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REGULAR PAPER

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Peripheral nerve extracellular matrix remodeling in Charcot-Marie-Tooth type I disease

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Abstract Charcot-Marie-Tooth type 1 disease (CMT1) is a group of inherited demyelinating neuropathies caused by mutations in genes expressed by myelinating Schwann cells. Rather than demyelination per se, alterations of Schwann cell-axon interactions have been suggested as the main cause of motor-sensory impairment in CMT1 patients. In an attempt to identify molecules that may be involved in such altered interactions, the extracellular matrix (ECM) remodeling occurring in CMT1 sural nerves was studied. For comparison, both normal sural nerves and sural nerves affected by neuropathies of different origin were used. The study was performed by immunohistochemical analysis using antibodies against collagen types I, III, IV, V, and VI and the glycoproteins fibronectin, laminin, vitronectin and tenascin. Up-regulation of collagens, fibronectin and laminin was commonly found in nerve biopsy specimens from patients affected by CMT1 and control diseases, but higher levels of overexpression were usually observed in CMT1 cases. On the other hand, vitronectin and tenascin appeared preferentially induced in CMT1 compared to other pathologies investigated here. Vitronectin, whose expression in normal nerves was limited to perineurial layers and to the walls of epineurial and endoneurial vessels, became strongly and diffusely expressed in the endoneurium in most CMT1 biopsy specimens. The expression of tenascin, confined to the perineurium, to vessel walls and to the nodes of Ranvier in normal nerves, was displaced and extended along the internodes of several nerve fibers in the majority of CMT1 nerves. Thus, compared with our pathological controls CMT1 seemed to determine the most extensive remodeling of peripheral nerve ECM.

Keywords Charcot-Marie-Tooth type I disease · Extracellular matrix · Vitronectin · Tenascin · Sural nerve

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Introduction

The hereditary motor and sensory neuropathies (HMSN) are degenerative diseases that, through different pathogenic mechanisms, lead to a progressive impairment of both motor and sensory functions. Among these, the type I HMSN or Charcot-Marie-Tooth type 1 disease (CMT1) is a genetically heterogeneous group, the underlying genetic lesions mapping to one of at least four different genes, sharing demyelinating features [12, 20, 32]. Accordingly, the mutations which have been proved to cause CMT1 affect genes expressed in myelinating Schwann cells, namely those encoding the peripheral myelin 22 protein (PMP22), the myelin protein zero (P0), the connexin-32 (Cx32) and the early growth response 2 (EGR-2) transcription factor [20, 32]. Importantly, in addition to myelination these genetic lesions have been shown to affect Schwann cell functions such as differentiation and cellular adhesion [14, 20]. On the other hand, the pathogenic mechanism that, starting from the molecular lesions, leads to the clinical manifestation of CMT1 remains poorly understood. Moreover, the picture is complicated by the finding that the clinical symptoms of the disease, rather than to the severity of demyelination, seem to be related to the progressive distal axonal degeneration observed in CMT1 patients. Therefore, an alteration of Schwann cell-axon interactions has been suggested as the main cause of motor-sensory impairment [11, 20, 22]. In this regard, it is of note that mutations in myelin-related proteins such as P0 and Cx32 can also cause axonal degeneration with rela-

tive preservation of the myelin sheaths [39, 40]. The characterization of Schwann cell microenvironment in CMT1 patients can thus help to identify molecules participating in altered Schwann cell-axon interactions or involved in the impeded regeneration of the damaged nerve fibers.

Extracellular matrix (ECM) molecules have been demonstrated to play a fundamental role in the development and maintenance of nervous tissues, and changes in ECM microenvironment can have a profound influence on cellular behavior [24, 31, 43]. Peripheral nerve ECM provides protection for nerve fibers from teasing and pressure and contributes to the formation of the blood-nerve barrier. In addition, it exerts several regulatory functions through specific interactions with integrin and non-integrin cell surface receptors [3]. ECM molecules have been proved to modulate axonal growth and pathfinding as well as Schwann cell proliferation and differentiation during both development and regeneration [3, 10, 24]. It has been demonstrated that both the availability and the activity of several growth factors present in nervous tissues can be modulated through the interaction with ECM molecules. Furthermore, the contact with an appropriate ECM is required by neurons from different sources to respond to survival factors, such as nerve growth factor or brain-derived neurotrophic factor [43].

ECM remodeling in peripheral nerves of CMT1-affected patients has been scarcely investigated, even though the endoneurial ECM is synthesized and deposited mainly by Schwann cells [3], which in this inherited disease exhibit an altered phenotype [14]. This observation prompted us to study the expression of ECM molecules, namely collagen types I, III, IV, V, and VI and the glycoproteins laminin, fibronectin, vitronectin and tenascin in sural nerve biopsy specimens from CMT1 patients, as well as in sural nerves from subjects with no evidence of neuropathy and from disease controls. This study highlights that some of the above molecules appear to be preferentially modulated in CMT1-affected nerves.

Materials and methods

Subjects

The study was performed on diagnostic sural nerve biopsy specimens obtained from 31 patients, after they had given their informed consent (Table 1). Of these subjects, 10 had a diagnosis of CMT1, according to the "Guidelines for the diagnosis of Charcot-Marie-Tooth disease and related neuropathies" by the ad hoc Working Group of the Italian Neurological Society [26]. Five of these patients were shown to have CMT1A with a chromosome 17p11.2 duplication by molecular analysis. Sural nerve biopsy specimens from patients with hereditary neuropathy with liability to pressure palsies with 17p11.2 deletion (HNPP, *n*=3), adrenomyeloneuropathy (AMN, *n*=2), chronic inflammatory demyelinating polyneuropathy (CIDP, *n*=8) and chronic idiopathic axonal neuropathy (*n*=8) were included as disease controls. Biopsies of sural nerve with no sign of neuropathy were obtained from organ donor control subjects (*n*=4).

Nerve biopsies

Sural nerve biopsy samples were obtained with standard procedures under local anesthesia. The portion of each specimen assigned to histological examination and to immunohistochemistry was frozen in isopentane cooled by liquid nitrogen and stored at –80°C until use. According to routine procedures, further portions were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer and postfixed in 1% osmium tetroxide in the same buffer. These portions were either dehydrated in acetone and embedded in Epon via propylene oxide, for semithin sections and electron microscopy, or macerated in glycerol, for teasing of single myelinated fibers under a stereomicroscope.

