participating centres approved the study.

Statistical analyses

Changes from baseline and from follow-up visits were compared with paired t-test or Wilcoxon signed-rank test. Pearson's correlation coefficient was used to test for correlation between scales. Their responsiveness was assessed by calculating the standardized response mean (SRM) as the mean baseline-to-12 months point change in score divided by the standard deviation (SD) of the individual's score change. The SRM threshold levels were defined as follows: ≥0.20 small, ≥0.50 moderate, and ≥0.80 good (Cohen J., n.d.)]. Responders were defined as patients with a less than

two-point increase in NIS-LL during treatment because it is the least degree of change a physician can recognize and because it was one of the co-primary end-points in the Tafamidis registration trial ("Diabetic Polyneuropathy in Controlled Clinical Trials: Consensus Report of the Peripheral Nerve Society" 1995; Coelho et al. 2012b)]Univariate logistic regression was performed with response to treatment at 12 months as outcome variable and stage of FAP, NIS, NIS-LL, mBMI >960 (corresponding to the median mBMI in our study cohort), presence of cardiac involvement, presence of dysautonomia, type of mutation (Val30Met vs other mutations), age, age at onset, disease duration and gender as independent variables. Variables significantly associated with the response to treatment at univariate analysis were tested in an age- and gender-adjusted multivariate logistic regression model.

RESULTS

We enrolled 61 patients. One patient with alcohol-related liver cirrhosis had developed ATTR-related neuropathy following domino liver transplantation received 14 years before enrolment from a donor with Val30Met *TTR* mutation. Baseline characteristics of study population are summarized in **Table 1**.

Table 1. Patients' demographics and baseline data

		N=61
Age (years)		62 ± 11 (31-81)
Age at onset (years)		59 ± 11 (30-79)
Disease duration (years)		3.4 ± 2.1 (0.6-10)
Male gender		42 (69%)
***TTR* genotype**		
Val30Met		17 (28%)
Phe64Leu		16 (26%)
Glu89Gln		14 (23%)
Thr49Ala		5 (8%)
Tyr78Phe		3 (5%)
Ala120Ser		2 (3%)
Glu54Gln		1 (2%)
Ile68Leu		1 (2%)
Ser77Tyr		1 (2%)
Ala45Thr		1 (2%)
Stage at enrolment		
Stage 1: walk unaided		44 (72%)
-sensory disturbances only (PND I)		26 (43%)
-sensory disturbances and motor weakness (PND II)		18 (29%)
Stage 2: use of a cane/walker		12 (20%)
- walking only with the help of one stick or crutch (PND IIIA)		9 (15%)
- walking with the help of two sticks or crutches (PND IIIB)		3 (5%)
Stage 3: wheelchair-reliant (PND IV)		5 (8%)
	Mean ± SD	**Median (min-max)**
NIS (0-244)	53 ± 38	44 (0-138.5)
NIS-LL (0-88)	28 ± 20	23 (0-74)
CMTES (0-28)	12 ± 7	11 (0-26)
CMTNS (0-36)	16 ± 9	15.5 (1-33)
Kumamoto Score (0-96)	20 ± 12	20 (2-53)
IVS (mm)	13.6 ± 3	13 (7-24)
NT-pro-BNP (pg/mL)	1047 ± 1576	260 (5-7136)
mBMI	978 ± 195	959 (568-1445)

Table 1 (continued): *Patients' characteristics at baseline are reported as number and percentage for categorical variable. Continuous variables are reported as mean±standard deviation or median (min, max), as appropriate. CMTES: Charcot-Marie-Tooth Examination Score; CMTNS: Charcot-Marie-Tooth Neuropathy Score; IVS: inter-ventricular septum; mBMI: modified Body Mass Index; NIS: Neuropathy Impairment Scale; NIS-LL: NIS-Lower Limb. PND: Polyneuropathy Disability Score*

The majority were males, with remarkably late mean age of onset and mean disease duration of 3.4 years. Notably, 44 (72%) subjects had TTR mutations other than Val30Met. A summary of baseline features of patients carrying one of the 3 most common mutations (Val30Met, Glu89Gln and Phe64Leu) is provided in **Table 2**. Forty-four patients (72%) were in stage 1 at study entry, whereas seventeen had already lost ambulatory independency, of whom 12 (20%) were in stage 2 and 5 (8%) in stage 3, and Tafamidis was prescribed according to the contemporary national regulation. There was a good correlation between PND score and NIS at enrolment (r=0.83), as also reported in a recent multinational study (David Adams et al. 2015)]. Forty-two (69%) patients had dysautonomia at enrolment, including 29 patients (48%) with diarrhoea, 37 patients (61%) with orthostatic hypotension, 18 patients (30%) with urinary retention, five patients with dry eye (8%) and 15 patients with dry mouth (25%). Out of 57 patients with available echocardiography at study entry, 34 (60%) had definite cardiac involvement with IVS > 12 mm. NT-proBNP was altered in 22/53 (42%) patients. ECG was abnormal in 27 (44%) mainly due to low limb voltages and conduction abnormalities. Of the 61 enrolled patients with baseline visit, 53 were evaluated also after 6 months (M6), 37 up to 12 months (M12), 34 up to 18 months (M18), 19 up to 24 months (M24), 12 up to 30 months (M30) and seven up to 36 months (M36).

Seven patients (11%) discontinued treatment: one patient underwent OLT, one moved to a different country where Tafamidis was not licensed, the subject who received domino-liver transplant lost eligibility to Tafamidis treatment following changes of the national regulation for Tafamidis prescription, another patient with Phe64Leu mutation died of cardiac failure, and three patients

discontinued because of disease progression (two patients showed cardiac disease progression at M12 and M24, respectively, and one patient discontinued because of neuropathy progression at M18).

Changes in neurological function

Both NIS and NIS-LL scores worsened as a mean during the follow-up period **(Table 2).**

Table 2. Change of neurological function after 12 and 18 months of Tafamidis treatment

	Change after 12 months of treatment (N=37)	**Change after 18 months of treatment (N=34)**
NIS	10.3 ± 17	15.6 ± 25.7
NIS-LL	5.9 ± 9.3	8 ± 13.7
CMTES	2.1 ± 3	3.4 ± 3.9
CMTNS	2.3 ± 3.8	NA
Kumamoto Scale	5.4 ± 6.1	7.3 ± 6.4

CMTES: Charcot-Marie-Tooth Examination Score; CMTNS: Charcot-Marie-Tooth Neuropathy Score; NIS: Neuropathy Impairment Scale; NIS-LL: NIS-Lower Limb. NA: not applicable

In 34 patients treated for at least 18 months, NIS increased by 15.6 points (**Figure 1A**). In patients treated for longer periods the neurological function continued to deteriorate in the subsequent months (**Figure 1B**). However, when examining the 6-month interval deterioration rate, we observed that NIS increase significantly declined from 8.3 ± 10.3 during the M0-M6 interval to 1.4 ± 12 in the subsequent M6-M12 interval (p=0.025), and remained relatively stable at 18 months (**Figure 1C**). Similar considerations apply to changes of NIS-LL (**Figure 1E**). Overall, one-third of patients showed stability of the neuropathy, as defined by NIS-LL increase < 2 points from baseline, across the whole duration of the study (**Figure 1D**). The percentage of responders after 12

months of treatment tended to be higher in patients in very early disease stage, whose neurological impairment was limited to sensory disturbances, compared with patients showing motor weakness and altered walking capability, 44% vs 29% respectively, although the difference was not significant (p=0.3). Patients with non-Val30Met mutations showed changes of neurological function similar to Val30Met patients and responders were distributed in similar rates across both Val30Met and other mutations.

The mean Kumamoto score, which evaluates impairment related to sensory-motor and autonomic neuropathy as well as cardiac conduction defects, steadily worsened throughout the duration of the study **(Figure 1F)**.

Regarding the 39 patients with evidence of dysautonomia at baseline, as assessed with the Kumamoto scale, and followed-up for at least 6 months, autonomic function remained stable in 13 (33%), worsened in 22 (56%), and improved in four (10%), including improvement of urinary retention (two cases), orthostatic hypotension (one case), eye and mouth dryness (two cases). Out of 15 patients without autonomic dysfunction at baseline, six later developed dysautonomia (orthostatic hypotension in four cases and diarrhoea in two cases. Two of these patients developed additional symptoms, namely dry mouth and urinary incontinence).

CMTES also showed a steady mean increase ranging from 1.7 to 2.6 per year (**Figure 1G**).

During the follow-up period, overall nine patients had progressed to a higher disability grade in FAP stage (seven patients progressed from stage 1 to 2, and three patients progressed from stage 2 to 3), although in six of them the progression occurred in the first six months, while only two patients progressed at M12 and one patient at M18. Seven patients with stable FAP disease stage progressed in terms of PND score, namely five patients with sensory disturbances only at baseline developed motor impairment and two patients with unilateral aid at walking at baseline required bilateral support later. Details of disease course in single patients are reported in **table 4.**

There was good correlation between NIS, CMTES and Kumamoto scores. Kumamoto Scale showed the highest responsiveness to change (SRM>0.8), followed by CMTES and NIS-LL, in both ambulatory independent patients and patients requiring walking aids (**Table 3**).

