Immunophenotypical characterization of paraneoplastic neurological syndrome patients: a multicentric study

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Paraneoplastic neurological syndromes (PNS) are a group of rare and severe immune-mediated disorders that affect the nervous system in patients with cancer. The best way to diagnose a paraneoplastic neurological disorder is to identify anti-onconeural protein antibodies that are specifically associated with various cancers. The aim of this multicentric study was to clinically and immunologically characterize patients with PNS and study their association with cancer. Patients suspected to have PNS were enrolled from various clinical centres and were characterized immunologically. This study population consisted of 112 patients. Onset of PNS was mainly subacute (76%). PNS patients had various neurological disorders and symptoms. PNS developed before the diagnosis of cancer in 28 definite PNS patients and in six suspected PNS patients. The most frequent autoantibodies detected in PNS patients were anti-Hu and anti-Yo. One definite PNS patient with cerebellar syndrome had anti-Tr antibody and seven patients had atypical antibodies. The literature associates these antibodies with various neurological disorders and cancers. Our observations confirm the important role of autoantibodies in PNS and their importance for the early diagnosis of cancer in PNS patients.

Keywords. Autoantibodies; cancer; immunophenotypical characterization; paraneoplastic neurological syndromes

1. Introduction

Paraneoplastic neurological syndromes (PNS) are rare immune-mediated neurological disorders due to systemic manifestations of distant tumours, characterized by rapid onset, severe clinical manifestations and focal neurological signs (Honnorat and Antoine 2007; Rosenfeld and Dalmau 2018). PNS develop in 1 out of 10000 cancer patients and may involve any part of the central and peripheral nervous systems (Honnorat and Antoine 2007; Darnell and Posner 2003). PNS precede the diagnosis of malignancy in about 50–80% of cases, although they can also manifest after the cancer has been diagnosed, and sometimes appear during its remission (Chan and Baehring 2019). Diagnosis of PNS requires the exclusion of other conditions related to cancer that could account for neurological symptoms, such as metastases, infections, nutritional or metabolic deficits and therapies (Honnorat and Antoine 2007; Rosenfeld and Dalmau 2018; Darnell and Posner 2003). Autoimmunity is considered fundamental in the pathogenesis of many PNS (Darnell and Posner 2003; Blaes 2012). The autoantibodies are regarded as the result of an immunological response to cancer and may cross-react with cells of the nervous system, causing neuronal damage. The immunological hypothesis was advanced after the finding of voltage-gated calcium channel antibodies in Lambert-Eaton myasthenic syndrome patients with small cell lung cancer (Gutmann *et al.* 1972). The potential for onconeural antibodies to cause disease depends on the epitope's subcellular localization (Blyakhman and Chakravarthy 2019; Zaborowski and Michalak 2013). Detection of autoantibodies assists the diagnosis of PNS (Lancaster 2017). Correlation of cancer with neuronal involvement from the molecular standpoint may therefore lead to rational tumour therapy. In the last 20 years, the field of autoimmune PNS has gained from great activity in the identification of new antibodies. There are currently more than 20 antibodies available for commercial testing (Lancaster 2017; Zidan *et al.* 2019). The main aims of this multicentric study were to identify the clinical characteristics associated with typical and atypical paraneoplastic autoantibodies, and to characterize those autoantibodies that react against neuronal and glial cells of PNS patients of the study population.

2. Methods

2.1 *Patient selection criteria and clinical examination*

The study population included 112 patients with suspected PNS who were seen at Pavia University Hospital (Pavia, Italy), Brescia University Hospital (Brescia, Italy), Chiari University Hospital (Chiari, Italy) and Clatterbridge Oncological Centre (Liverpool, UK) between October 2000 and June 2009. We used the criteria of the Paraneoplastic Neurologic Syndrome Euronetwork guidelines (Graus *et al.* 2004) for diagnosis and classification as possible or definite PNS. Patients were divided according to clinical presentation, primarily on the basis of one of the following neurological disorders:

- cerebellar degeneration (Dalmau and Rosenfeld 2008);
- encephalomyelitis or limbic encephalitis (Asztely and Kumlien 2012);
- polyneuropathy (England and Asbury 2004);
- retinal degeneration (Gordon and Dinkin 2019);
- motor neurone disease (Mélé *et al.* 2018);
- opsoclonus-myoclonus (Armangué *et al.* 2016);
- peripheral nervous disorders including sensory neuro-/neuronopathy, dysautonomia and neuromyotonia (Antoine and Camdessanchè 2007);
- Lambert-Eaton myasthenia syndrome, polymyositis and necrotizing myelopathy (Dean *et al.* 2018).