Table 1 Summary of subjects included in the study (*CMT1* Charcot-Marie-Tooth type 1 disease, *HNPP* hereditary neuropathy with liability to pressure palsies with 17p11.2 deletion, *AMN* adrenomyeloneuropathy, *CIDP* chronic inflammatory demyelinating polyneuropathy, *Axonal* chronic idiopathic axonal neuropathy)

	No. of cases	Sex (M:F)	Mean age (years)	Range (years)
Control	4	1:3	44	30–56
CMT1	10	6:4	45.5	16–70
HNPP	3	2:1	40.3	22–72
AMN	2	2:0	36	33–39
CIDP	8	5:3	63	33–78
Axonal	8	8:0	55.1	19–74

Immunohistochemistry

Immunohistochemical detection of ECM molecules was performed on cryostat sections obtained from frozen sural nerve specimens (7 μm-thick transverse sections and 8 μm-thick longitudinal sections), using the Histostain-SP kit (Zymed Laboratories, San Francisco, Calif.) according to the manufacturer's protocol. Briefly, the sections were fixed with cold acetone (10 min) and endogenous peroxidase activity was quenched with a 3% H_20_2 solution (10 min, room temperature). All subsequent procedures were performed at room temperature. After a blocking step with non-immune goat serum (20 min), the sections were incubated 1 h with the following PBS-diluted primary antibodies: polyclonal anti-collagen type I and anti-collagen type III (AB745 and AB747, respectively, Chemicon International, Temecula, Calif.; 1:100 diluted), polyclonal anti-collagen types IV, V, and VI (AB748, AB763, AB7822, respectively, Chemicon; 1:250), polyclonal anti-laminin (L9393, Sigma Chemical Co., St. Louis, Mo.; 1:100), monoclonal anti-fibronectin (F7387, Sigma; 1:200), monoclonal anti-vitronectin (MAB1945, Chemicon; 1:100), and monoclonal anti-tenascin (T2551, Sigma; 1:4,000). The sections were then washed in PBS and overlaid with the appropriate biotinylated secondary antibody (goat anti-rabbit or anti-mouse IgG, 30 min), followed by extensive PBS washing, a streptavidin-peroxidase conjugate incubation (10 min), PBS rinsing and finally by the substrate-chromogen (AEC) solution (3–5 min, depending on the primary antibody). Hematoxylin was used for counterstaining. Negative controls were performed replacing the primary antibody with rabbit or mouse non-immune serum. At the end of the procedure the sections were observed and photographed with a Zeiss Axiophot photomicroscope.

Nerve fiber morphometry and evaluation of histopathological features in nerve biopsy specimens

Transverse semithin sections obtained from selected sural nerve biopsy specimens were stained with toluidine blue and used to quantify the density of myelinated fibers (MF/mm^2), using a Leica Quantimet 500 image analyzer, and to evaluate the prevalence of onion bulbs, tomacula, axonal ovoids and clusters of axonal regeneration. Teased fibers preparations obtained from the same biopsy samples were used to evaluate the presence of demyelinated or re-

Table 2 Immunodetection of ECM molecules in normal sural nerves and in nerves affected by CMT1 or by other neuropathies: HNPP, AMN, CIDP, chronic idiopathic axonal neuropathy; (*ECM* extracellular matrix, Ep_S epineurial stroma, *P* perineurium, En_S endoneurial stroma, *SC* Schwann cells, *MF* myelinated fibers, – no staining, + moderate staining, ++ intermediate staining, +++ strong staining)

ECM molecule	Localization	Control	CMT1	Other neuropathies
Type I collagen	Ep_S	+	+	Perineurial expression similar to control nerves; endoneurial expression variably increased, generally to a lower extent than in CMT1
	P	++	+++[a]	
	En_S	+	++	
	SC	++	+++	
Type III collagen	Ep_S	+	+	
	P	++	+++[a]	
	En_S	++	+++	
Fibronectin	Ep_S	+	+	
	P	++	+++[a]	
	En_S	+	+	
	SC	++	+++	
Type IV and V, laminin	Ep_S	–	–	
	P	++	+++[a]	
	En_S	–	–	
	SC	++	+++	
Type VI collagen	Ep_S	+	+	
	P	++	+++[a]	
	En_S	+	+	
	SC	++	+++	
Vitronectin	Ep_S	–	–[b]	Expression similar to control nerves except in 2/2 AMN and 1/8 CIDP with endoneurial expression similar to CMT1
	P	+	++[a]	
	En_S	–	+++[c]	
Tenascin	Ep_S	–	–	Expression similar to control nerves except in 2/3 HNPP and 1/8 CIDP with endoneurial expression similar to CMT1
	P	+	++[a]	
	En_S	–	–	
	MF	–[d]	++[e]	

[a]In 5/10 nerve biopsy specimens
[b]Occasionally positive
[c]In 7/10 nerve biopsy specimens
[d]Negative except in correspondence with the nodes of Ranvier
[e]In 7/10 nerve biopsy specimens, a variable number of myelinated fibers is immunostained

myelinated internodes. The prevalence of these histopathological figures was classified as moderate (+), intermediate (++), or marked (+++), according to an arbitrary scale.

Results

The results of the immunohistochemical characterization of ECM molecules expression in normal and pathological sural nerves are summarized in Table 2.

Collagens, fibronectin and laminin

In healthy sural nerves, type I and type III collagen, the major stromal collagens, were expressed in the epineurium, perineurium and endoneurium. In the latter the immunostaining for type III collagen was distributed diffusely, whereas type I staining appeared less homogeneous, being particularly evident around axon-myelin units. This finding is consistent with previous reports in which a high type III to type I ratio of intrafascicular collagen was described [1, 35]. Both collagens were also expressed in association with epineurial and endoneurial vessels. The anti-fibronectin antibody stained the three peripheral nerve compartments diffusely, with the perineurium, particularly the inner perineurial layers, showing the highest immunoreactivity. The intrafascicular stroma was moderately immunolabeled, while Schwann cells and blood vessel walls displayed a stronger staining. Type IV and type V collagens shared a similar pattern of distribution: neither of the two collagens was detected in the general epineurial or endoneurial stroma, while perineurial layers and Schwann cells were strongly immunolabeled, as well as epineurial and endoneurial vessels. The previous finding of a higher type V collagen expression in the inner perineurial layers versus the outer ones was also confirmed [1]. The distribution of type VI collagen closely resembled that of type IV and V. However, unlike type IV and type V, a moderate labeling for type VI collagen was also present in both the epineurial and endoneurial stroma, and a stronger staining was found in association with epineurial fibroblasts (Fig. 1). The distribution of laminin also paralleled that of type IV and type V collagen. As reported for type V collagen, the expression of this glycoprotein appeared stronger in the inner perineurial layers than in the outer ones.

