Morphometric analysis of sural nerve biopsies
in Familial Amyloid Polyneuropathy
Armindo Picão Fernandes

2018
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Original Paper

Dissertação do Mestrado Integrado em Medicina

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Porto, Maio 2018
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AGRADECIMENTOS

Ao Doutor Ricardo Taipa, meu orientador, por todos os ensinamentos, pela disponibilidade e pelo apoio, e por desde cedo no meu percurso académico ter contribuído para o meu entusiasmo pelas Neurociências.

À Dr.ª Teresa Coelho, minha coorientadora, por me ter apresentado o mundo da PAF e pelos preciosos conhecimentos transmitidos.

Ao Prof. Doutor Pedro Oliveira pela importante ajuda no tratamento estatístico dos dados.

À minha família e amigos, por todo o amparo, pela compreensão e pela confiança incondicionais ao longo destes anos.

À Rita, minha namorada, por continuar a ser uma pedra basilar no trilho deste percurso.

A todos, bem hajam por terem contribuído para a realização desta dissertação.
Morphometric analysis of sural nerve biopsies in Familial Amyloid Polyneuropathy

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ABSTRACT

Introduction: Familial amyloid neuropathy (FAP) with transthyretin Val30Met mutation (ATTR-FAP) is the most common type of FAP. Although several authors have previously reported a size-dependent fiber loss in ATTR-FAP, predominantly involving unmyelinated and small diameter myelinated fibers (MF), the mechanisms of nerve fiber loss have not been fully understood.

Objective: To establish the morphometric pattern of peripheral neuropathy in hereditary ATTR-FAP (symptomatic and asymptomatic subjects) and acquired form of ATTR-FAP, and to compare pathological and clinical features between these groups.

Methods: We analyzed sural nerve biopsies from 98 patients with Val30Met ATTR-FAP, 37 ATTR-FAP asymptomatic mutation carriers, and 10 patients with acquired ATTR-FAP, aged between 17 and 84 years, which were performed between 1981 and 2017 at Centro Hospitalar do Porto.

Results: The mean age at nerve biopsy was 32.2 ± 13.3 years for asymptomatic mutation carriers, 47.5 ± 14.8 years for ATTR-FAP patients, 62.4 ± 3.7 years for de novo ATTR-FAP, and 46.5 ± 18.4 years for controls. The mean duration between nerve biopsy and symptoms onset was 9.2 ± 7.3 years (range from 1 to 27 years) in the asymptomatic carriers, and from liver transplantation to disease onset was 7.1 ± 2.7 years (range from 4 to 13 years) in the acquired ATTR-FAP cases. ATTR-FAP patients had loss of all fiber type modalities compared to both asymptomatic carriers and controls (p<0.001), whereas asymptomatic carriers showed loss of small myelinated fibers when compared to controls (p<0.05). Acquired ATTR-FAP patients revealed similar pathological findings to their inherited counterparts, but with lesser degrees of fiber loss for the same disease clinical stage (p<0.01). There was a positive correlation between myelinated fiber density and time gap to symptoms onset in the asymptomatic carriers that developed early-onset form of the disease, being this correlation only statistically significant for the large MF (r=0.52, p<0.01). Subjects with amyloid deposition in sural nerve biopsies developed symptoms earlier than those with no amyloid. No statistical differences were observed regarding the MF density and the amyloid deposition between the early- and late-onset cases. The loss of MF increased with disease progression, and patients in more advanced clinical stage showed more frequent amyloid deposition in the nerve.
Conclusions: This study confirms that small fiber size loss is an initial event in ATTR-FAP, already present in asymptomatic gene carriers, starting several years before symptoms onset. We show for the first time that large MF loss and amyloid deposition are pathological features that correlate independently with short period to symptoms onset for asymptomatic carriers that developed early-onset form of the disease.