Paraneoplastic neurological syndromes were also distinguished as acute, subacute and chronic (Dankó *et al.* 2009). Demographics, clinical presentation, prior history of malignancy and any related ancillary testing, including brain MRI, EEG, EMG/nerve conduction studies and paraneoplastic testing, were recorded for all patients. Informed consent was obtained from each patient. The study was conducted in accordance with the Declaration of Helsinki and was approved by the local ethics committee.

2.2 *Immunohistochemistry*

Adult Sprauge-Dawley rats were anesthetized and sacrificed by decapitation. The brain was removed and dissected sagittally. Unfixed tissues were obtained by immersing the sagittal blocks in optimal cutting temperature (OCT) compound and subsequent freezing in isopentane and liquid nitrogen. Paraformaldehyde (PFA)-fixed tissues were obtained by immersing the sagittal brain blocks in 4% PFA for ten days in sucrose, then in 30% phosphate buffered saline (PBS) solution for 48 hours for cryoprotective purposes, and finally in OCT compound. These were then frozen by immersion in isopentane and liquid nitrogen. Fixed and non-fixed PFA blocks of tissue were stored at − 80°C until use.

Seven µm-thick sections of non-fixed tissues and tissues fixed in PFA were obtained by means of a cryotome and used for immunohistochemical study. The unfixed sections were then incubated in acetone or methanol-acetone at 4°C for 10 min. Anti-Hu and anti-Yo positive sera were used as positive controls. Endogenous peroxides in tissue were quenched by immersion of slides in a bath of 1.5% hydrogen peroxide for 10 min. Non-specific binding was reduced by pre-incubation sections with 10% Bovine Serum Albumin (BSA) in Phosphate Buffer Solution-Tween (PBS-T) at room temperature for 30 min. Patient and control sera were applied at a dilution of 1:500 in PBS-T 1% BSA for 1 hour at room temperature. After three washes with PBS-T, the secondary biotinylated antibody, goat-anti-human IgG-biotin (Invitrogen, Carlsbad, CA), was added at a dilution of 1:200 for 30 min (PBS-T 1% BSA) at room temperature. The tertiary layer was streptavidin-peroxidase conjugate (1:1000 in PBS-T 1% BSA). Sections were incubated with streptavidin complex for 30 min at room temperature and developed to visualize staining.

2.3 *Immunoblotting*

Immunoblotting was performed using recombinant proteins (Milenia Biotech, Gießen, Germany) in cases testing positive for typical antibodies by immunohistochemistry. In cases testing positive for atypical antibodies by immunohistochemistry, immunoblot was performed using protein-extracted from the rat brain. Positive-testing controls (anti-Hu and anti-Yo positive sera) and negative control sera obtained from 40 healthy volunteers without any pathological immunohistochemical or immunoblot pattern were used. Protein extracts from rat brain were run on a 10% sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE). Samples and molecular weight markers (Sigma) were dissolved in final sample buffer (FSB). Gels were run at a constant current of 40 mA for approximately 90 min. The proteins were transferred to nitrocellulose membranes (Hybond-C pure, Amersham Bioscience, Little Chalfont, UK) using the transblot semi-dry technique (Biorad), run at a constant current of 15 volts for 35 min. The proteins were visualized with Ponceau staining. After blocking for non-specific binding with 5% non-fat milk powder in PBS-T for 30 min at RT, membranes were cut into 5-mm strips which were incubated overnight with patient serum (diluted 1:1000 in goat serum) in PBS-T with 1% BSA. The secondary antibody, horseradish peroxidase conjugated rabbit anti-human IgG (Santa Cruz, CA, USA) was diluted 1:1000 in PBS-T and added for 2 hours. Membranes were incubated with colour developer (amino-ethyl carbazole) for 20 min.