In the endoneurium of nerves affected by CMT1 or by the other pathological conditions listed in Table 1, a variable increase of type I and type III collagen was detected in the stromal compartment, whereas the deposition of laminin, fibronectin, type IV, V and VI collagen (Fig. 1b) appeared

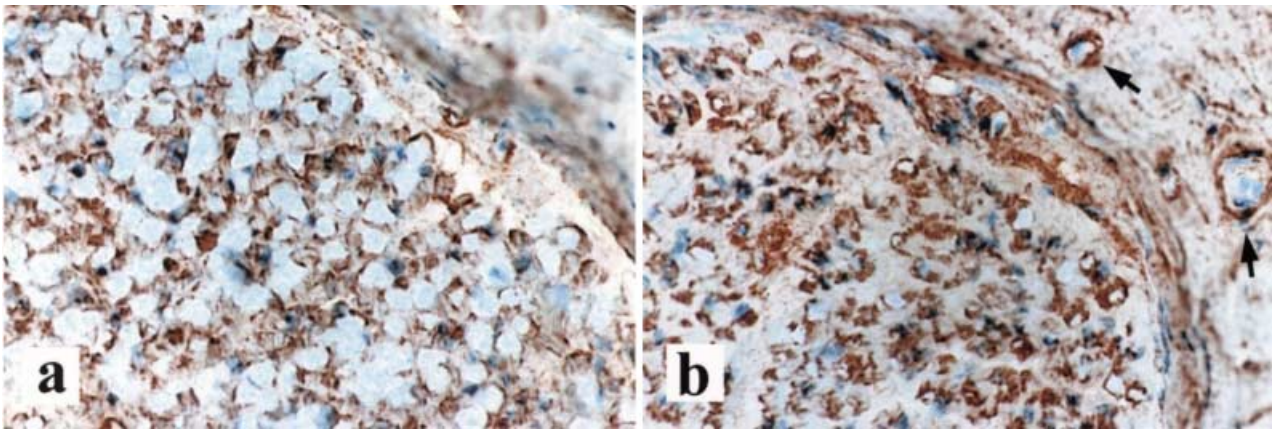

Fig. 1 Immunohistochemical detection of type VI collagen in transversally sectioned sural nerves from a control subject (**a**) and a CMT1 case (**b**). In CMT1-affected nerves type VI collagen deposition appears increased in association with Schwann cells, especially around some myelinated fibers. The *arrows* in **b** indicate epineurial blood vessels. **a**, **b** ×280

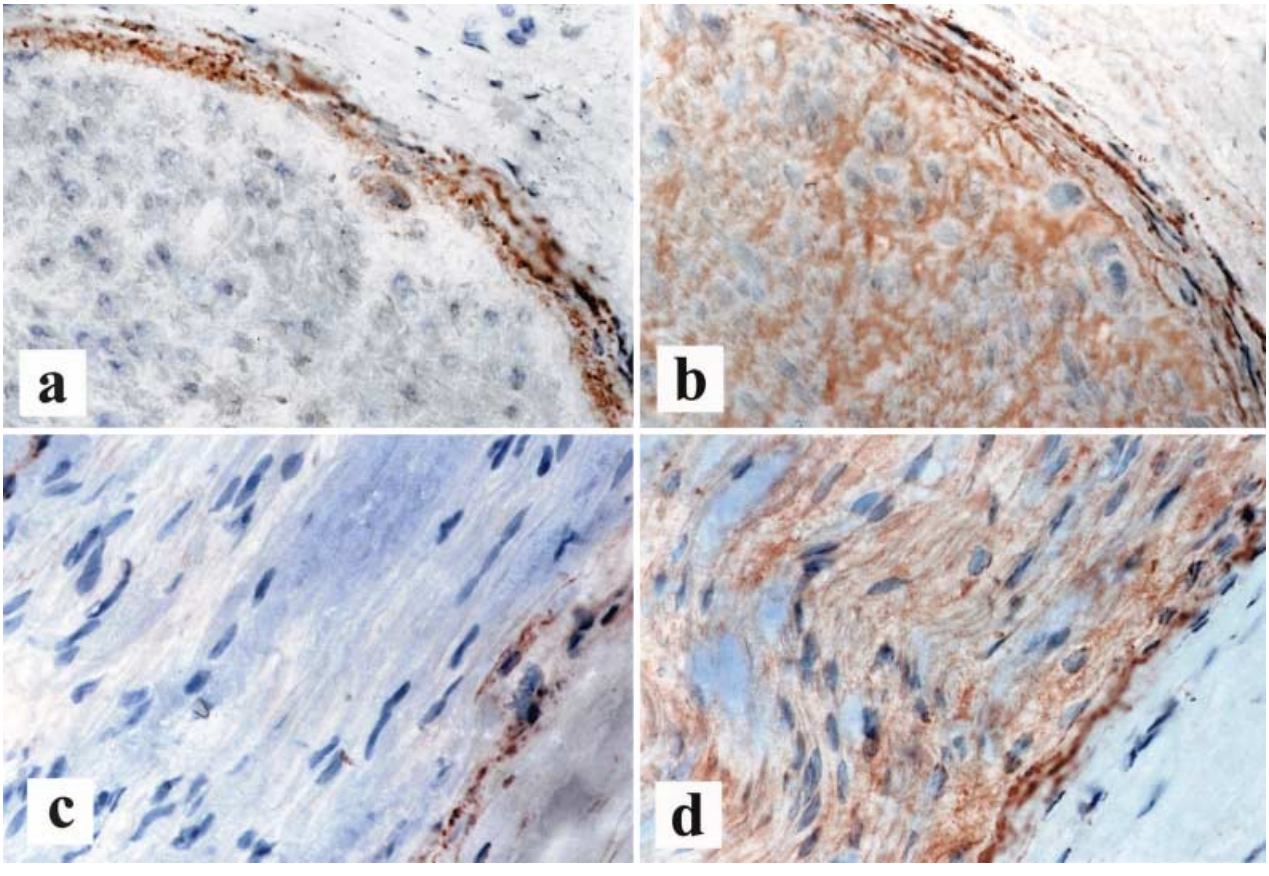

Fig. 2 Immunohistochemical detection of vitronectin in transversally (**a**, **b**) and longitudinally sectioned (**c**, **d**) sural nerves from a control subject (**a**, **c**) and two CMT1 cases (**b**, **d**). Both CMT1 nerves display a strong endoneurial immunostaining for vitronectin, but only the nerve in **b** shows epineurial positivity in association with fibroblasts. **a–d** ×280