Keywords: amyloidosis; familial amyloid neuropathy; nerve fibers; pathology; sural serve; transthyretin
Análise morfométrica de biópsias de nervo sural na Polineuropatia Amiloidótica Familiar

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RESUMO

Introdução: A polineuropatia amiloidótica familiar (PAF) associada à mutação Val30Met no gene da transtirretina (PAF-ATTR) é a forma mais comum de PAF. Vários autores descreveram previamente uma perda de fibras tamanho dependente, com envolvimento preferencial das fibras não mielinizadas e das fibras mielinizadas (FM) de pequeno diâmetro, contudo esse mecanismo de perda não está totalmente esclarecido.

Objetivo: Estabelecer o padrão morfométrico de neuropatia periférica na forma hereditária (em sintomáticos e assintomáticos) e adquirida de PAF-ATTR, e comparar as características patológicas e clínicas entre os grupos.

Métodos: Analisámos biópsias de nervo sural de 98 doentes com a mutação Val30Met de PAF-ATTR, de 37 portadores assintomáticos e de 10 doentes com forma adquirida de PAF-ATTR, com idades entre os 17 e os 84 anos, que foram realizadas entre 1981 e 2017 no Centro Hospitalar do Porto.

Resultados: A idade média, em anos, à data da biópsia foi de 32.2 ± 13.3 nos portadores assintomáticos, 47.5 ± 14.8 nos doentes com PAF-ATTR, 62.4 ± 3.7 nos doentes com PAF-ATTR adquirida e 46.5 ± 18.4 nos controlos. A duração média, em anos, entre a biópsia de nervo e o início de sintomas foi de 9.2 ± 7.3 (variando entre 1 e 27) nos portadores assintomáticos, e entre a transplantação hepática e o início de sintomas foi de 7.1 ± 2.7 (variando entre 4 e 13) nos doentes com PAF-ATTR adquirida. Os doentes com PAF-ATTR apresentaram perda de todas as classes de fibras, comparando com os portadores assintomáticos e os controlos (p<0.001), enquanto que os portadores assintomáticos apresentaram perda das pequenas FM quando comparados com os controlos (p<0.05). Os doentes com PAF-ATTR adquirida revelaram achados patológicos idênticos aos homólogos hereditários, embora com uma taxa inferior de perda de fibras para os mesmos estádios clínicos (p<0.01). Encontrámos uma correlação positiva entre a densidade de fibras mielinizadas e o intervalo de tempo até ao início de sintomas nos portadores assintomáticos que desenvolveram a forma precoce da doença, sendo que esta correlação apenas se mostrou estatisticamente significativa para as FM de grande diâmetro (r=0.52, p<0.01). Indivíduos com depósitos de amiloide na biópsia desenvolveram sintomas mais precocemente do que os sem depósitos de amiloide. Não foram observadas diferenças estatísticas entre os doentes com forma precoce e os
doentes com a forma tardia de PAF-ATTR. A perda de FM aumentou com a progressão clínica da doença, e doentes em estádios clínicos mais avançados apresentaram deposição de amiloide na biópsia com maior frequência.
Conclusão: Este estudo confirma que a perda de pequenas fibras é um evento inicial na PAF-ATTR, estando já presente em portadores assintomáticos, e que começa anos antes do início de sintomas. Mostramos, pela primeira vez, que a perda de grandes FM e a deposição de amiloide são características patológicas que se correlacionam de forma independente com períodos mais curtos para o início de sintomas em portadores assintomáticos que desenvolveram a forma precoce da doença.