Table 3. Sensitivity to change of neurological function after 12 months of treatment expressed as SRM, standardized response mean.

	Stage 1	**Stage 2 & 3**
NIS	0.56	0.67
NIS-LL	0.61	0.66
CMTES	0.7	0.75
Kumamoto Scale	0.83	0.89

CMTES: Charcot-Marie-Tooth Examination Score; CMTNS: Charcot-Marie-Tooth Neuropathy Score; NIS-LL: Neuropathy Impairment Score-Lower Limb

Figure 1 (continued). Changes of neurological function during Tafamidis treatment.

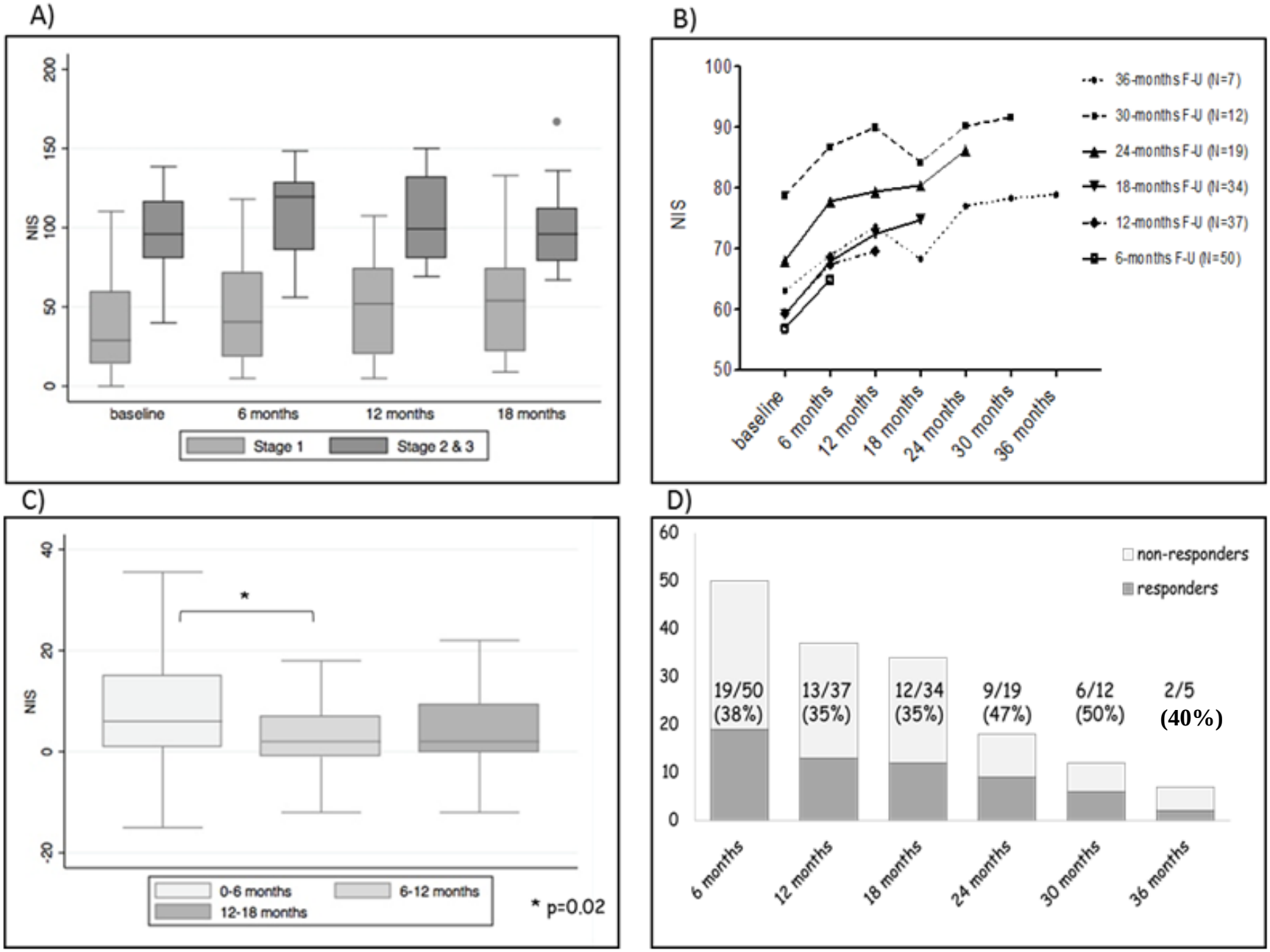


Figure 1 (continued)

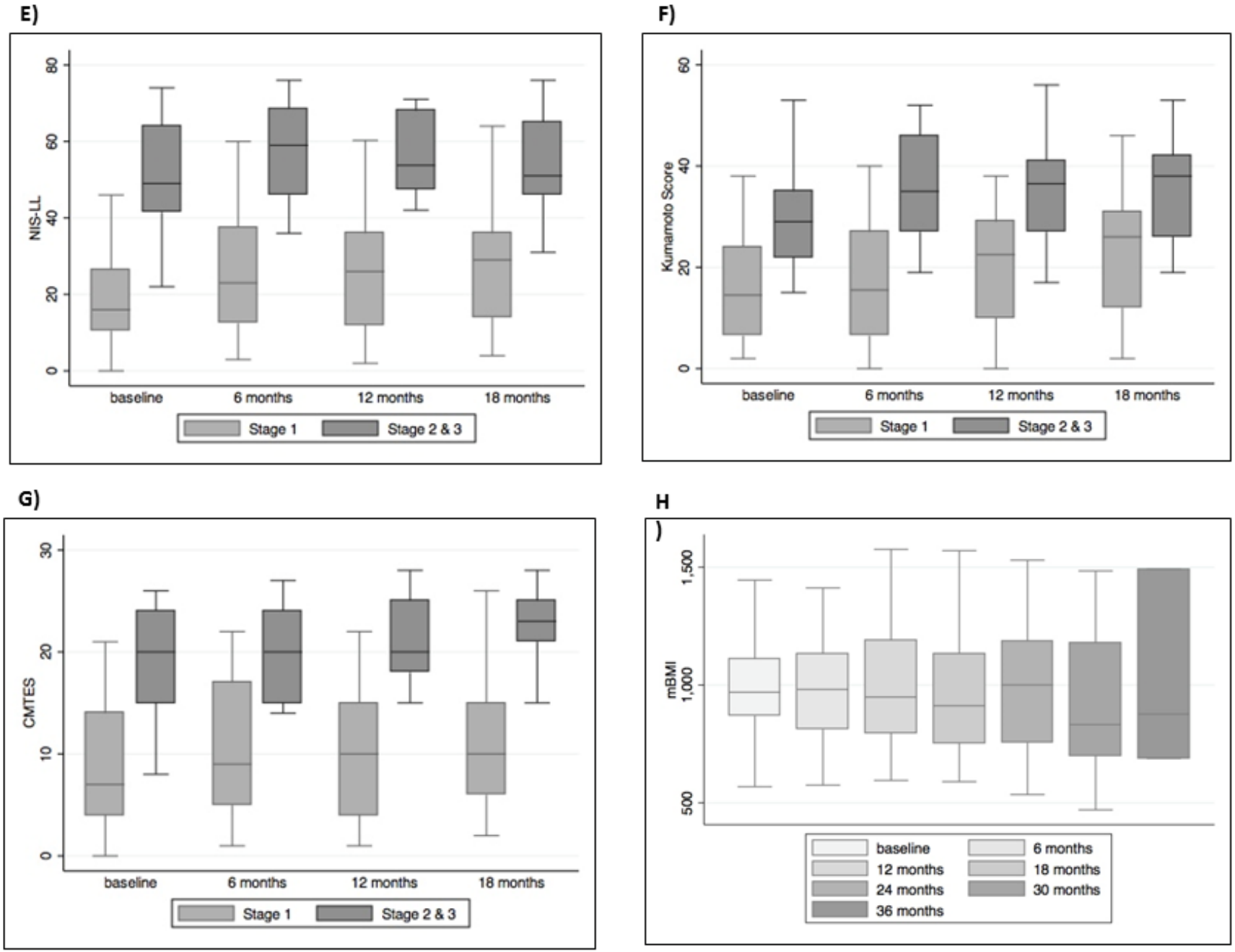


Figure 1 (continued). (A) *Change from baseline of NIS over 18 months of treatment in early (stage 1, N=21) and late (stage 2&3, N=13) stages of the disease.* ***(B)*** *Progression of neurological impairment in patients followed up over 36 months of treatment (note that patients with longer follow up had more severe disease due to inclusion of patients in stage 2-3 in the early phases of the study). (C) Six-month change of NIS over 18 months of treatment (N=35).* ***(D)*** *Percentage of responders (change of NIS from baseline NIS-LL<2 points) over 36 months of treatment.* *Change from baseline of NIS-LL* ***(E),*** *Kumamoto Score* ***(F)*** *and CMTES* ***(G)*** *over 18 months of treatment in early (stage 1, N=21) and late (stage 2&3, N=13) stages of the disease.* ***(H)*** *Preservation of mBMI over 36 months of treatment.* *CMTES: Charcot-Marie-Tooth Examination Score, mBMI: modified Body Mass Index, NIS-LL: Neuropathy Impairment Score-Lower Limb. F-U: follow-up.*

Changes in cardiac function

In the 34 patient with cardiac involvement at baseline, mean IVS increased from baseline by 0.6 ± 1.6 mm at M12 and 1.05 ± 2.0 mm at M24. Five/34 (15%) patients showed echocardiographic evidence of cardiac disease progression (1 at M6, 2 at M12, 1 at M18 and 1 at M30). In three of them increase in IVS was paralleled by consistent increase of NT-proBNP by ≥30% from baseline. Eight (35%) of 23 patients without cardiac involvement at baseline later developed cardiomyopathy, as defined by IVS>12 mm (see supplementary methods), (four at M6, two at M12, one at M18, one at M30) and three showed a concomitant increase of NT-proBNP by≥30%. Overall, 12 patients progressed to a higher NYHA heart failure class (one at M6, four at M12, five at M18, one at M24 and one at M30). One patient required pace-maker implantation after 18 months of treatment.

mBMI

Remarkably, mBMI did not change during the 36 months of observation while on treatment **(Figure 1H**).