to increase to a variable extent in association with Schwann cells, especially around myelinated fibers. The expression of the aforementioned ECM molecules was thus quantitatively affected, while their distribution reflected that observed in control nerves. The highest levels of increased deposition were found in CMT1 and in some CIDP nerve biopsy specimems. Furthermore, five of ten nerves affected by CMT1 displayed perineurial hypertrophy and the expression of perineurial ECM molecules was accordingly increased. In these nerves the immunolabeling for fibronectin, collagen type V and laminin was often of the same intensity in the outer and inner perineurial layers.

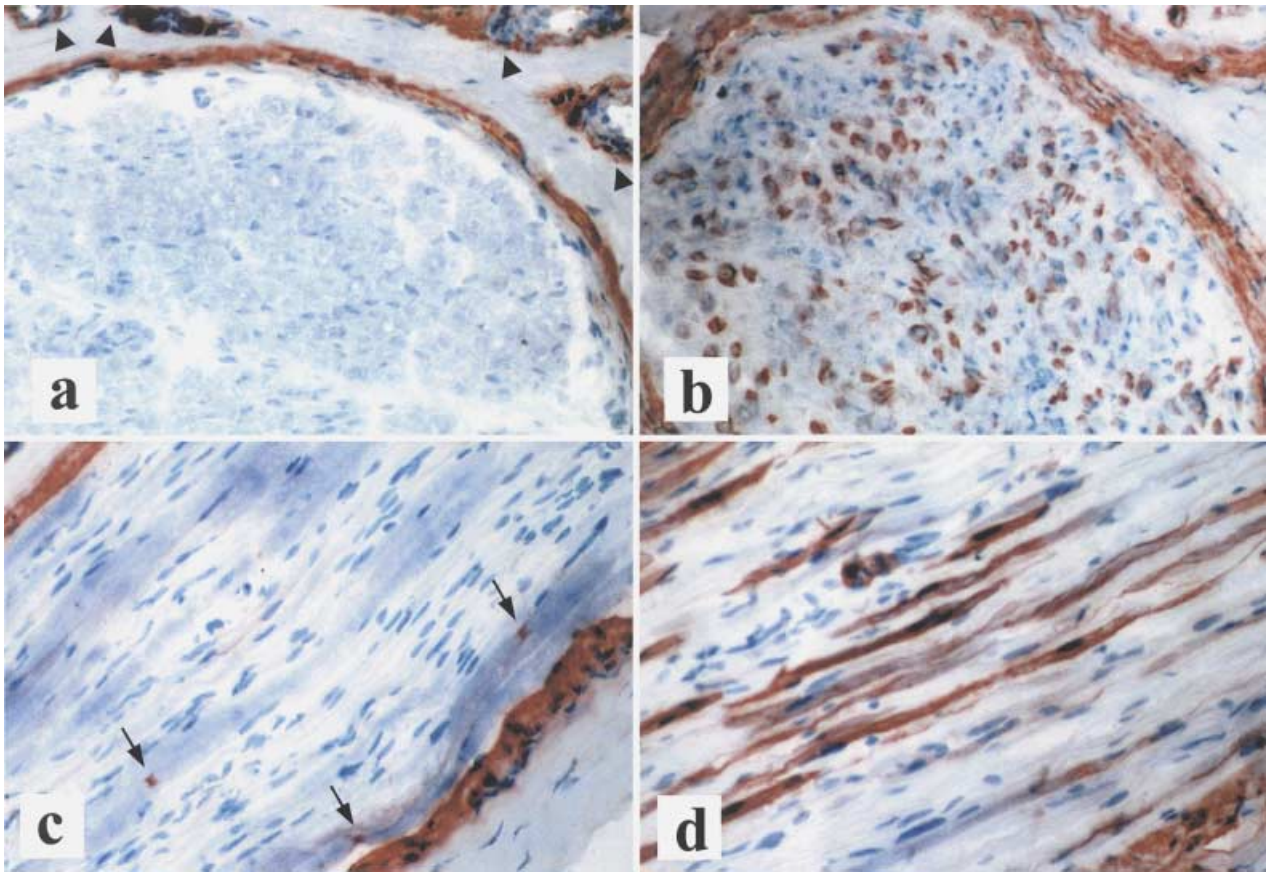

Fig. 3 Immunohistochemical detection of tenascin in transversally (**a**, **b**) and longitudinally sectioned (**c**, **d**) sural nerves from a control subject (**a**, **c**) and a CMT1 case (**b**, **d**). In normal nerve, a strong staining of the perineurium (**a**, **c**) and of epineurial vessels (*arrowheads* in **a**) is evident. Moreover, nodes of Ranvier are clearly immunodecorated (*arrows* in **c**). In a sural nerve from a CMT1 patient, the majority of myelinating Schwann cells are stained, as well as the hypertrophic perineurium (**b**); in longitudinal section, the immune reaction outlines several serial internodes (**d**). **a–d** ×180

Vitronectin

In control nerves vitronectin immunostaining was restricted to a punctated pattern distributed along perineurial layers and associated with epineurial and endoneurial blood vessels (Fig. 2a, c). The appearance of a strong endoneurial immunostaining for vitronectin was a feature of the majority (seven of ten) of CMT1 nerves. This immunostaining displayed a diffused distribution, suggesting that in the endoneurium the spatial organization of the molecule can be completely distinct from that of the perineurium and vessel walls (Fig. 2b, d). An endoneurial up-regulation similar to that observed in CMT1 nerves was found in the two AMN nerves included in the study, a lower intrafascicular increase was occasionally observed in nerves affected by CIDP (one of eight cases), and no clear increase was seen in HNPP or in axonal neuropathy biopsies. Regardless of the presence of intrafascicular vitronectin, in some CMT1 and CIDP nerve biopsy specimens the expression of this glycoprotein was also found in association with epineurial fibroblasts. An increase in its perineurial deposition was also detectable in those CMT1 nerve samples displaying perineurial hypertrophy. Therefore, a pathological vitronectin increase could be found in each of the three peripheral nerve compartments, epineurial, perineurial and endoneurial, but the increase in one compartment did not imply that the others were similarly affected (Fig. 2b, d).