2.4 *Statistical analysis*

For statistical analysis, proportion was used as a descriptive statistic for categorical and ordinal variables, the median and interquartile range for ordinal and continuous variables and the mean for continuous variables. Statistical analysis was performed using the chi-square or Fisher's test for the categorical variables and the Student T-test for analysis of differences in average values. A p value < 0.05 was considered significant. All statistical calculations were performed with SPSS software package version 25.0 (Chicago, IL, USA).

3. Results

3.1 *Demographic data and clinical findings*

This study population consisted of 112 PNS patients: 52 with definite PNS and 60 with possible PNS. The demographic and clinical data of the patients is shown in table 1. The median age of PNS cases was 63 years (range 39–86) with no significant difference between the two groups (0.91). Males and females had substantially the same proportions of cases with definite and possible PNS ($p = 0.93$).

Onset of PNS was mainly subacute (76%) and did not differ significantly in the two groups ($p = 0.19$). Definite and possible PNS patients had various neurological disorders and symptoms. In the former, there was a prevalence of cerebellar syndromes and encephalomyelitis (37/52), whereas polyneuropathies prevailed (35/60) in possible PNS patients ($p = 0.00003$).

Among definite PNS cases, neurological dysfunction developed an average of 6 months (range 3–12 months) before tumour diagnosis in 28 patients, and an average of 6 months (range 3–12 months) after tumour diagnosis in 18 patients. In possible PNS cases, neurological dysfunction developed an average of 6 months (range 3–12 months) before tumour diagnosis in 6 patients, and an average of 6 months (range 3–12 months) after tumour diagnosis in 15 patients. The main site of cancer was the lung in definite PNS patients, whereas possible PNS patients showed no particular prevalence ($p = 0.0002$).

Table 1. Demographic and clinical characteristics of patients with definite and possible PNS

	Patients with definite PNS (n = 52)	Patients with possible PNS (n = 60)	Total patients with PNS (n = 112)
Age (years, median and range)	64 (39–85)	63 (48–86)	63 (39–86)
Gender (number females/males)	23/28	28/32	51/60
Acute (number)	10	4	14
Subacute	36	49	85
Chronic	6	7	13
Neurological syndromes (number of patients)			
Cerebellar syndromes	20	14	34
Encephalomyelitis	17	5	22
Polyneuropathy	7	35	42
Retinal degeneration	2	–	2
Associated encephalomyelitis and polyneuropathy	2	2	4
Motor neurone disease	2	–	2
Limbic encephalitis	1	–	1
Multiple mononeuropathy	1	–	1
Opsoclonus-myoclonus	1	2	3
Necrotizing myelopathy	–	1	1
Neuromyotonia	–	1	1
Symptoms and signs (number of patients)			
Ataxia	18	17	35
Memory loss	8	3	11
Confusional state	6	–	6
Dysesthesia	4	12	16
Dysesthesia and weakness	–	2	2
Diplopia	2	1	3
Nystagmus and slurry speech	2	15	17
Sensory loss	2	2	4
Sensory loss and weakness	2	–	2
Memory and sensory loss	–	1	1
Visual defect	2	–	2
Fasciculation	2	1	3
Opsoclonus	1	–	1
Parkinson	1	–	1
Motor weakness	1	–	1
Cranial palsy	1	2	3
Slurry speech	1	2	3
Pathological findings in neurological investigation (number of patients)			
MRI brain	10/52	3/54	13/106
MRI spinal cord	–	2/54	2/54
CT	2/52	–	2/52
EEG	3/12	–	3/12
EMG	12/21	30/54	42/54
CSF	3/3	–	3/3
Diagnosis of cancer (number of patients)			
Lung	**22**	**7**	**29**
Carcinoma	16/22		
Small cell lung cancer (SCLC)	3/22		
Recurrent SCLC	1/22		
Adenocarcinoma associated with SCLC	1/22		
Bronchogenic carcinoma associated with mesothelioma	1/22		
Bronchial carcinoma			
Urogenital tract	**11**	**2**	**13**
Ovary	5/11	–	
Breast	4/11	2/2	
Fallopian tubes	1/11	–	

Table 1 (continued)