Table 4

ID #	TTR Mutation	Gender	Age of onset	PND baseline	PND last follow-up	NYHA baseline	NYHA at last follow-up	PND progression	NYHA progression	PND or NYHA progression	Echo cardio progression	PND or Echo cardio progression
1	Ala120Ser	F	63	2	4	1	1	progressed	stable	progressed	developed ACM	progressed
2	Ala120Ser	F	59	1	NA	1	NA	NA	NA	NA	NA	NA
3	Ala45Thr	M	56	2	2	2	2	stable	stable	stable	stable	stable
4	Glu54Gln	F	48	1	2	2	2	progressed	stable	progressed	stable	progressed
5	Glu89Gln	M	45	1	1	1	1	Stable	stable	stable	stable	stable
6	Glu89Gln	F	52	2	3	2	2	progressed	stable	progressed	stable	progressed
7	Glu89Gln	F	62	1	NA	3	NA	NA	NA	NA	NA	NA
8	Glu89Gln	F	55	2	NA	2	NA	NA	NA	NA	NA	NA
9	Glu89Gln	F	52	5	5	0	3	stable	progressed	progressed	progressed	stable
10	Glu89Gln	M	45	1	2	1	2	progressed	progressed	progressed	progressed	progressed
11	Glu89Gln	F	52	1	1	1	1	stable	stable	stable	stable	stable
12	Glu89Gln	M	51	5	5	2	3	stable	progressed	progressed	progressed	progressed
13	Glu89Gln	F	43	1	2	2	3	progressed	progressed	progressed	stable	progressed
14	Glu89Gln	M	54	3	3	1	2	stable	progressed	progressed	developed ACM	progressed
15	Glu89Gln	M	51	5	5	3	4	stable	progressed	progressed	progressed	progressed
16	Glu89Gln	M	53	1	1	2	2	stable	stable	stable	stable	stable
17	Glu89Gln	M	53	1	1	1	1	stable	stable	stable	stable	stable
18	Glu89Gln	M	42	1	1	1	1	stable	stable	stable	stable	stable
19	Ile68Leu	M	61	1	1	3	3	stable	stable	stable	stable	stable
20	Phe64Leu	M	68	2	2	1	1	stable	stable	stable	stable	stable
21	Phe64Leu	M	61	2	2	1	1	stable	stable	stable	stable	stable
22	Phe64Leu	M	57	2	NA	1	NA	NA	NA	NA	NA	NA
23	Phe64Leu	M	68	2	2	1	1	stable	stable	stable	stable	stable
24	Phe64Leu	M	67	2	2	1	1	stable	stable	stable	stable	stable
25	Phe64Leu	M	71	5	5	0	0	stable	stable	stable	stable	stable

26	Phe64Leu	M	72	2/3	4/5	0	3	progressed	progressed	progressed	developed ACM	progressed
27	Phe64Leu	M	63	5	5	1	2	stable	progressed	progressed	progressed	progressed
28	Phe64Leu	M	75	2	3	1	1	progressed	stable	progressed	developed ACM	progressed
29	Phe64Leu	F	65	3	3	2	3	stable	progressed	progressed	stable	stable
30	Phe64Leu	M	60	4	5	3	4	progressed	progressed	progressed	stable	progressed
31	Phe64Leu	M	68	1	2	1	1	progressed	stable	progressed	stable	stable
32	Phe64Leu	M	58	4	5	2	1	progressed	improved	progressed/improved	stable	progressed
33	Phe64Leu	F	72	4	4	1	1	stable	stable	stable	NE	stable
34	Phe64Leu	M	68	1	1	2	2	stable	stable	stable	stable	stable
35	Phe64Leu	M	72	3	NA	1	NA	NA	NA	NA	NA	NA
36	Ser77tyr	F	67	1	1	2	2	stable	stable	stable	stable	stable
37	Thr49Ala	M	36	1	1	1	1	stable	stable	stable	stable	stable
38	Thr49Ala	F	43	1	1	1	1	stable	stable	stable	stable	stable
39	Thr49Ala	F	30	1	1	1	1	stable	stable	stable	stable	stable
40	Thr49Ala	F	46	2	2	1	2	stable	progressed	progressed	stable	stable
41	Thr49Ala	M	55	1	1	0	0	stable	stable	stable	stable	stable
42	Tyr78Phe	M	59	2	2	0	0	stable	stable	stable	developed ACM	progressed
43	Tyr78Phe	M	67	1	NA	1	NA	NA	NA	NA	NA	NA
44	Tyr78Phe	M	67	3	4	1	1	progressed	stable	progressed	developed ACM	progressed
45	Val30Met	M	64	2	3	3	3	progressed	stable	progressed	stable	progressed
46	Val30Met	M	68	2	2	2	2	stable	stable	stable	stable	stable
47	Val30Met	M	43	1	NA	1	NA	NA	NA	NA	NA	NA
48	Val30Met	M	68	3	3	2	2	stable	stable	stable	stable	stable
49	Val30Met	M	72	1	1	2	2	stable	stable	stable	stable	stable
50	Val30Met	F	65	3	3	1	1	stable	stable	stable	stable	stable
51	Val30Met	F	67	1	1	1	1	stable	stable	stable	stable	stable
52	Val30Met	M	61	2	3	1	1	progressed	stable	progressed	stable	progressed
53	Val30Met	M	42	1	1	1	1	stable	stable	stable	stable	stable

54	Val30Met	M	67	3	4	2	3	progressed	progressed	progressed	NE	progressed
55	Val30Met	M	68	3	3	1	1	stable	stable	stable	NE	stable
56	Val30Met	F	79	2	3	1	1	progressed	stable	progressed	stable	progressed
57	Val30Met	F	73	3	3	1	1	stable	stable	stable	stable	stable
58	Val30Met	M	57	1	1	1	1	stable	stable	stable	developed ACM	progressed
59	Val30Met	M	58	2	NA	1	NA	NA	NA	NA	NA	NA
60	Val30Met	M	72	1	2	1	1	progressed	stable	progressed	stable	progressed
61	Val30Met	M	66	1	1	1	1	stable	stable	stable	developed ACM	progressed

NA not available. ACM amyloid cardiomyopathy, PND polyneuropathy Disability Score, NYHA New York Health Association Score

Adverse events

AEs were reported by eight (13%) patients: two had urinary tract infections, one diarrhoea, one gastroenteritis, and one angular stomatitis. Three patients had serious AEs: one patient died of cardiac failure and two had rapid cardiac function worsening, consistent with disease-associated cardiac morbidity and mortality and unlikely to be related to treatment, which led to Tafamidis discontinuation in one of them. No patient discontinued because of treatment-related AE.

Predictors of response to treatment

A higher mBMI at baseline, but not disease stage, NIS, NIS-LL, presence of cardiac involvement, presence of dysautonomia, mutation type, onset age, disease duration, age or gender, was significantly associated (p=0.02) with response to treatment after 1 year. In a multivariable age- and gender-adjusted logistic regression model, mBMI >960 was associated with a 7-fold higher probability of stability of NIS-LL after 12 months of treatment compared with subjects with lower mBMI [OR 6.8, p= 0.02, CI 1.3-34.7].

DISCUSSION

To date, one double-blind placebo-controlled study (Coelho et al. 2012b)] and three single-arm interventional or observational studies [8–10] provided information about safety and efficacy of Tafamidis for ATTR **(table 5).**

In the first 18-month trial, Tafamidis slowed neurologic impairment progression in Val30Met patients, although the primary endpoints were not reached. Overall, 45% of treated patients had stable NIS-LL after 18 months. Nutritional status significantly improved in Tafamidis-treated subjects, but worsened in placebo-controls (Coelho et al. 2012b)].