Tenascin

Tenascin expression was also deeply affected in the majority of CMT1 nerve biopsy specimens. The immunostaining for this glycoprotein, which was limited to perineurial layers, to the nodes of Ranvier and to blood vessel walls in normal sural nerves (Fig. 3a, c), became strikingly evident in the form of numerous endoneurial foci resembling axon-myelin units in several transversally sectioned CMT1 nerves (Fig. 3b). The immunohistochemistry performed on longitudinally sectioned CMT1 nerves confirmed that the anti-tenascin antibody specifically marked nerve fibers, myelinated to a variable extent, and further evidenced that in these fibers tenascin expression was extended along the internodes (Fig. 3d). This pattern of endoneurial tenascin expression was found, to a different degree, in seven of ten CMT1 cases. Importantly, these cases included only some of the CMT1 cases displaying intrafascicular vitronectin. A similar pattern of expression,

Table 3 Histopathological features of selected sural nerves in relation to the endoneurial expression of tenascin: density of myelinated fibers (MF/mm^2) and de-remyelination figures, onion bulbs, tomacula, axonal ovoids, regeneration clusters (– none; + moderate, ++ intermediate, +++ marked presence)

Case	Age/sex	Diagnosis	Tenascin-positive nerve fibers	Myelinated fibers density (MF/mm^2)	Other histological features	
					Type	Degree
1	56/F	CMT1	+++	1,895	De-remyelination	+++
					Onion bulbs	+
					Ovoids	–
					Clusters	+
2	35/M	CMT1	+++	1,936	De-remyelination	+++
					Onion bulbs	++
					Ovoids	–
					Clusters	+
3	70/M	CMT1	+++	1,921	De-remyelination	+++
					Onion bulbs	+++
					Ovoids	–
					Clusters	++
4	28/F	CMT1	–	1,850	De-remyelination	++
					Onion bulbs	++
					Ovoids	–
					Clusters	–
5	70/M	CMT1	–	1,750	De-remyelination	+++
					Onion bulbs	+++
					Ovoids	–
					Clusters	+
6	27/M	HNPP	++	4,424	De-remyelination	+++
					Tomacula	++
					Onion bulbs	–
					Ovoids	–
					Clusters	+
7	70/M	CIDP	++	3,392	De-remyelination	+++
					Onion bulbs	–
					Ovoids	–
					Clusters	+

but with a minor number of tenascin-positive fibers, was also observed in two of three HNPP nerves. On the other side, only one of the eight nerves affected by CIDP (different from the one with increased vitronectin), presented a milder but more diffused increase in intrafascicular tenascin, and no increase was observed in AMN or axonal neuropathy nerve biopsies. Due to perineurial hypertrophy, an increased deposition of tenascin was observed in this compartment in some CMT1-affected nerves, as reported for the other ECM molecules.

In the attempt to find a possible relation between the presence of tenascin-positive nerve fibers and specific histopathological features, the density of myelinated fibers and the prevalence of de-remyelination figures, onion bulbs, ovoids and clusters of axonal regeneration were evaluated in semithin sections and teased fibers preparations from CMT1 biopsies either highly positive (n=3) or negative (n=2) for tenascin-expressing fibers. The same analysis was performed in control disease samples displaying increased endoneurial tenascin expression. As shown in Table 3, no evident relation was found between the increased deposition of the molecule and the parameters taken into account. The presence of tenascin-negative axonal ovoids was also observed in two pathological nerve sections immunostained with the anti-tenascin antibody (data not shown).

Discussion

In this study we compared the expression and distribution of ECM molecules in normal and CMT1-affected sural nerves, as well as in nerves from patients affected by HNPP, AMN, CIDP and chronic idiopathic axonal neuropathy, as control diseases. Other authors have described the expression of some ECM molecules in normal peripheral nerve, either human or animal, and our results are generally in agreement with their reports [1, 3, 27, 33]. More interestingly, we show that, in comparison with our pathological controls, CMT1 disease seems to determine the most extensive quantitative and qualitative remodeling of peripheral nerve ECM. In fact, if the collagens, fibronectin and laminin were up regulated in all the pathological conditions investigated, the extent of this up-regulation was generally higher in CMT1 cases. Furthermore, ECM molecules such as vitronectin and tenascin appeared preferentially overexpressed in CMT1 compared to other pathologies here studied.

We report a conspicuous increase in type I and III collagen in CMT1 biopsy specimens and, to a minor degree, in our control disease cases. Therefore, we interpret this finding as due to both the severity of nerve fiber loss and the duration of the disease, rather than to an intrinsic metabolic feature of Schwann cells harboring CMT1 genetic mutations. In fact the intrafascicular up-regulation of these interstitial fibrillar collagens is generally considered as a reaction of endoneurial cellular elements to nerve fiber degeneration, as it has been previously described in studies on human neuropathies as well as in animal studies after peripheral nerve injury [1, 35]. It is worth noting that denervated Schwann cells, which are particularly abundant in CMT1 nerves, express transforming growth factor-beta 1, which in turn can alter and enhance the expression of ECM molecules [36]. Molecules that are up regulated in the vicinity of nerve injury are likely to be involved in the regulation of axonal re-growth, suggesting that the increased collagen deposition could be induced to support axonal regeneration. This view is indirectly corroborated by experimental evidences showing that fibrillar collagens are able to promote neurite outgrowth in vitro [2, 31, 43] and to support peripheral nerve regeneration in vivo [41, 44, 45]. On the other hand, it is possible that taking the disease and the consequent collagen deposition a chronic course, as in the case of CMT1, the high collagen content could represent a mechanical barrier and thus hamper the regeneration process [1, 35, 44].