	Patients with definite PNS (n = 52)	Patients with possible PNS (n = 60)	Total patients with PNS (n = 112)
Uterus	1/11	–	
Prostate	**5**	**6**	**11**
Kidneys	**1**	–	**1**
Gastrointestinal tract	**4**	**2**	**6**
Stomach	2/4	–	
Liver	1/4	1/2	
Colon	1/4	1/2	
Lymphoma	**2**	**3**	**5**
Hodgkin	1/2	1/3	
Non-Hodgkin (B-lymphoma)	1/2	2/3	

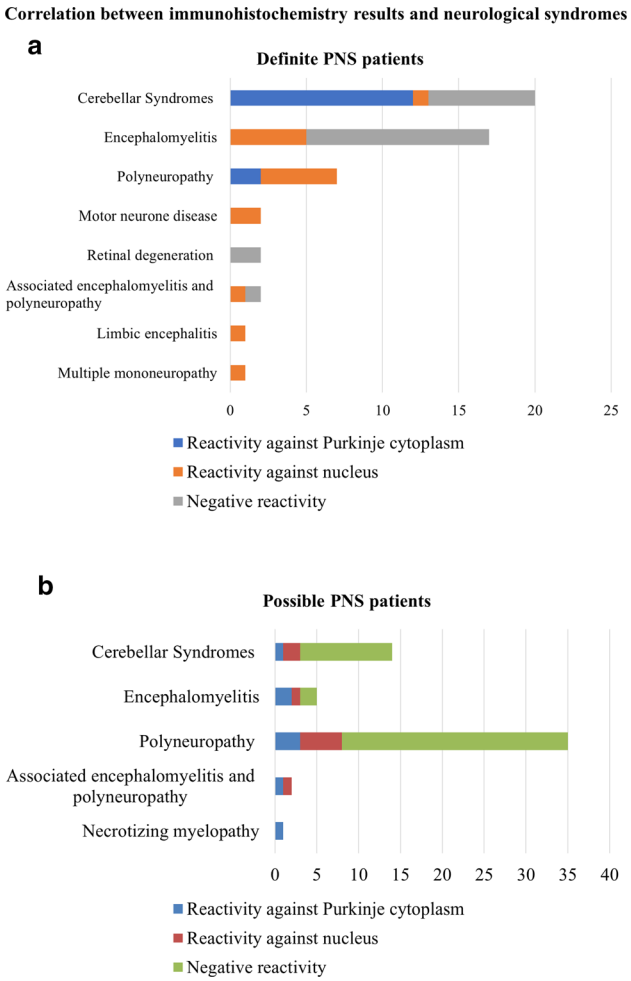


Figure 1. Immunohistochemistry results in definite PNS patients (**a**) and in possible PNS patients (**b**) in relation to neurological syndromes.

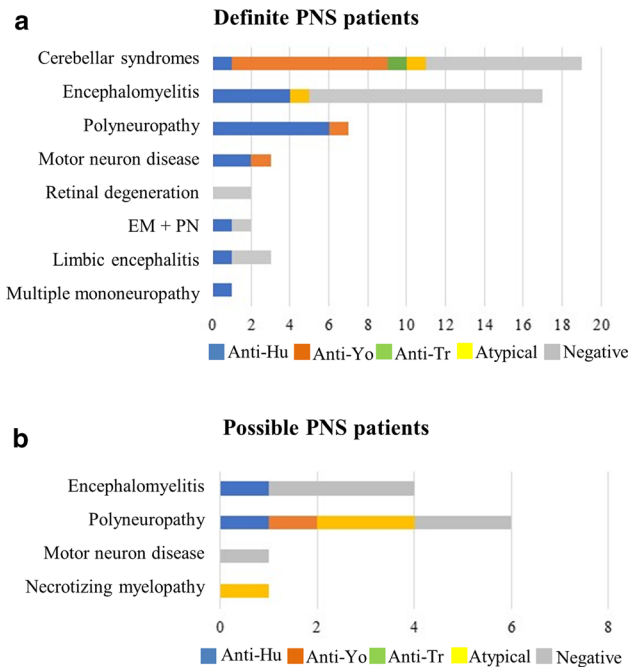


Figure 2. Immunoblot results in definite PNS patients (**a**) and in possible PNS patients (**b**) in relation to neurological syndromes. EM = encephalomyelitis; PN = polyneuropathy