Palavras-chave: amiloidose; fibras nervosas; nervo sural; neuropatia amiloidótica familiar; patologia; transtirretina
LIST OF ABBREVIATIONS

ATTR – Transthyretin
ATTR-FAP - Familial Amyloid Polyneuropathy with transthyretin mutation
CHP – Centro Hospitalar do Porto
DLT – Domino liver transplantation
FAP – Familial Amyloid Polyneuropathy
MF – Myelinated fibers
PND – Polyneuropathy disability
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INTRODUCTION

Familial amyloid polyneuropathy (FAP) is a hereditary multisystemic disease, with autosomal dominant transmission, characterized by the extracellular deposition of insoluble fibril aggregates, predominantly affecting unmyelinated and small diameter myelinated fibers. FAP can be divided into three main types, considering the precursor of the amyloid protein – transthyretin (ATTR), apolipoprotein A-1 (AApoAI) and gelsolin (AGel). The amyloidosis resulting from the deposition of mutated transthyretin is, among all the forms, the most common and has the worst prognosis, leading to patients’ death within an average period of 10 years after diagnosis.\textsuperscript{1, 2}

Corino de Andrade first described FAP in 1952\textsuperscript{3}, and later the genetic basis was identified: the substitution of methionine for valine at position 30 (Val30Met).\textsuperscript{4} Currently, over 150 mutations of the TTR gene are known, most of them amyloidogenic, producing a broad range of phenotypes.\textsuperscript{5}

Clinically, FAP with transthyretin mutation (ATTR-FAP) is characterized by an axonal, sensorimotor and autonomic polyneuropathy, with onset of symptoms during third and fifth decades of life in early-onset patients, and decades later in the late-onset FAP patients.\textsuperscript{1, 6-8} These two groups differ in some clinical and pathological features; nonetheless, the reason for that has not yet been fully elucidated.\textsuperscript{6-10} Extra-neurological manifestations, including, renal, ocular, cardiac, hematological, and metabolic abnormalities may also be present, increasing morbidity and mortality in these patients.\textsuperscript{5, 11-13} Even though the Nomenclature Committee of International Society of Amyloidosis (ISA) recommends the use of the protein name to refer the disease, FAP is also considered an appropriated term as it is widely used in the neurology literature.\textsuperscript{14}

Recently, several authors have described a form of acquired ATTR amyloidosis in patients undergoing Domino Liver Transplantation (DLT), a surgical procedure in which the liver from an ATTR-FAP patient (functionally and anatomically normal) is removed and transplanted into another recipient, whom starts developing \textit{de novo} amyloid neuropathy after a median of 7 years following transplantation.\textsuperscript{15-18}

Morphometry of sural nerve biopsies is an important diagnostic tool of peripheral neuropathies as it provides quantitative and objective details, and enables us to evaluate the progression of disease and the treatment response.\textsuperscript{19, 20} However, this is an invasive procedure, nowadays reserved for selected cases only. Three compartments form part of the basic structure of the sural nerve – the epineurium, perineurium and endoneurium, and five to fifteen nerve fascicles are usually present in it.\textsuperscript{21, 22} The normal density of myelinated axons is between 7000 and 10000 fibers/mm\textsuperscript{2} in young adults, with an age-
dependent decrease. The diameter of the myelinated fibers ranges from 2 to 17µm, showing a bimodal distribution with peaks between 3-6µm and 9-12µm. The g-ratio (ratio of axon diameter to fiber diameter) is useful to assess the severity of demyelination, and in normal conditions varies between 0.5 and 0.7.

Previous morphological and morphometric studies of sural nerve biopsies in ATTR-FAP have already reported the pathological changes in nerve fibers, but the exact mechanism of nerve fiber loss remains unknown. The primary objective of this study was to establish the morphometric pattern of peripheral neuropathy in hereditary and acquired ATTR amyloidosis, and to assess if there is any relation between the density of myelinated fibers and the clinical characteristics of the disease. Some of these results had been previously reported. We extended the number of analyzed cases in the asymptomatic gene carriers and ATTR-FAP patients, added follow-up clinical data for analysis, and studied for the first time patients with acquired ATTR amyloidosis. The establishment of the morphometric pattern, as well as the identification of potential clinical correlations, is therapeutically relevant, as it would allow for a better interpretation of the role of disease-modifying agents in ATTR-FAP.
MATERIALS AND METHODS