Type IV and V collagens and the multi-domain glycoproteins fibronectin and laminin are also induced after peripheral nerve injury and have been proved to play important roles for neurite elongation and for ensheathment and myelination of axons by Schwann cells [2, 3, 8, 10, 24, 43]. The deposition of these ECM molecules, which were variably increased in all the pathological conditions included in this study, again appeared to reach the highest levels in CMT1 nerve biopsy specimens, especially in areas encircling myelinated fibers. Similar levels of up-regulation were also found in some CIDP biopsy samples. This conspicuous deposition can again be related to the abundance of denervated Schwann cells, as well as to the presence of onion bulb formations. Moreover, it is possible that due to the recurrent remyelination processes occurring in these neuropathies, especially in CMT1, Schwann cells are in a metabolically activated state, which may render them incline to deposit increased amounts of basement membrane components and associated molecules. In our material an increased deposition of type VI collagen paralleled that of type IV and type V. In agreement with our results, the expression of this microfibrillar collagen has been previously reported in normal human peripheral nerve in association with Schwann cells tubes, perineurial and epineurial cells [1, 27]. However, according to our findings, type VI collagen displayed a partially different distribution compared to that reported by other authors since, unlike type IV and V, it appeared to be expressed diffusely, even if in limited amounts, in the endoneurial stroma of normal nerve. This quantitative discrepancy is probably imputable to the different sensitivity of the antibody or of the technique utilized in this study. Increased type VI collagen deposition is a common finding in several fibrotic processes such as neurofibroma and keloid [27, 28]. On the other hand, little information is available with respect to the functional role of this collagen in the physiopathology of the peripheral nervous system (PNS). It has been reported that type VI collagen is expressed during development of both animal and human nerves, being regarded as an organizer of ECM architecture, and that in some experimental models it acts as a non-permissive substrate for neuritogenesis [2, 16, 28, 29]. Of note, most of the studies regarding type VI collagen expression in peripheral nerve deal with its increased deposition in the perineurium and endoneurium of nerves affected by diabetic polyneuropathy, and even in this pathology it is still uncertain whether and how such an increase could contribute to the impairment of neuronal functions [1, 28].

Vitronectin is a multifunctional glycoprotein present both in plasma and in the ECM of several tissues. It is implicated in cell adhesion, migration, proliferation and differentiation, as well as in processes such as coagulation, fibrinolysis, regulation of the complement- and perforin-mediated cytolysis and, through its ability to bind collagen and glycosaminoglycans, in the organization of the extracellular microenvironment. Moreover, its expression has been found to rise in pathological conditions such as neoplasia and in fibrotic and necrotic areas [37, 38]. Nonetheless, vitronectin expression in normal and pathological peripheral nerve has received little attention. In agreement with our findings, its distribution in the perineurium and vessel walls of normal peripheral nerve has been marginally reported in studies whose main object was the role of the complement system in neuropathies of different origin [34]. In fact, since vitronectin is an inhibitor of the complement membrane attack complex, its presence is considered an indicator of the complex functional state [38]. An increase in its deposition, associated with increased expression of complement proteins, has actually been found in endoneurial vessels of patients with diabetic and amyloid AL neuropathy [34, 46]. However, to our knowledge, there are no reports of such a widespread and marked endoneurial immunolabeling for vitronectin as the one we observed in CMT1- and AMN-affected nerves. Due to the abundance of this glycoprotein in normal plasma, we cannot rule out that the high amount of vitronectin we found in the endoneurium of pathological nerve biopsy specimens is of vascular origin, instead of being synthesized by endoneurial cells, although our strong and uniform vitronectin immunostaining seems to argue against this hypothesis. A further element inconsistent with this interpretation is that the intrafascicular deposition of plasma vitronectin would most likely require an alteration of the blood-nerve barrier. Therefore, this glycoprotein should be conspicuous in the endoneurium of nerves affected by inflammatory neuropathies, in which blood-nerve barrier alterations are more frequent and severe. On the contrary, in our material a moderate vitronectin immunolabeling was observed in only one out of eight CIDP nerve biopsy specimens, while a strong immuno-

staining was found in the majority of CMT1- and AMN-affected nerves. These observations indicate that the high vitronectin content we observed in pathological nerves is probably due to local synthesis. It is worth underlining that the intensity of the endoneurial vitronectin immunolabeling did not seem to be related to the extent of type I and type III collagen endoneurial expression, as revealed by the comparison of our vitronectin positive (9/31) and negative (22/31) pathological nerves. This finding suggests that vitronectin induction may not be simply related to the general fibrotic response accompanying nerve fiber loss. Vitronectin has been found to stimulate axonal growth from different types of neurons in vitro [13, 17, 25], and integrin receptors for vitronectin are expressed by Schwann cells [7]. Therefore, the induction of this molecule in the endoneurial microenvironment could influence both axonal and Schwann cell behavior. Moreover, vitronectin could indirectly participate in the remodeling of the endoneurial microenvironment, through the modulation of proteases active on ECM components [5, 37]. Further studies are thus necessary to establish which is the source and what role could be played by this glycoprotein in the pathogenesis of CMT1 and other human neuropathies.

In the past years, from research performed on animal models, tenascin has emerged as a mediator of neuron-glia interactions in both the central nervous system and the PNS [22, 33]. The expression of this ECM glycoprotein, characterized by adhesive and counter-adhesive properties, is transiently induced during development, being subsequently down regulated so that in the adult it is low and restricted to limited regions. It has been found to rise again, however, if a remodeling process such as tissue repair or tumorigenesis occurs [18, 19]. This general paradigm is well represented in the PNS. In fact, high levels of tenascin expression are found in developing peripheral nerves and myelinating Schwann cells, whereas a restricted tenascin expression, limited to the perineurium and to the nodes of Ranvier, is typical of the adult nerve [4, 19, 33]. The reported distribution concurs with our results on normal nerve biopsy samples. Conversely, endoneurial tenascin up-regulation and diffusion along the internodes was a frequent finding in our CMT1 and HNPP nerve specimens and, even if less prominently, was also a feature of one of the eight CIDP biopsy samples included in this study. Until recently tenascin-C was considered to be the only isoform expressed in the mammalian PNS; however, lately it has been demonstrated that tenascin-R and -X are also present during peripheral nerve development, and that tenascin-R is induced after injury of the mouse sciatic nerve [18, 19, 30]. Since the antibody used in this study does not discriminate between tenascin-C, -R, and -X, it would be interesting to ascertain whether specific tenascin variants are selectively overexpressed in CMT1 and in other human neuropathies.