Table 5. Comparison with previous Tafamidis studies for patients' mutation type and NIS-LL progression rate

	Tafamidis				**Placebo**
	Present study	**Coelho et al**(Coelho et al. 2012b)	**Lozeron et al**(Lozeron et al. 2013)	**Merlini et al**(Merlini et al. 2013)	**Coelho et al**(Coelho et al. 2012b)
N	61	64	37	21	64
Type of study	observational	RCT	Open-label	Open-label	RCT
Non-Val30Met	72%	0%	0%	100%	0%
Age at onset (years)	59 ± 11	39.8 ± 12.7	62.8 (16)	63.1 ± 9.9	38.4 ± 12.9
Disease duration (months)	40.8 ± 25.2	47.0 ± 48.40	48.4 (30.4)	64.7 ± 60.8	34.7 ± 32.88
NIS-LL					
baseline	28 ± 5	8.4 ± 11.40	27.6 ± 17.2	27.6 ± 24.7	11.4 ± 13.5
month 6	+4.5 ± 6.3; 62%	NA	+4.8 ± 3.1	NA	NA
month 12	+5.9 ± 9.3; 65%	NA	+6.6	+2.5 (-1.2, 6.2)	NA
month 18	+8.0 ± 13.7; 65%	+2.81; 54.7%	NA	NA	+5.83; 71.5 %

Continuous data are reported as mean ± standard deviation or median (95% confidence interval); when information is available, patients showing neurologic progression (NIS-LL≥2) are reported in %. NA: not available. NIS-LL: Neuropathy Impairment Score-Lower Limb. RCT: randomized controlled trial.

The beneficial treatment effect was maintained in the following 12-month open-label extension study, and no significant concern about its safety ensued. NIS-LL steadily increased by 0.96-1.32 points/year, with higher benefit when treatment started early (Coelho et al. 2013)].

However, a much lower responder rate, with only two out of 37 patients (7%) showing stable NIS-LL after 12 months and 55% of patients progressing to a higher disability stage, was observed in a single centre open-label study in Val30Met patients with more advanced neuropathy (Lozeron et al. 2013)].

Tafamidis effectively stabilized TTR tetramers in plasma from patients with both Val30Met and non-Val30Met mutations (Merlini et al. 2013). Nonetheless, after 12 months of treatment, benefit on neurological function in non-Val30Met subjects (Merlini et al. 2013)] was less marked than in Val30Met patients (Coelho et al. 2012b)].

Our study provides long-term follow-up data in a large cohort of patients with ATTR from a non-endemic area, including subjects with more advanced disease and non-Val30Met mutations.

Since 2010 and until drug registration, Tafamidis was available in Italy under a special accession program for the treatment of ATTR with symptomatic polyneuropathy, whatever the disease stage and mutation type, and patients started treatment, making the current study possible. The distribution of mutations in recruited patients, with 72% of non-Val30Met mutation, of which Phe64Leu and Glu89Gln were the most common, reflects the high genetic heterogeneity of ATTR in Italy (Mazzeo, Russo, and Di Bella in press; Russo et al. 2012; Cappellari et al. 2011; Luigetti et al. 2013; Rapezzi et al. 2013)]. Of note, patients with Glu89Gln mutation had earlier onset of the disease and more prominent heart involvement, while patients with Phe64Leu mutation showed more aggressive neuropathy. Such data are in keeping with recent observation in a partly overlapping cohort of patients with ATTR from endemic regions in Sicily, thus confirming earlier heart dysfunction in Glu89Gln mutation and more severe peripheral neuropathy in Phe64Leu mutation (Mazzeo, Russo, and Di Bella in press)].

Tafamidis proved safe and well-tolerated over 36 months and no patient had treatment-related serious AEs. In keeping with previous reports (Suhr et al. 2014)], we did not observe significant changes of mBMI over 3 years of treatment. More than one third of patients showed no meaningful NIS-LL increase along 36 months of treatment, independently from mutation type. Notably, both patients in early and late disease stages responded to treatment, although patients in very early stage without motor impairment (PND1) tended to have a higher responder rate.

This observation confirms previous understanding of a higher efficacy of anti-amyloidogenic treatments if given early in disease course suggesting that treatment should be started as soon as possible (Merlini et al. 2013)]. Moreover, our study suggests a possible benefit of Tafamidis also in ATTR patients with more advances disease.

Notably, the worsening of neurologic function, according to NIS, NIS-LL and PND, occurred mainly in the first 6 months of treatment and became subsequently less prominent. Such observation, in keeping with previous experience by Lozeron *et al*. (Lozeron et al. 2013)] would encourage continuing Tafamidis administration for at least 1 year. In fact, although tetramer stabilization if often reached by 6 weeks of treatment (Merlini et al. 2013)], we observed that it can take up to 12 months before clinically significant changes are detected, thus making inappropriate an earlier discontinuation based on apparent inefficacy.

Overall, Tafamidis was not able to prevent functional progression of the disease in 23 (43%) subjects, including 16 patients who worsened in their walking ability and 12 patients who reached a higher NYHA score during the follow-up period. Two-thirds of subjects increased their neurological impairment according to NIS-LL, 17% reached a higher disability FAP stage and in 30% the PND score worsened. Similarly, one-third of patients without heart involvement developed amyloid-related cardiac disease, septal thickness significantly increased in 15% of patients with cardiomyopathy at baseline, and 22% of patients reached a higher NYHA score. Mean NIS-LL increase after 18 months was 3-fold higher in comparison with the progression rate reported by Coelho *et al.*(Coelho et al. 2012b)] **(table 5)**.

The discrepancy between the two studies reflects fundamental differences in the characteristics of the patient cohorts, with a majority of early-onset slowly-progressive Val30Met patients from endemic regions for ATTR in the Tafamidis registration trial (Coelho et al. 2012b)] compared to a large proportion of late-onset and non-Val30Met patients with faster progression in our study. In fact, both later age of onset and presence of non-Val30Met mutation are known negative prognostic factors associated with a more aggressive disease course (Rapezzi et al. 2013; Misu et al. 1999b; H. Koike et al. 2004; Haruki Koike et al. 2012)].

Neurologic deterioration in non-responders could be due to limited tetramer stabilization, suggesting a role for tetramer stabilization assay in non-responding patients who may possibly benefit from higher doses of the drug. Other pathogenic mechanisms, including continued deposition of transthyretin and toxicity of amyloid precursors, template-effect of deposited amyloid fibrils by exposure of aggregation-prone regions, impaired amyloid clearance, and sustained activation of endoplasmic reticulum stress may also be involved in disease progression (H. Koike et al. 2004; Sousa et al. 2001)]. Remarkably, also in Amyloid Light-chain amyloidosis nerve damage can progress despite complete hematologic response to chemotherapy and sustained M-protein reduction (Rajkumar, Gertz, and Kyle 1998).

Efficacy of Tafamidis on dysautonomia was also partial with 26/54 patients (48%) showing normal or stable autonomic function at last follow up.

Overall, such observations confirm that OLT, which proved to be able to stop neurologic progression in 76% of stage 1 patients with Val30Met mutation, is still an important option in the treatment of selected cases of ATTR [(Suhr, Friman, and Ericzon 2005; Ericzon et al. 2015)]. Moreover, it highlights the need of further research on treatments for FAP, including RNA interference-based therapies (anti-sense oligonucleotides and small interfering RNAs).

We next investigated which factors could predict a better response to treatment. We tested whether NIS-LL change after 12 months of treatment was influenced by mutation type, onset age, symptoms duration, neurological, cardiologic and nutritional status at baseline. In our study, the only factor

able to predict treatment response and preservation of neurologic function was a higher mBMI at treatment onset. The odds of having a stable NIS-LL after 1 year of treatment was 7-fold higher in patients with mBMI >960 as compared to patients with lower mBMI.

There is no obvious explanation for this observation. Weight loss is common in ATTR although its ultimate cause is unknown. A low BMI was associated with reduced survival in patients with ATTR following liver transplant (D. Adams et al. 2000; Ericzon et al. 2015)]. The effect of a reduced mBMI on neuropathy progression was not previously reported. It is possible that malabsorption plays a role, i.e. by negatively affecting Tafamidis bioavailability. Also, a detrimental effect of poor nutritional status on unfolded protein response mediated cellular stress may be hypothesized (Palorini et al. 2013)].

Concerning the different employed scales, CMTES and CMTNS showed good correlation with NIS and Kumamoto scale, and CMTES and Kumamoto scale proved to be more responsive to changes of neurological function after 1 year, which may warrant its consideration in future trials in ATTR.

The main limitation of our observational study is that we have no control population for direct comparison which, together with the contemporary unavailability of data about pre-treatment progression rate in treated patients, limits the evaluation of treatment efficacy.

In conclusion, neuropathy and cardiomyopathy progressed in a significant proportion of patients with ATTR despite treatment. However, after the first 6 months of treatment the worsening of neurological function slowed across all stages for the entire study duration. This observation, together with the experience of patients with non-Val30Met mutation and high disability who remained neurologically stable even at long-term follow-up, entail that also subjects with more advanced neuropathy, as well as patients with non-Val30Met mutation, may benefit from Tafamidis treatment. Body weight preservation is an important favourable prognostic factor.

11. APPENDIX 2

ALTERED TDP43-DEPENDENT SPLICING IN *HSPB8*-RELATED DISTAL HEREDITARY MOTOR NEUROPATHY AND MYOFIBRILLAR MYOPATHY

INTRODUCTION

Mutations in the small heat shock protein 22 gene (*HSPB8*) are associated with Charcot-Marie-Tooth type 2L (CMT2L) and distal hereditary motor neuronopathy type IIa (dHMN2A) (Irobi et al. 2004; Tang et al. 2005).