Patients

We analyzed sural nerve biopsies from 98 patients with Val30Met ATTR-FAP, 37 ATTR-FAP asymptomatic mutation carriers, and 10 patients with acquired ATTR amyloidosis, aged between 17 and 84 years, which were performed between 1981 and 2017 at hospital Centro Hospitalar do Porto (CHP). Patients with an onset of symptoms before the age of 50 were classified as early-onset cases, and those with an onset age over 50 years were classified as late-onset cases.8,9,29

Thirty-one sural nerve biopsies from subjects who were classified as normal by experienced neuropathologists have been selected from the Neuromuscular Database of the Neuropathology Unit of CHP and used as control. Additionally, clinical records were reviewed to exclude history of any neurological disorder that could affect the peripheral nerve composition.

All aspects associated with the current study were approved by the Ethics Committee of CHP.

Clinical and demographic assessments

Clinical and demographic data were obtained from the database of the Corino de Andrade Unit and by consulting the patients’ clinical records. The following data were collected: gender, age at onset of symptoms, age at the time of sural nerve biopsy, age at the time of DLT, presence of family ATTR amyloidosis history, and presence of comorbidities. The family ATTR amyloidosis history was considered to be positive in cases with the presence of one first or second-degree relative with symptomatic ATTR-FAP.

The Polyneuropathy Disability (PND) score, an useful disease staging tool in the evaluation of peripheral sensory and motor disturbances in FAP patients12, was calculated by consulting patients’ medical records, in order to establish the stage of disease at the time of nerve biopsy. ATTR-FAP patients were stratified by the following PND stages: stage I – sensory disturbances with preserved walking capability; stage II – sensory and motor deficits but ability to walk without any support; stage IIIA – walking only with the help of one stick or crutch; stage IIIB – walking with the help of two sticks or crutches; stage IV - confined to a wheelchair or bedridden.

Sural nerve specimens

The sural nerve was exposed at the level of lateral malleolus, under local anesthesia, and a fascicular nerve biopsy was performed.30, 31 The sural nerve specimens were divided into two portions. The first one was fixed in 2,5% glutaraldehyde on phosphate-
buffered saline solution at pH 7.4, dehydrated with numerous alcohol passages and embedded in epoxy resin. Semithin transverse sections (1 µm thick) were cut and stained with 1% toluidine blue solution for morphometric study. The second portion of the specimen was fixed in 10% formalin solution and embedded in paraffin. Sections were cut by routine methods and stained with hematoxylin and eosin and Congo red. The presence of amyloid deposits was detected by Congo red staining, exhibiting an apple-green birefringence under polarized light (figure 1).

**Morphometric analysis**

A semi-automated analysis of the density of myelinated fibers was carried out using the Leica Application Suite (LAS) V4.5 and Fiber software. For each nerve, 3 to 4 digital images were captured by a digital camera (Leica MC170 HD) incorporated on a light microscope (Leica DM 4000 B), under a 63x objective, in order to cover the maximum nerve area (figure 2). The total, the large (>7 µm), and the small (≤ 7 µm) myelinated fiber (MF) densities were calculated, as also as the mean fiber diameter, the mean g-ratio and the ratio of small to large MF densities for patients with a total myelinated fiber density ≥100/mm².

**Statistical analysis**

Subjects’ data are reported as numbers and percentages for categorical variables, and as medians with interquartile range medians or as means ± SD for continuous variables.

Due to asymmetrical distribution and non-homogeneity of variances of the majority of the parameters, statistical analyses were performed with the non-parametric tests Pearson's Chi-square, Mann-Whitney test, and Kruskal-Wallis test for independent samples, using IBM SPSS® Statistics24 software. Values of $p<0.05$ were considered significant.
Figure 1. Representative characteristics of amyloid deposition in the sural nerve from a ATTR-FAP patient. (A) Hematoxylin and eosin staining. (B) Immunostaining with anti-human transthyretin (TTR) antibody. (C) Congo red staining. (D) Congo red staining under polarized light.