No evident relation was found between the presence of tenascin-positive nerve fibers and the prevalence of histopathological features such as de-remyelination figures, onion bulbs, ovoids of axonal degeneration and regeneration clusters. Moreover, axonal ovoids were not immunostained in sections displaying tenascin-positive nerve fibers. Interestingly, the lack of a univocal relation between tenascin overexpression and the presence of specific histopathological figures has also been reported by Fruttiger et al. [9] in the myelin-associated glycoprotein (MAG)-deficient mouse peripheral nerve. In fact in their study tenascin was detected by immunoelectron microscopy in the ECM surrounding Schwann cells devoid of axonal contact, in association with some, but not all, the onion bulb formations, as well as in the basal lamina of a few morphologically normal myelinating Schwann cells [9]. A tenascin up-regulation similar to that observed here has also been described in other animal models of CMT1, such as the *trembler* mouse and the P0-deficient mouse, but not in human neuropathies [9, 23, 33]. Research performed on these models and nerve transection studies suggest that the presence of tenascin-expressing nerve fibers can be indicative of regenerative events [9, 10, 23, 33]. That tenascin can have a pro-regenerative role in the PNS is sustained by the observation that in the distal stump of transected nerves its content rises during the degeneration phase, to return to background levels after reinnervation, and by the demonstration that this ECM molecule is able to promote neurite outgrowth from peripheral neurons in vitro [4, 6]. In addition, it has been proposed that tenascin could mediate the clustering of the voltage-gated sodium channels at the nodal regions during both development and remyelination [22, 42].

We found a variable number of tenascin-expressing nerve fibers in the majority, but not in all, the CMT1 and HNPP nerve biopsy specimens, and in one CIDP nerve sample. These findings, coupled with the reported literature, underline that tenascin up-regulation is not specifically associated with a type of neuropathy, but rather reflects a particular functional stage of Schwann cells involved in axonal regeneration and/or remyelination processes. However, it is possible that Schwann cells harboring genetic mutations such as those typical of CMT1 and HNPP could remain longer in this functional stage which is consequently more represented in these than in other neuropathies. This hypothesis is consistent with reports suggesting that an abnormal Schwann cell differentiation is a feature of CMT1 disease [14]. Importantly, as previously reported, molecules that are transiently upregulated to support regenerative functions can, on the other hand, interfere with regeneration processes if chronically accumulated in the endoneurial microenvironment [28]. It is noteworthy that tenascin can also induce matrix metalloproteases, which in turn have recently been implied in several neurological disorders and have been shown to degrade myelin proteins, thereby possibly contributing to myelin damage [15, 19, 21].

In this report we have shown that CMT1 is characterized by complex ECM changes. These are probably secondary to degenerative events occurring in the endoneurium, as it is in other neuropathies. On the other hand in CMT1, at variance with other neuropathies, Schwann cells have been shown to exhibit an impaired phenotype and an abnormal differentiation, which can lead to an altered ECM

remodeling. Functional studies should further investigate whether specific ECM molecules chronically overexpressed in CMT1 nerves can contribute to nerve fiber damage or hamper an efficient regeneration.