More recently, heterozygous *HSPB8* mutations (p.K141E and p.P173SfsX43) have been reported in two unrelated families with distal myopathy and motor neuropathy (Ghaoui et al. 2016). In these cases, muscle biopsy showed alterations consistent with myofibrillar myopathy (MFM) along with protein aggregates and TDP43 inclusions.

Here, we report a third family with autosomal dominant dHMN and MFM/protein aggregate myopathy (PAM). Using muscle biopsy tissue from a patient carrying the K141E mutation in *HSPB8* we have also investigated whether the presence of protein aggregates was paralleled by altered TDP43 function, a finding of possible pathogenic relevance to myofibre and nerve degeneration.

METHODS

Patients Standard Protocol Approvals, Registrations, and Patient Consents

Patients were referred to the inherited neuropathy clinic of the IRCCS Foundation, "C.Besta" Neurological Institute, Milan (IT) (I-1, II-1, II-3) or the National Hospital for Neurology and Neurosurgery, London (UK) (II-2). Clinical, neurophysiological and, if performed, pathological data were thoroughly reviewed. Muscle strength was scored using the MRC grading scale.

Institutional review boards approved the study and all examined family members gave written informed consent.

Muscle pathology

Patient II-1 and I-1 underwent quadriceps femoris muscle biopsy. Specimens were processed according to standard procedures. Ultrastructural examination of muscle sample was carried out as previously described (Ripolone et al. 2015).

Genetic analysis

Genomic DNA was extracted from peripheral lymphocytes using standard techniques. All exons and their franking intronic regions of *HSPB8, BAG3, DES, CRYAB, MYOT, ZASP, FLNC, VCP, DNAJB6,* and *FHL1* were sequenced directly using an ABI PRISM 3130 automated sequencer (PE Applied Biosystems). Primer sequences are available on request.

Sequence variants were assessed by using publically available databases including 1000 Genomes Project database (http://www.1000genomes.org/), NHLBI Exome Sequencing Project 5400 database (http://evs.gs.washington.edu/EVS/), dbSNP135 (http://www.ncbi.nlm.nih.gov/SNP/), and Human Gene Mutation Database (http://www.hgmd.cf.ac.uk/).

RNA purification and RT-PCR analysis and quantitative real time PCR analysis and SYSH-SY5 cell line siRNA

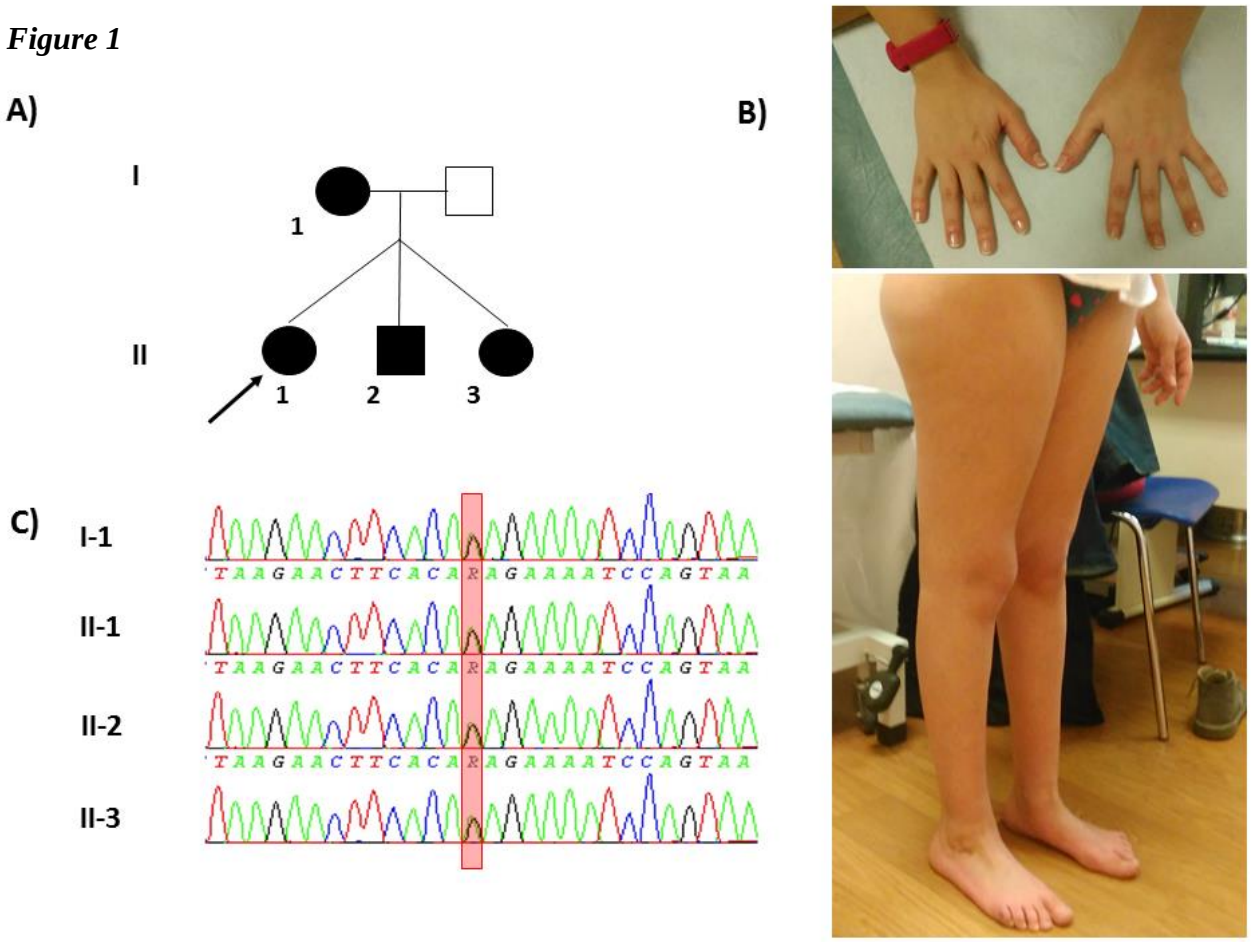
Methods were illustrated in the previous chapter "". In addition to POLDIP3, MADD, FNIP, splicing of the transcript BRD8 was evaluated. The following primers were used: BRD8 Rv 5′-ctcagagagaaagtggaggaggttc-3′.

RESULTS

Family pedigree

The pedigree was consistent with an autosomal dominant neuromuscular disorder, with three trizygotic twins and their mother affected (**Figure 1A**).

Figure 1



*(**A**) Pedigree. (**B**) Proximal and distal lower limb atrophy with relatively preserved muscle bulk at upper limbs. (**C**) Electropherograms showing the c.421A>G change (red highlighted) of HSPB8 gene in affected family members.*

Case 1 (II-1)

The index case, a female patient of 31 years, reported normal birth and developmental milestones. She underwent surgery for pes planus at age 6 years. At age 20, she started complaining of calf muscle cramps and progressive walking difficulties. She reported repeated ankle sprains and

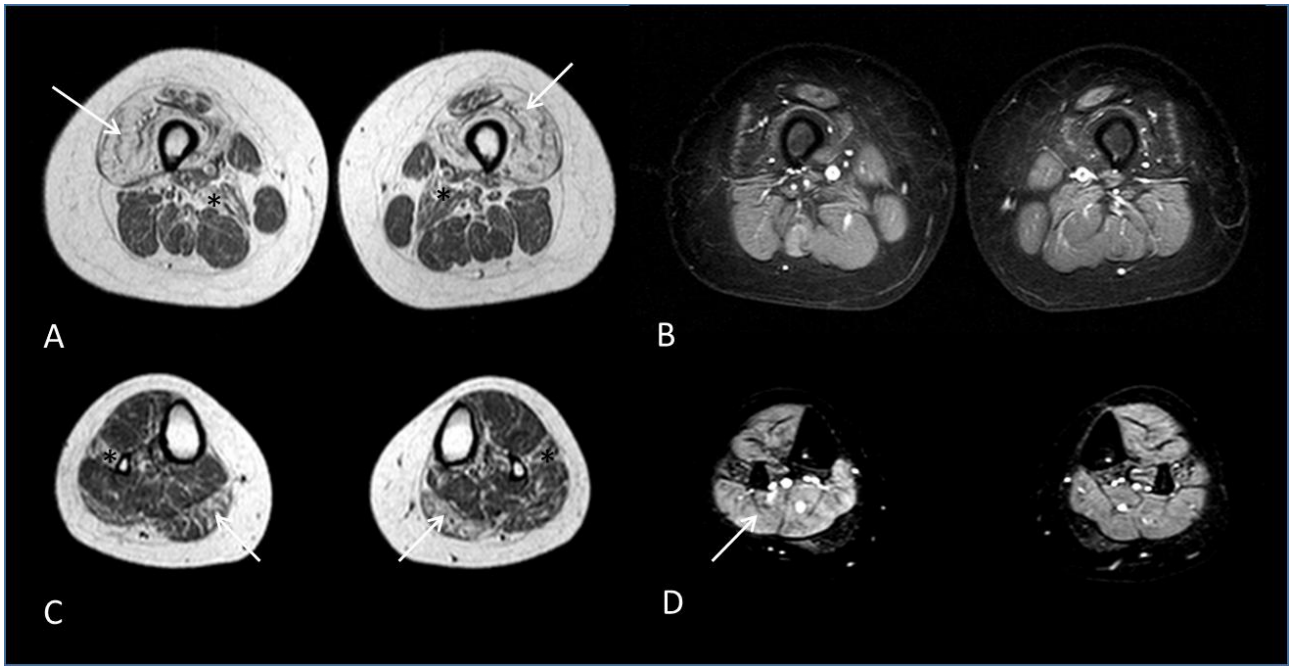
frequent falls in her late 20s, causing repeat bone fractures of fibular and peroneal bones. She was referred to our service at the age of 31.