3.2 *Immunohistochemistry and immunoblot results*

The results of immunohistochemistry are shown in figure 1 and those of immunoblot analysis in figure 2 and table 2. Immunoblot analysis detected 29 onconeural antibodies (16 anti-Hu, 10 anti-Yo, one anti-Tr and two atypical) in definite PNS patients and 6 onconeural antibodies (two anti-Hu, one anti-Yo and three atypical) in possible PNS patients with a statistically significant difference between the two groups ($p < 0.00001$) and associations with various neurological disorders and cancers. The cut-off was established on the basis of band intensity compared with positive controls. Definite PNS patients with anti-Hu and anti-Yo antibodies were more numerous than patients with other antibody positivity

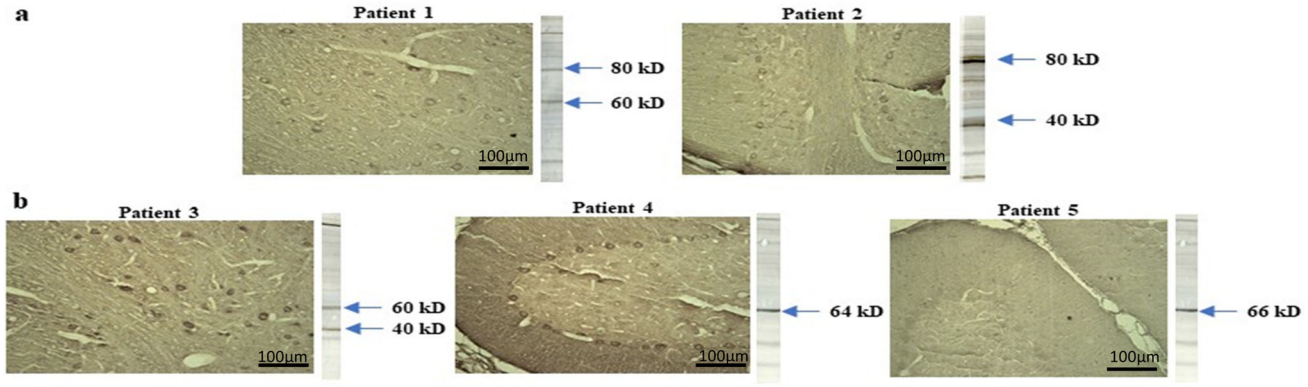
Table 2. Results of sera tested for PNS antibodies and their correlation with neurological disorders and cancers

Antibody	Total PNS patients (n = 112)	Definite PNS patients (n = 52)	Associated neurological disease (n/n total)	Associated cancer (n/n total)	Possible PNS patients (n = 60)	Associated neurological disease (n/n total)	Associated cancer (n/n total)
Anti-Hu	18 (16%)	16 (31%)	Polyneuropathy (6/16) Encephalomyelitis (4/16) Motor neuron disorders (2/16) Cerebellar syndrome (1/16) Polyneuropathy associated with encephalomyelitis (1/16) Limbic disorders (1/16) Mononeuropathy (1/16)	Lung cancer (6/16) Prostate cancer (4/16) Small cell lung cancer (2/16) Gynaecological cancers: 1 fallopian cancer, 1 ovarian cancer (2/16) Total cancer patients (14/16)	2 (3%)	Polyneuropathy (1/2) Encephalomyelitis (1/2)	Prostate cancer (2/2) Total cancer patients (2/2)
Anti-Yo	11 (10%)	10 (19%)	Cerebellar syndrome (8/10) Polyneuropathy (1/10) Motor neuron disorders (1/10)	Gynaecological cancers: 1 breast cancer, 2 ovarian cancer, 1 uterine cancer (4/10) lung cancer (2/10) Total cancer patients (6/10)	1 (2%)	Polyneuropathy (1/1)	Non-Hodgkin lymphoma (1/1) Total cancer patients (1/1)
Anti-Tr	1 (1%)	1 (2%)	Cerebellar syndrome (1/1)	Hodgkin lymphoma (1/1) Total cancer patients (1/1)	–	–	–
Atypical	5 (5%)	2 (4%)	Cerebellar syndrome (1/2) Encephalomyelitis (1/2)	Cancer lung (1/2) Non-Hodgkin lymphoma (1/2) Total cancer patients (1/1)	3 (5%)	Polyneuropathy (2/1) Necrotizing myelopathy (1/3)	Non-Hodgkin lymphoma (1/3) Prostate cancer (1/3) Bronchial carcinoma (1/3) Total cancer patients: 3/3

($p = 0.012$). Atypical antibodies were identified in both definite and possible PNS groups without any significant difference ($p = 0.79$). The atypical paraneoplastic autoantibodies that react against neuronal and glial cells identified in PNS patients are shown in figure 3.