Figure 2. Semi-automated morphometry. Representative semithin cross-section of a sural nerve’s endoneurial area from an ATTR-FAP patient, under a x63 objective. (A to D). (B) Manual correction to remove the wrongly delineated areas. (C and D) Myelin sheaths marked in blue and axons marked in red.
RESULTS

1. Pathological findings in ATTR-FAP asymptomatic mutation carriers, ATTR-FAP patients and de novo ATTR amyloidosis patients.

Demographic and morphometric data are summarized in table I. The mean age at nerve biopsy was 32.2 ± 13.3 years for asymptomatic mutation carriers, 47.5 ± 14.8 years for ATTR-FAP patients and 62.4 ± 3.7 years for de novo ATTR amyloidosis patients. In the asymptomatic carriers, the mean duration between nerve biopsy and symptom onset was 9.2 ± 7.3 years, ranging from 1 to 27, and the mean time from symptom onset to patients’ death was 12.3 ± 4.0. In ATTR-FAP patients, the average time from onset of neuropathy to nerve biopsy was 3.4 ± 3.1 years, and the mean time from onset of symptoms to death was 10.8 ± 6.3 years. Except for the asymptomatic mutation carriers, the proportion of men was higher in the ATTR-FAP patients and control groups, although these differences were not statistically significant.

The de novo ATTR amyloidosis group included 9 men and 1 woman, none of whom had symptoms of polyneuropathy previously to DLT. The indications for liver transplantation were alcoholic cirrhosis (n=6), hepatocellular carcinoma (HCC) (n=1), hepatitis B virus (HBV) cirrhosis (n=1), hepatitis C virus (HCV) cirrhosis (n=1), and hemochromatosis (n=1). These patients underwent DLT between 2001 and 2007. The mean age at DLT was 54.2 ± 4.9; the mean duration between DLT and onset of symptoms was 7.1 ± 2.7 years (range from 4 to 13 years). The mean time from onset of neuropathy to nerve biopsy was 1.1 ± 1.3 years, and the mean time between onset of symptoms and patients’ death was 3.8 ± 1.7 years.

Table I – Demographic, clinical and morphometric features of ATTR-FAP, acquired ATTR and control subjects

<table>
<thead>
<tr>
<th>Features</th>
<th>Controls (n=31)</th>
<th>Asymptomatic mutation carriers (n=37)</th>
<th>ATTR-FAP patients</th>
<th>Acquired ATTR amyloidosis patients (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male sex, % (n)</td>
<td>58.1 (18)</td>
<td>48.6 (18)</td>
<td>55.1 (54)</td>
<td>90 (9)</td>
</tr>
<tr>
<td>Age at onset, yr</td>
<td>NA</td>
<td>39.6 ± 13.4³</td>
<td>44.1 ± 14.3</td>
<td>61.1 ± 3.5</td>
</tr>
<tr>
<td>Age at nerve biopsy, yr</td>
<td>46.5 ± 18.4</td>
<td>32.2 ± 13.3</td>
<td>47.5 ± 14.8</td>
<td>62.4 ± 3.7</td>
</tr>
<tr>
<td>Pathology of the sural nerve</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total myelinated fiber density, n/mm²</td>
<td>9072 ± 1967</td>
<td>7766 ± 1286</td>
<td>1742 ± 2167</td>
<td>4138 ± 2815</td>
</tr>
<tr>
<td>Large myelinated fiber density, n/mm²</td>
<td>3940 ± 1031</td>
<td>4427 ± 1013</td>
<td>1060 ± 1211</td>
<td>2299 ± 1374</td>
</tr>
<tr>
<td>Small myelinated fiber density, n/mm²</td>
<td>5131 ± 1430</td>
<td>3339 ± 792</td>
<td>686 ± 1072</td>
<td>1839 ± 1543</td>
</tr>
<tr>
<td>Ratio small/large</td>
<td>1.2 [1.0-1.6]</td>
<td>0.8 [0.6-0.9]</td>
<td>0.6 [0.3-1.1]</td>
<td>0.6 [0.5-1.3]</td>
</tr>
<tr>
<td>Fiber diameter, µm</td>
<td>7.5 ± 0.6</td>
<td>8.6 ± 0.7</td>
<td>8.7 ± 1.8</td>
<td>8.4 ± 0.9</td>
</tr>
<tr>
<td>g-ratio⁵</td>
<td>0.52 ± 0.05</td>
<td>0.50 ± 0.06</td>
<td>0.49 ± 0.06⁵</td>
<td>0.48 ± 0.05</td>
</tr>
</tbody>
</table>