Neurological examination showed waddling and steppage gait. She was unable to stand from kneeling or sitting and she needed help to sit from the lying position. There was proximal and distal atrophy in the lower limbs. Strength was reduced in proximal muscle groups (hip flexion 4 bilaterally, knee extension 3 on the right, 2 on the left) and distal muscles (foot dorsiflexion 4 bilaterally, foot plantar flexion 4 on the right and normal on the left, toe extension 3 on the right and 2 on the left). Deep tendon reflexes (DTR) were present in the upper limbs and absent in the lower limbs. Neck flexors were also weak (3). Examination of cranial nerves and upper limbs was normal. Sensation was normal throughout.

CK was slightly raised (274 U/l; n.v. = 24-150). Nerve conduction studies showed markedly reduced CMAP amplitudes in the lower limbs (peroneal nerve: 0.2 mV, tibial nerve: 2 mV) and borderline values in the upper limbs (ulnar nerve: 6 mV, median nerve: 7 mV). Motor and sensory conduction velocities and sensory action potentials were normal. EMG showed mixed myopathic and neurogenic changes in proximal and distal muscle groups.

Muscle MRI showed a selective pattern of muscle fatty degeneration with prominent involvement of quadriceps, adductor magnus, medial gastrocenmius and peroneus muscle groups (**Figure 2**).

Figure 2. MR axial T1-weighted (A, C) and T2-STIR (B, D) sequences at the level of the thigh (A, B) and calf (C, D), case II-1.



*In the thigh (**A, B**), a nearly complete fatty substitution of the quadriceps muscle is evident as hyper intensity bilaterally (**A**, arrows); the adductor magnus is also involved, though to a lesser extent (**A**, black*). No edema-like alterations are documented in the T2-STIR sequences (**B**). In the calf (**C, D**) there is selective fatty substitution at the level of the peroneal (**C**, black*) and both gemini muscles, predominantly the medial ones (**C**, arrows). A slight hyper intensity is also evident in the T2-STIR sequence (**D**) at the level of the right soleus (arrow).*

A quadriceps muscle biopsy showed the presence of marked fibre size variability, split fibres, internal nuclei and grouping of hypotrophic fibres belonging to both fibre types, pointing to a primary myopathy. Rimmed vacuoles, subsarcolemmal masses and core-like structures on oxidative stains were also observed. In immunofluorescence analysis, several fibres contained protein aggregates which were positive for AB-crystallin and myotilin (**Figure 3 A-F**). Stains for desmin and myosin had normal distribution. At electron microscopy, Z line streaming was observed in rare sarcomeres.

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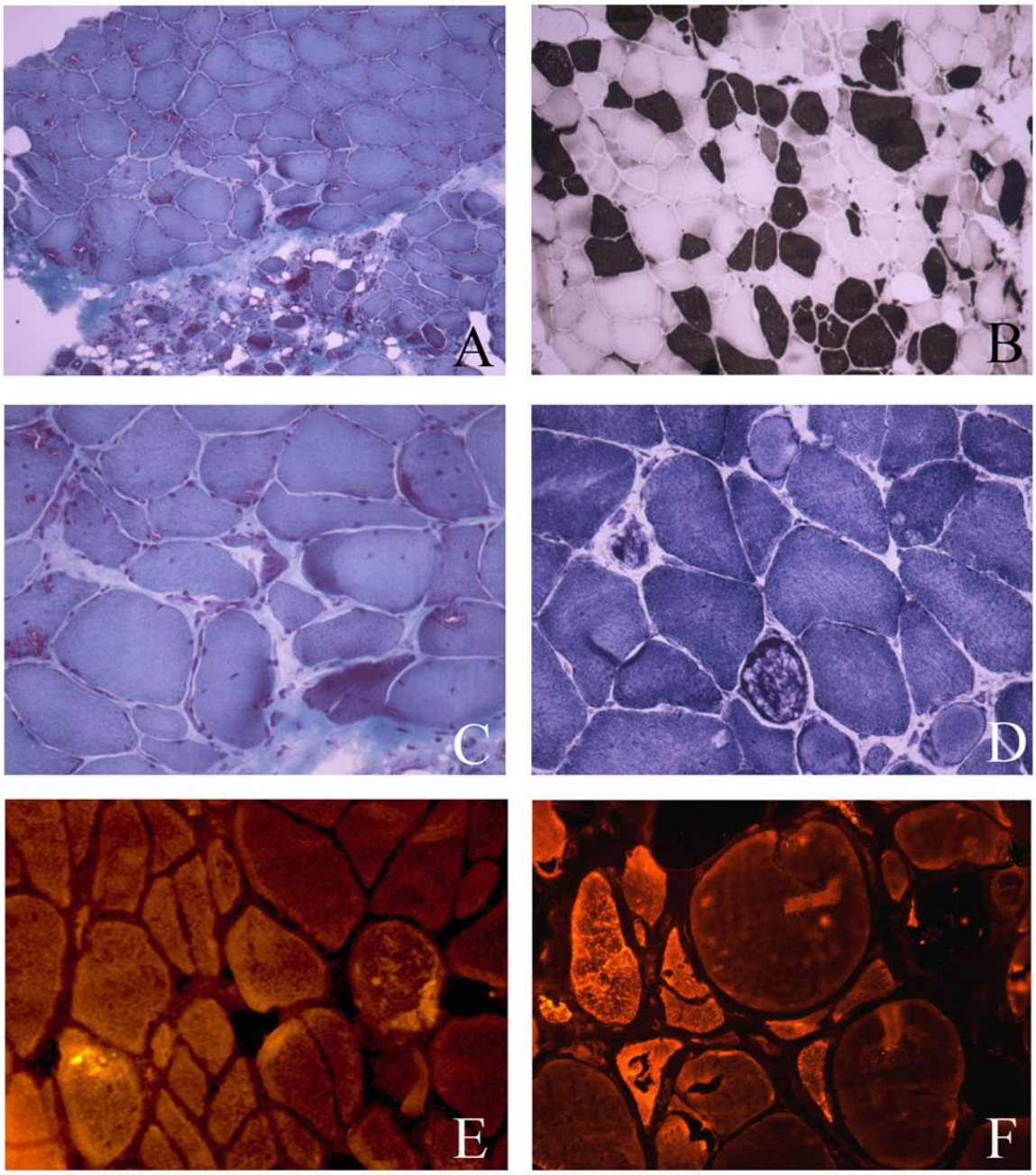
Figure 3. Muscle pathology



*(**A**) Gomori's Trichrome 100x: marked fiber size variability including small groups of hypotrophic fibres; several splittings and internal nuclei; a few fibres with rimmed vacuoles. (**B**) ATPase pH 4.3 100x: grouping of hypotrophic fibres belonging to both fibre types. (**C**) Gomori's Trichrome 400x (detail of picture A): some fibres with hyperchromic cytoplasmic areas. (**D**) NADH 400x: a few fibres with irregular distribution of enzymatic activity, including core-like aspects. (**E**) Anti Myotilin 400x: positivity in few cytoplasmic areas. (**F**) Anti alphaB-crystallin 400x: cytoplasmic positivity in several fibres.*

Case 2 (II-2)

This patient had normal birth and milestones. Onset of symptoms occurred during adolescence, when he noticed he could not sprint during basketball matches. In his 20s, he noticed he could not stand from squatting and had difficulty jumping and climbing stairs. He stopped running at 24 years of age. Since then, he has experienced progressive difficulty standing up and frequent falls. He denied symptoms in upper limbs.

Examination at age 31 showed difficulty standing from the chair. He could stand on heels and toes, but had a waddling gait. Cranial nerves were normal. Tone and strength were normal in upper limbs. There was no scapular winging. In lower limbs, he had severe proximal and distal wasting more pronounced proximally, proximal weakness with hip flexion grade 4 on the right and 2 on the left, knee flexion grade 4 bilaterally, and knee extension 4 on the right and 2 on the left. Ankle dorsiflexion was 4 on the right and normal on the left, toe extension was grade 4 bilaterally, while plantarflexion was preserved. Truncal weakness was also present. Reflexes were brisk in upper limbs and present in lower limbs. Sensory examination was normal.

CK level was 656 IU/L (n.v. 38-204 UI/L). NCS showed absent tibial CMAPs bilaterally and reduced peroneal CMAP amplitudes with normal conduction velocities. Sensory nerve conductions were normal. EMG showed myopathic changes in proximal lower limb muscles and bilateral medial gastrocnemious in a patchy distribution. Chronic neurogenic changes with large MUAP were recorded at tibialis anterior muscles, bilaterally.