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References

1. Bradley JL, King RHM, Muddle JR, Thomas PK (2000) The extracellular matrix of peripheral nerve in diabetic polyneuropathy. Acta Neuropathol 99:539–546
2. Carri NG, Rubin K, Gullberg D, Ebendal T (1992) Neuritogenesis on collagen substrates. Involvement of integrin-like matrix receptors in retinal fibre outgrowth on collagen. Int J Dev Neurosci 10:393–405
3. Chernousov MA, Carey DJ (2000) Schwann cell extracellular matrix molecules and their receptors. Histol Histopathol 15:593–601
4. Chiquet M, Wehrle-Haller B (1994) Tenascin-C in peripheral nerve morphogenesis. Perspect Dev Neurobiol 2:67–74
5. Ciambrone GJ, McKeown-Longo PJ (1992) Vitronectin regulates the synthesis and localization of urokinase-type plasminogen activator in HT-1080 cells. J Biol Chem 19:13617–13622
6. Daniloff KJ, Crossin KL, Pincon-Raymond M, Murawsky M, Rieger F, Edelman GM (1989) Expression of cytotactin in the normal and regenerating neuromuscular system. J Cell Biol 108:625–635
7. Detrait E, Laduron S, Meremans V, Baron-Van Evercooren A, Bosch de Aguilar P van den, Knoops B (1999) Expression of integrins by murine MSC80 Schwann cell line: relationship to cell adhesion and migration. Neurosci Lett 267:49–52
8. Fernandez-Valle C, Fregien N, Wood PM, Bunge MB (1993) Expression of the protein zero myelin gene in axon-related Schwann cells is linked to basal lamina formation. Development 119:867–880
9. Fruttiger M, Montag D, Schachner M, Martini R (1995) Crucial role for the myelin-associated glycoprotein in the maintenance of axon-myelin integrity. Eur J Neurosci 7:511–515
10. Fu SY, Gordon T (1997) The cellular and molecular basis of peripheral nerve regeneration. Mol Neurobiol 14:67–117
11. Gabreels-Festen AA, Bolhuis PA, Hoogendijk JE, Valenttijn LJ, Eshuis EJ, Gabreels FJ (1995) Charcot-Marie-Tooth disease type 1A: morphological phenotype of the 17p duplication versus PMP22 point mutations. Acta Neuropathol 90:645–649
12. Gabreels-Festen AAWM, Gabreels FJM, Jennekens FGI (1993) Hereditary motor and sensory neuropathies. Present status of types I, II and III. Clin Neurol Neurosurg 95:93–107
13. Grabham PW, Gallimore PH, Grand RJ (1992) Vitronectin is the major serum protein essential for NGF-mediated neurite outgrowth from PC12 cells. Exp Cell Res 202:337–344
14. Hanemann CO, Muller HW (1998) Pathogenesis of Charcot-Marie-Tooth 1A (CMT1A) neuropathy. Trends Neurosci 21: 282–286
15. Hartung HP, Kieseier BC (2000) The role of matrix metalloproteinases in autoimmune damage to the central and peripheral nervous system. J Neuroimmunol 107:140–147
16. Hirvonen OM, Peltonen SA, Hirvonen HE, Bjorkstrand AS, Peltonen JT (1996) Expression of laminin and collagen genes by cells cultured from developing human sciatic nerves. J Peripher Nerv Syst 1:42–52
17. Isahara K, Yamamoto M (1995) The interaction of vascular endothelial cells and dorsal root ganglion neurites is mediated by vitronectin and heparan sulfate proteoglycans. Brain Res Dev Brain Res 84:164–178
18. Joester A, Faissner A (2001) The structure and function of tenascins in the nervous system. Matrix Biol 20:13–22
19. Jones FS, Jones PL (2000) The tenascin family of ECM glycoproteins: structure, function, and regulation during embryonic development and tissue remodeling. Dev Dyn 218:235–259
20. Kamholz J, Menichella D, Jani A, Garbern J, Lewis RA, Krajewski KM, Lilien J, Scherer SS, Shy ME (2000) Charcot-Marie-Tooth disease type I. Molecular pathogenesis to gene therapy. Brain 123:222–233
21. Leppert D, Hughes P, Huber S, Erne B, Grygar C, Said G, Miller KM, Steck AJ, Probst A, Fuhr P (1999) Matrix metalloproteinase upregulation in chronic inflammatory demyelinating polyneuropathy and nonsystemic vasculitic neuropathy. Neurology 53:62–70
22. Martini R (2001) The effects of myelinating Schwann cells on axons. Muscle Nerve 24:456–466
23. Martini R, Zielasek J, Toyka KV, Giese KP, Schachner M (1995) Protein zero (P0)-deficient mice show myelin degeneration in peripheral nerves characteristic of inherited human neuropathies. Nat Genet 11:281–286
24. Mirsky R, Jessen KR (1999) The neurobiology of Schwann cells. Brain Pathol 9:293–311
25. Neugebauer KM, Emmett CJ, Venstrom KA, Reichardt LF (1991) Vitronectin and thrombospondin promote retinal neurite outgrowth: developmental regulation and role of integrins. Neuron 6:345–358
26. Pareyson D (1999) Guidelines for the diagnosis of Charcot-Marie-Tooth disease and related neuropathies. Ad hoc Working Group of the Peripheral Nervous System Study Group, Italian Neurological Society. Ital J Neurol Sci 20:207–216
27. Peltonen J, Jaakkola S, Hsiao LL, Timpl R, Chu ML, Uitto J (1990) Type VI collagen. In situ hybridizations and immunohistochemistry reveal abundant mRNA and protein levels in human neurofibroma, schwannoma, and normal peripheral nerve tissues. Lab Invest 62:487–492
28. Peltonen JT, Kalliomaki MA, Muona PKM (1997) Extracellular matrix of peripheral nerves in diabetes. J Peripher Nerv Syst 2:213–226
29. Perris R, Kuo HJ, Glanville RW, Bronner-Fraser M (1993) Collagen type VI in neural crest development: distribution in situ and interactions with cells in vitro. Dev Dyn 198:135–149
30. Probstmeier R, Nellen J, Gloor S, Wernig A, Pesheva P (2001) Tenascin-R is expressed by Schwann cells in the peripheral nervous system. J Neurosci Res 64:70–78
31. Reichardt LF, Tomaselli KJ (1991) Extracellular matrix molecules and their receptors. Annu Rev Neurosci 14:531–570
32. Reilly MM (1998) Genetically determined neuropathies. J Neurol 245:6–13
33. Rieger F, Daniloff JK, Pincon-Raymond M, Crossin KL, Grumet M, Edelman GM (1986) Neuronal cell adhesion molecules and cytotactin are co-localized at the node of Ranvier. J Cell Biol 103:379–391
34. Rosoklija GB, Dwork AJ, Younger DS, Karlikaya G, Latov N, Hays AP (2000) Local activation of the complement system in endoneurial microvessels of diabetic neuropathy. Acta Neuropathol 99:55–62
35. Salonen V, Lehto M, Vaheri A, Aro H, Peltonen J (1985) Endoneurial fibrosis following nerve transection. An immunohistological study of collagen types and fibronectin in the rat. Acta Neuropathol (Berl) 67:315–321
36. Scherer SS (1997) The biology and pathobiology of Schwann cells. Curr Opin Neurol 10:386–397
37. Schvartz I, Seger D, Shaltiel S (1999) Vitronectin. Int J Biochem 31:539–544
38. Seiffert D (1997) Constitutive and regulated expression of vitronectin. Histol Histopathol 12:787–797
39. Senderek J, Hermanns B, Bergmann C, Boroojerdi B, Bajbouj M, Hungs M, Ramaekers VT, Quasthoff S, Karch D, Schroder JM (1999) X-linked dominant Charcot-Marie-Tooth neuropathy: clinical, electrophysiological, and morphological phenotype in four families with different connexin32 mutations. J Neurol Sci 167:90–101

40. Senderek J, Hermanns B, Lehmann U, Bergmann C, Marx G, Kabus C, Timmerman V, Stoltenburg-Didinger G, Schroder JM (2000) Charcot-Marie-Tooth neuropathy type 2 and P0 point mutations: two novel amino acid substitutions (Asp61Gly; Tyr119Cys) and a possible "hotspot" on Thr124Met. Brain Pathol 10:235–248
41. Siironen J, Vuorinen V, Taskinen HS, Roytta M (1995) Axonal regeneration into chronically denervated distal stump. 2. Active expression of type I collagen mRNA in epineurium. Acta Neuropathol 89:219–26
42. Srinivasan J, Schachner M, Catterall WA (1998) Interaction of voltage-gated sodium channels with the extracellular matrix molecules tenascin-C and tenascin-R. Proc Natl Acad Sci USA 95:15753–15757
43. Venstrom KA, Reichardt LF (1993) Extracellular matrix. 2. Role of extracellular matrix molecules and their receptors in the nervous system. FASEB J 7:996–1003
44. Vuorinen V, Siironen J, Roytta M (1995) Axonal regeneration into chronically denervated distal stump. 1. Electron microscope studies. Acta Neuropathol 89:209–218
45. Yoshii S, Oka M (2001) Peripheral nerve regeneration along collagen filaments. Brain Res 888:158–162
46. Zanusso GL, Moretto G, Bonetti B, Monaco S, Rizzuto N (1992) Complement neoantigen and vitronectin are components of plaques in amyloid AL neuropathy. Ital J Neurol Sci 13:493–499