4. Discussion

Autoantibodies are an important finding in the diagnosis of PNS and occult tumours, because paraneoplastic neurological symptoms often precede tumour diagnosis (Zis *et al.* 2017; Zoccarato *et al.* 2017).



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Patient	Sex	Age	PNS	Cancer	Immunohistochemistry	WB
1	Female	68	Paraneoplastic encephalomyelitis	B-Lymphoma	Cytoplasm of neurones	60-80 kDa
2	Male	76	Paraneoplastic cerebellar degeneration	Bronchial carcinoma and mesothelioma	Cytoplasm of neurones	40 -80 kDa
3	Male	69	Necrotising myelitis	Bronchial carcinoma	Cytoplasm of neurones	40-60 kDa
4	Male	59	Motor- sensory polyneuropathy	Non-Hodgkin Lymphoma	Cytoplasm of neurones and molecular layer	66 kDa
5	Male	68	Motor- sensory polyneuropathy	Prostate	Nucleus of neurons	60 kDa

Figure 3. Characterization of atypical paraneoplastic neurological autoantibodies that react against neuronal and glial cells identified in definite PNS patients (**a**; 1 and 2 patients) and possible PNS patients (**b**; 3, 4 and 5 patients) with immunohistochemistry of rat brain (scale bar 100 µm) and immunoblot (**a, b**). Table summarising association of atypical autoantibodies with PNS and cancer (**c**).

Classic onconeural antibodies in PNS are directed against intracellular neuronal antigens such as Hu, Yo, Ri, amphiphysin, CV2, Ma1, Ma2/Ta, PCA-2, Tr and SOX1. If a patient tests positive for these antibodies, the probability of a tumour is greater than 95% (Zoccarato *et al.* 2017; Vincent 2005; Berger *et al.* 2015). According to current guidelines, autoantibodies in PNS should always be determined using unrelated laboratory methods, for example indirect immunohistochemistry and immunoblot (Zoccarato *et al.* 2017; Sæther *et al.* 2017). As all PNS are rare, it is essential to recruit PNS patients from different hospitals or to collect clinical data from different centres to define new onconeural antibodies, syndromes and cancer associations (Van Coevorden-Hameete *et al.* 2016). The aim of this multicentric study was to characterize PNS induced by various tumours in 112 patients, 52 with definite and 60 with possible PNS. The tumours that induced PNS were lung cancers, female reproductive tract cancers, gastrointestinal tract cancers, lymphoma, prostate cancer and kidney cancer. In 18 definite and six possible PNS patients, the tumour was diagnosed after manifestation of neurological symptoms. In our population, onset of PNS was mainly subacute (76%). Most PNS patients had acute or subacute onset, whereas noninflammatory neurodegenerative disorders are chronically progressive (Graus *et al.* 2004).

In this analysis, we identified autoantibodies in 32% of PNS patients. In PNS patients, autoantibodies that react against neuronal nuclear antigens may be anti-Hu or anti-neuronal nuclear antibody 1 (ANNA-1), generally associated with encephalomyelitis and subacute sensory neuropathy, in turn often associated with small cell lung carcinoma, and rarely with ovarian, breast, prostate, cervical cancer, thymoma, Hodgkin's lymphoma or transitional cell bladder carcinoma (Daroff *et al.* 2016; Graus *et al.* 2001). They may also be anti-Ri or anti-neuronal nuclear antibody 2 (ANNA-2), associated with opsoclonus-myoclonus, in turn generally associated with neuroblastoma in children and small cell lung carcinoma in adults (Lukacs *et al.* 2012; Armangué *et al.* 2016). They may also be anti-Ma 1, associated with paraneoplastic brainstem encephalitis, in turn associated with breast, lung, colon and parotid cancer (Graus and Dalmau 2019). Finally they may be anti-Ma 2 or anti-Ta, associated with brainstem encephalitis, in turn associated with testicular cancer (Graus and Dalmau 2019; Ortega Suero *et al.* 2018). In the population of the present study, anti-Hu was detected in 16 definite PNS patients (6 with polyneuropathies, 4 with encephalomyelitis, 2 with