Muscle MRI showed variable degree of fatty degeneration with the adductor magnus muscle being completely degenerated and the quadriceps femoris showing a variable degree of involvement. The sartorius, gracilis, semimembranosus, semitendinosus as well as biceps muscles were less involved. At calves, tibialis posterior muscle showed a marked degree of fatty degeneration and volume loss. The medial head of the gastrocnemius muscles and the *extensor hallucis longus* (EHL) and *extensor digitorum lungus (EDL)* were also involved bilaterally. Increased water content was also noted in residual muscular tissue of left vastus muscles and right EHL and EDL.

Case 3 (II-3)

Onset of symptoms, encompassing difficulties with rising from sitting position and impossibility to run, occurred in her late 20s. At age 31 years, neurologic examination showed pes cavus with hammertoes, moderate wasting of the thighs, but only minimal atrophy of the legs. There was mild weakness, grade 4, of neck flexion, hip flexion and abduction, knee extension and flexion, foot dorsiflexion and toe extension. DTRs were normal throughout, except for ankle DTRs, which were brisk. She had a steppage gait but walking was only slightly affected., She was able to rise on toes but could not walk on heels. She needed to push herself with her arms in order to get up from a chair and found it also difficult to sit from the lying position. The remainder of the examination was unremarkable.

Case 4 (1-1)

The mother of the trizygotic twins reported difficulties with climbing stairs since the age of 30. Thereafter, walking progressively worsened and she needed a stick by the age of 52 and two crutches three years later. She has needed a wheelchair for longer stretches since then. Neurologic examination at age 58 showed diffuse atrophy of lower limbs, back-knee, pes cavus and hammertoes. Independent walking was not possible. There was reduced power of truncal, upper and lower limb muscles (neck flexion, abductor pollicis brevis, first digital interosseous and extensor digitorum, all grade 4, hip flexion 4 on the right, hip extension 2 bilaterally, knee extension 4 on the right and 3 on the left, knee flexion 2 bilaterally). No active movements of feet and toes were possible. DTRs were present in the upper limbs and absent in the lower limbs. There was no sensory loss.

Genetic analysis

A heterozygous c.421A>G (p.Lys141Glu) change in exon 2 of *HSPB8* was identified in all four affected family members (**Figure 1C**). This is the same change as previously reported in

dHMN2A/CMT2L and distal myopathy and motor neuropathy (Evgrafov et al. 2004; Ghaoui et al. 2016). In addition, a heterozygous c.743A>G (p.His248Arg) change (rs369947845) was found in exon 3 of *BAG3* only in cases I-1 and II-2. No mutation was found in *DES, CRYAB, MYOT, ZASP, FLNC, VCP, DNAJB6, and FHL1*.

Altered TDP43-dependent splicing in HSPB8^{K141E} muscle tissue

TDP43 positive aggregate were reported to be present in numerous fibres from patients with HSPB8-related dHMN/MFM and are also a common finding of other PAM (Ghaoui et al. 2016; Andrea Cortese et al. 2014; Olivé et al. 2009b). Therefore, we investigated in the muscle biopsy of HSPB8^{K141E} case (II-1) if the presence of protein aggregates was paralleled by changes of *TDP43* expression and loss of function of TDP43. This was evaluated by assessment of TDP43 mRNA expression levels and analysis of alternative exon splicing of 4 validated TDP43-dependent target genes, as described in previous works by some of us (De Conti et al. 2015).

TDP43 mRNA was significantly lower in HSPB8^{K141E} muscle biopsy than in muscle from normal controls (**Figure 4A**). Moreover, in HSPB8^{K141E} muscle tissue splicing of three out of four assessed transcripts showed changes of exon alternative splicing consistent with TDP43 loss of function, and in particular enhanced inclusion of *BRD8* exon 20 and exclusion of *POLDIP3* exon 3 and *FNIP1* exon 7 compared to normal muscle (**Figure 4B**). Reported as a control, the same level of changes observed in the patient tissue was also observed in TDP43 depleted SH-SY-5Y cell line using siRNA. Differently from cell line results, however, no changes were noted as regards MADD exon 31 splicing in HSPB8^{K141E} muscle.

Figure 4.

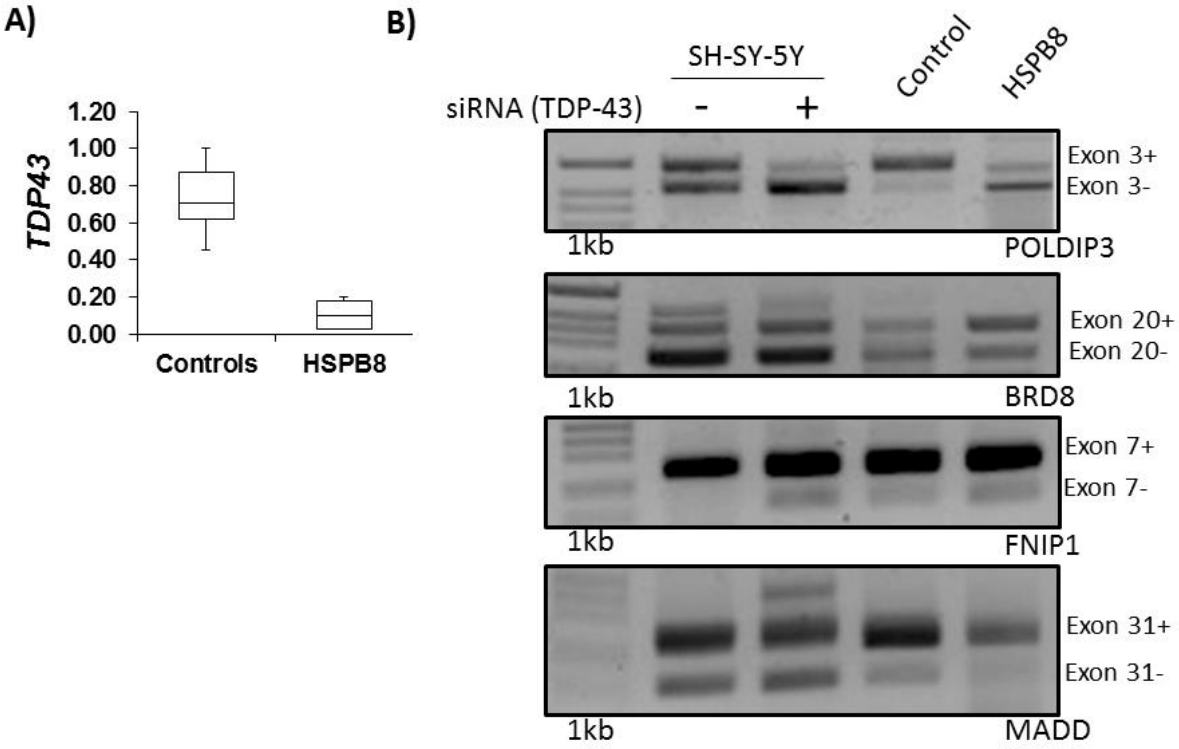


Figure 4. (A). Box plots of TDP43 mRNA levels measured using qPCR of the Controls and of the HSPB8 Proband as ddCt values*. The box whisker plots visualize the minimum (end of the bottom whisker), the first quartile (bottom border of the box), the median (line through the box), the third quartile (top border of the box), and the maximum (end of the top whisker) of the distribution. The RPL13a housekeeping gene was used for normalization of TDP43 expression for the upper graph, and GAPDH for the lower graph****. (B). Alternative splicing of selected genes in controls vs. the HSPB8 patient II-1.*** *For each gene, as comparison with a TDP43 loss-of-function situation we used SH-SY-5Y cell lines treated either with control siRNA or TDP43 siRNA (lane – and +, respectively, on the left). Standard RT-PCR amplifications were then performed for the POLDIP3, BRD8, FNIP1, MADD transcripts. The labeling on the right shows the affected exons in each case. Alternative splicing of these exons in one representative normal muscle (three were used) and in the proband (II-1) is shown on the right (Lanes Control and HSPB8, respectively). Significance level were indicated as *** for P ≤ 0.001; as ** for P ≤ 0.01 and as * for P ≤ 0.05. NS stands for not statistically significant.*

DISCUSSION

HSPB8 is a member of a family of 10 small heath shock proteins (HSP) which are molecularly defined by the presence of a highly conserved alfa-crystallin domain and are functionally involved in chaperone-mediated autophagy (Benndorf et al. 2014).

Mutations in other HSPBs, including HSPB1, HSPB3 and HSPB5, also known as AB-crystallin, are associated with various forms of neuropathy and myopathy. In particular, mutations in HSPB8 have been reported in families with autosomal dominant dHMN and CMT2 (Tang et al. 2005; Irobi et al. 2004). The HSPB8-related neuropathy typically has an onset in the II-III decade, has a slow progression and is characterized by distal atrophy and weakness, with no or mild sensory involvement. Mutations in HSPB1 are also causative of autosomal dominant dHMN/CMT2, with a phenotype which was reported to be indistinguishable from that caused by mutations in HSPB8 (Evgrafov et al. 2004). On the other hand, mutations in AB-crystallin (HSPB5) are responsible for an infrequent form of myofibrillar myopathy characterized by proximal and distal limb weakness, respiratory failure, cardiomyopathy, and cataract (Vicart et al. 1998). Notably, peripheral neuropathy can also be associated.