ATTR-FAP – familial amyloid polyneuropathy with transthyretin mutation; ATTR – transthyretin; NA – not applicable

³ Values are expressed as mean ± SD;

⁴ Values are expressed as median [interquartile range];

⁵ Results are presented for patients with total myelinated fiber densities ≥100/mm²

⁶ Results are presented for patients with total myelinated fiber densities ≥100/mm²

⁷ n=32 (from asymptomatic TTR mutation carriers who developed symptoms during the 27 years of clinical follow-up); *n=87; *n=54; *n=33
1.1 Comparison between ATTR-FAP asymptomatic mutation carriers, ATTR-FAP patients and controls

ATTR-FAP patients had a total MF density of $1742 \pm 2167$ fibers/mm$^2$, exhibiting loss of large (Z= −7.11; $p < 0.001$) and small MF (Z= −6.88; $p < 0.001$) when compared to controls and asymptomatic mutation carriers (figure 3). These patients also presented greater MF diameters compared to both controls and asymptomatic mutation carriers ($p < 0.001$) and lower mean g-ratio (Z= −2.94; $p = 0.01$) than controls.

The asymptomatic mutation carriers presented depletion of small MF (Z= −2.46; $p=0.041$) and greater mean MF diameter (Z= 4.29; $p < 0.001$) compared to controls. The g-ratio did not differ from control group.

The medians of the ratios of small to large MF densities of both ATTR-FAP patients and asymptomatic carriers were lower than the one of the controls (Z= −5.65, $p<0.001$; Z= −4.00, $p < 0.001$, respectively), meaning that the majority of these subjects had a higher proportion of large than small MF in relation to controls (figure 4).

Amyloid deposition was found in a significantly lower proportion of asymptomatic carriers than of ATTR-FAP patients (32% versus 86%, respectively; $p < 0.001$).

![Figure 3](image_url)

**Figure 3.** – Density of myelinated fibers (total, large and small) and distribution of the mean g-ratio in controls, asymptomatic mutation carriers and ATTR-FAP patients. ***$p\leq0.01$ ***$p\leq0.001$ (Kruskal-Wallis test)
Figure 4a. – Normal morphometric findings in sural nerve specimen. (A to C) Photomicrographs of 1µm transversal sections from a control’s sural nerve (stained with toluidine blue). Graphic D shows a diameter frequency histogram of myelinated fibers in sural nerve from a control, exhibiting a typical bimodal distribution of fiber diameters, with higher proportion of small myelinated fiber than of large myelinated fibers.