motor neuron disorders, 1 with cerebellar syndrome, 1 with polyneuropathy associated with encephalomyelitis, 1 with limbic disorders and 1 with mononeuropathy), two of whom had small cell lung carcinoma, in line with the literature. Anti-Hu was also detected in two possible PNS patients (one with encephalomyelitis and one with polyneuropathy) who had prostate cancer. Antibodies that react against neuronal cytoplasm, anti-Yo or anti-Purkinje cell antibody-1 (APCA-1), may be associated with paraneoplastic cerebellar degeneration and usually occur in women with gynaecological tumours (Dalmau *et al.* 2004). Another antibody, anti-Tr, is associated with paraneoplastic cerebellar degeneration and occurs in patients with Hodgkin's lymphoma (Venkatraman and Opal 2016). Our immunohistochemistry results showed reactivity against Purkinje cytoplasm, which was associated with cerebellar syndrome, whereas reactivity against the nucleus was associated with encephalomyelitis in definite PNS patients. Ten definite PNS patients with anti-Yo, associated in eight cases with cerebellar syndrome, in one case with polyneuropathy and in another case with motor neuron disease, and one possible PNS patient with anti-Yo associated with polyneuropathy, were detected by immunoblot analysis. Four definite PNS patients with anti-Yo had gynaecological tumours in line with the literature (2 ovarian cancer, 1 uterine cancer and 1 breast cancer). Two definite PNS patients with anti-Yo had lung cancer and the others were without tumours. On the other hand, the possible PNS patient with anti-Yo had non-Hodgkin lymphoma. Only one definite PNS patient with cerebellar syndrome had anti-Tr and Hodgkin's lymphoma, in line with the literature. In this study, none of the PNS patients had cancer-associated retinopathy antibodies, which are associated with paraneoplastic degeneration correlated with small cell lung carcinoma (Bernal *et al.* 2003), or anti-amphiphysin antibodies, which are associated with stiff-person syndrome and small cell lung carcinoma (Kazarian and Laird-Offringa 2011). In line with data in the literature, the most frequent autoantibodies detected in definite PNS patients were anti-Hu and anti-Yo, associated with encephalomyelitis and cerebellar syndrome (Van Coevorden-Hameete *et al.* 2016; Murinson and Guarnaccia 2008; Kanikannan *et al.* 2015). Most of our PNS patients had already been diagnosed with cancer, although the neurological manifestations are known to precede those of cancer. So except in cases with anti-Yo that generally manifest PNS after discovery of the cancer (Chan and Baehring 2019), more efficient cancer-screening programs, which also consider neurological symptoms, are needed to enable early diagnosis of cancer and start appropriate immunotherapy as soon as possible. There is some evidence that immune-checkpoint inhibitors may be used effectively in cancer treatment (McKeon *et al.* 2011), although cancer patients may be at increased risk of PNS when thus treated (Longo *et al.* 2019). However, the number of controlled studies on the treatment of paraneoplastic syndromes have been few, due to the rarity of PNS (Graus and Dalmau 2019).

We found two uncharacterised antibodies in definite PNS patients, one with lung cancer and one with non-Hodgkin B-lymphoma, while in possible PNS patients three atypical antibodies were associated with bronchial carcinoma, non-Hodgkin lymphoma and prostate cancer. Concretely, only well characterised onconeural antibodies that have known patterns, well-characterised association with neurological syndromes, low frequency in patients without cancer and high frequency in cancer patients, should be used to classify the associated disorders (Van Coevorden-Hameete *et al.* 2016). Overall, our observations confirm an important role of autoantibodies in PNS associated with cancer and their importance in early diagnosis of cancer in PNS patients. The characterization of atypical autoantibodies may be an important step in the increasingly detailed diagnosis of patients with PNS. Further research is warranted to determine whether these and other atypical autoantibodies may provide useful information for prognosis and for specific treatment of PNS.

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