More recently, p.K141E and p.P173SfsX43 mutations in HSPB8 were identified in 2 families with autosomal dominant distal myopathy, along with a neurogenic component both on biopsy and neurophysiologic studies. The K141E mutant HSPB8 led to increased aggregation score in a GFP-120Q-huntingtin co-transfected cell lines (Ghaoui et al. 2016).

Here, we report a second family with the K141E mutation in *HSPB8*. Their clinical phenotype is similar to that of the previously reported family and is characterized by onset in the II-III decade of life with progressive weakness of distal and proximal lower limb muscles, in particular ankle dorsiflexion, knee extension and flexion and hip flexion. Asymmetry was possible in early stages. Muscle MRI in our patients showed also comparable findings with prominent involvement of gastrocnemius, peroneus and quadriceps muscles.

Differently from the cases reported by Ghaoui et al, our patients consistently showed neck flexor and truncal muscle involvement with early difficulties sitting from the lying position. Notably, in contrast to other causes of myofibrillar myopathy, none of the cases so far reported with HSPB8-related disease had evidence of respiratory or cardiac muscle involvement.

The list of genes and conditions responsible for combined or alternative muscle and nerve involvement is expanding (**Table 1**). Interestingly, mutations in other genes involved in protein degradation and chaperone-assisted selective autophagy, including *VCP, MATR3* and *BAG3* itself, which directly interact with HSPB8, all show heterogeneous clinical expressivity ranging from myopathic to neuropathic, neuronopathic and mixed phenotypes (Jaffer et al. 2012, 3; Senderek et al. 2009, 3; Watts et al. 2004; Johnson et al. 2010; Gonzalez et al. 2014). Moreover, neurogenic changes at electrodiagnostic studies are reported in patients with myofibrillar myopathy , sporadic inclusion body myositis and, occasionally, also in patients with oculopharyngeal muscular dystrophy (A Cortese et al. 2013; Selcen 2011; Piccolo et al. 2011; Olivé, Kley, and Goldfarb 2013). Since the presence of protein aggregates is a common pathologic finding of all these disorders, we speculate that protein aggregation and impaired protein aggregates disposal might represent a common pathogenic pathway underlying both peripheral nerve and muscle damage.

the K141E mutation in *HSPB8*, as well as the majority of missense mutations in *HSBP1* and AB-crystallin (*HSPB5*) so far reported, are localized in the conserved alfa-crystallin domain of the HSPBs, which is essential for formation of stable small HSP dimers (Kasakov et al. 2007; Benndorf et al. 2014). Substitution of a positively charged Arginine or Lysine residue with a different amino acid inside this domain interferes with alfa-crystallin mediated oligomerization of HSPs and can lead to impaired chaperoning function.

In addition to the dominant-negative effect by altered oligomer formation of HSPs, other possible pathogenic mechanisms of mutation in HSPBs have been identified, including a negative effect of mutated HSPBs on the cytoskeleton abnormally organized intermediate filaments in neurons and

muscle, increased enzymatic activity and impaired interaction with the survival motor neuron complex (Benndorf et al. 2014).

Table 1. Genes and condition associated with contemporary or alternative muscle and nerve involvement

Genes and conditions responsible for contemporary nerve and muscle involvement
HSPB8 (Ghaoui et al. 2016) BAG3 (Jaffer et al. 2012) GBE1 (DiMauro and Spiegel 2011) (Adult polyglucosan body disease) Mitochondrial disorders(Horga et al. 2014) HSPB1 (Frasquet M, Muelas N, Sivera R, Barreiro M, Velasquez-Costa JF, Chumillas MJ, Espinos C, LUpo V, Vilchez JJ, Sevilla T, n.d., 1)
Genes and conditions associated with myopathy. Possible neurogenic changes on EDx study
MYOT (Olivé, Kley, and Goldfarb 2013; Selcen 2011) ZASP (Olivé, Kley, and Goldfarb 2013; Selcen 2011) CRYAB (HSPB5) (Selcen and Engel 2003) PABPN1 (Piccolo et al. 2011) sIBM (A Cortese et al. 2013; Felice and North 2001)
Genes associated with neuropathy/neuronopathy. Possible muscle involvement or significantly raised CK
MATR3 (Müller et al. 2014), AR (Kennedy's disease) (Orsucci et al. 2014), XK (Mc Leod syndrome) (Marsh et al. 1981)
Other causes of alternative muscle or nerve involvement (variable expressivity depending on different mutations)
LMNA (Bonne et al. 1999; De Sandre-Giovannoli et al. 2002; Muchir et al. 2000), DNM2 (Züchner et al. 2005; Bitoun et al. 2005) , VCP (Johnson et al. 2010; Watts et al. 2004; Gonzalez et al. 2014), *HNRNPA1* (Mademan I, Deconinck T, Geuens T, Kochanski A, Van den Bergh P, Timmerman V, DE Jonghe P, Baets J, n.d.)

AR, Androgen Receptor; BAG3, BCL2 Associated Athanogene 3; CK: creatin kinase; CRYAB, AB-crystallin; DNM2, Dynamin 2; EDx: electrodiagnostic;

Table 1 (continued) *HNRNPA1, Heterogeneous Nuclear Ribonucleoprotein A1; HSPB1, small heat shock protein 27; HSPB8, small heat shock protein 22; LMNA, Lamin A/C; MYOT, myotilin; PABPN1, Poly(A) Binding Protein Nuclear 1; sIBM: sporadic inclusion body myositis; VCP, Valosin Containing Protein; XK, X-Linked Kx Blood Group; ZASP, ZO-2 Associated Speckle Protein MYOT*

Our study identified a role of impaired RNA metabolism as a novel possible contributor to HSPB8-pathology, and as a result of impaired TDP43 function.

TDP43 is a RNA binding protein, involved in different stages of RNA processing, including splicing regulation, and was first described in 2006 as being the major component of pathological inclusions present in the brain of patients affected by amyotrophic lateral sclerosis and frontotemporal lobar degeneration (Neumann et al. 2006; Buratti and Baralle 2012). Later studies found TDP43 mislocalization from the nuclei and its cytoplasmic aggregation in muscle fibres of patients with myopathies with protein aggregates, either acquired (e.g., sporadic inclusion body myositis) or hereditary (myofibrillar myopathies, DNAJB6-related limb-girdle muscular dystrophy, oculopharyngeal muscular dystrophy) (Weihl et al. 2008; Olivé et al. 2009a; Salajegheh et al. 2009; Hernandez Lain et al. 2011; Andrea Cortese et al. 2014; Harms et al. 2012). Moreover, TDP43-positive inclusions seem also to be a hallmark of muscle pathology in HSPB8^{K141E} related myopathy (Ghaoui et al. 2016).

Unfortunately, we did not have any muscle tissue left to confirm the presence of TDP43 aggregates in the patient muscle biopsy. However TDP43 inclusions were present in the myofibres of previously reported patients with HSPB8 related dHMN/MFM. Moreover, TDP43 inclusions have been consistently reported in other myopathies with protein aggregates.

Numerous studies demonstrated that changing the expression level of TDP43 is sufficient to alter the expression and splicing of hundreds RNAs *in vitro* (Polymenidou et al. 2011; Tollervey et al. 2011). Moreover, changes of RNA processing have been identified in iPS cell lines derived from

patients with amyotrophic lateral sclerosis carrying *TDP43* mutations and muscle fibres from patients with sporadic inclusion body myositis (Cortese et al. 2014; Egawa et al. 2012).

Here we show a consistent alteration of TDP43 dependent splicing in 3 out of 4 previously validated direct TDP43 target transcripts (*POLDIP3, FNIP* and *BRD8*). The only TDP43-controlled splicing event that was not altered belonged to the *MADD* gene. However, this is not necessarily surprising as the splicing process is very often a combinatorial event that is heavily dependent on cellular context (De Conti et al. 2015), and the cellular environment in muscle is understandably very different from that in neurons. In this respect, therefore, the observation of reduced *TDP43* mRNA levels and altered splicing of 3 out of 4 genes tested are strong indicators that TDP43 function in the patient muscle is impaired compared to healthy control muscle. Although there is little literature to suggest a straightforward connection between altered splicing in the *POLDIP3*, *FNIP*, and *BRD8* transcripts and pathology these results strongly suggest that other genes relevant to nerve and muscle survival may be abnormally spliced as a consequence of TDP43 loss of function.

In conclusion, our study confirms the role of mutated HSPB8 as a cause of a combined neuromuscular disorder encompassing dHMN and MFM/PAM. It will be important for clinicians to consider the possibility of both neuropathy and myopathy being present in any patient with a HSPB8 mutation. Moreover, we identified in muscle tissue from a patient carrying the $HSPB8^{K141E}$ mutation an altered splicing of TDP43 target genes, suggesting that impaired RNA metabolism, probably secondary to TDP43 loss of function, can play a role in muscle and nerve degeneration in HSPB8-related disease.

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