Figure 4b. – ATTR-FAP morphometric findings in sural nerve specimen. Photomicrographs of 1 µm transversal sections from a ATTR-FAP patient’s sural nerve (A) and an asymptomatic mutation carrier’s sural nerve (B) (stained with toluidine blue). In A, there is a severe depletion of both small and large myelinated fibers; in B there is a moderate loss of small myelinated fibers. Graphic C shows a diameter frequency histogram of myelinated fibers in sural nerve from a ATTR-FAP patient, exhibiting loss of the bimodal distribution of fiber diameters. Graphic D shows a diameter frequency histogram of myelinated fibers in sural nerve from an asymptomatic mutation carrier, presenting a higher peak of large than of small myelinated fibers, in contrast to the fiber distribution in controls.
2. Clinicopathological correlations
2.1. Asymptomatic mutation carriers

Correlation between MF density, amyloid deposition and time to disease onset

When considering the total group of asymptomatic mutation carriers, no correlation was found between the morphometric variables and the time gap to disease onset. Considering the subgroup that developed early-onset form of the disease, there was a positive correlation between total MF density and time to disease onset ($r = 0.52; p<0.01$). As shown in figure 5A, a higher density of MF at the time of nerve biopsy allowed a longer period free-of-disease. Interestingly, when analyzing this correlation separately according to the myelinated fiber size, this correlation was only significant for large MF density (figure 5B).

In this group, 12 cases presented amyloid in their sural nerve biopsy (32%). These cases developed symptoms earlier (the mean time to disease onset 4.4 ± 3.4 years) compared to the asymptomatic subjects without amyloid deposition (11.4 ± 7.6 years; $Z = −2.84, p=0.003$). This group showed younger age at symptom onset (32 years in amyloid group vs 43 in the non-amyloid group). Interestingly, when considering only the cases that developed the early-onset form of the disease, the time to disease onset remained shorter for the subjects with amyloid deposition (4.4 vs 8.1 years; $Z = −2.06, p = 0.041$) despite the similar age of onset between the groups (32 vs 35 years). Nevertheless, two patients with amyloid deposits in nerve biopsy started developing symptoms after a period of 10 years.

There were no differences in any of the evaluated morphometric parameters when comparing asymptomatic mutation carriers with or without amyloid deposits, considering either all subjects or the group that develop early-onset form of disease.

A multiple regression model, with the time gap from nerve biopsy to disease onset as the dependent variable, and myelinated fiber density, amyloid deposition and sex as predictors, showed that all these variables had a statistically significant contribution, with an explanatory power of $R^2=56\%$ of the observed variance. Moreover, amyloid deposition and sex had negative coefficients, meaning that the time gap between nerve biopsy and disease onset decreased with the increasing of the values of amyloid deposition, and was smaller for the male sex. On the other hand, a higher myelinated fiber density (with a small positive coefficient) implied an increasing of the dependent variable.
2.2. ATTR-FAP patients

**Correlation between MF density, amyloid deposition and clinical stages**

The distribution of the ATTR-FAP patients by clinical stages is summarized in table II. The PND score was assessed in 92 patients: 48.9% had only sensory disturbances (stage I), 44.6% exhibited impaired walking capability but without requiring any support (stage II), and 5.5% were able to walk only with help (stage III). One patient was confined to a wheelchair at time of nerve biopsy (stage IV). To establish a correlation between the total MF density and the clinical stage we considered only ATTR-FAP patients at PND stages I and II, due to the low number of patients in other clinical stages.

Patients in clinical stage I of PND score presented a mean density of MF per mm² of 2886 ± 2483, which revealed to be higher than of the patients in clinical stage II of PND score (720 ± 1313 MF/mm²; Z=-5.84, p<0.001). The same difference between the two clinical stages was also observed for the large (Z= -5.64, p<0.001) and small MF densities (Z= -5.61, p<0.001). The mean time between onset of symptoms and nerve biopsy was higher in patients with stage II compared to patients with stage I (4.8 ± 3.3 vs 1.9 ± 2.5 years, respectively; Z= 5.14, p<0.001). In the same direction, there was a weak negative correlation between the time from symptom onset to nerve biopsy and total MF densities (r= -0.266, p < 0.05). This negative correlation was stronger for large myelinated

**Figure 5.** – (A) Correlation between the total myelinated fiber density and the time to symptoms onset in ATTR-FAP patients who developed early form of disease (r=0.52; p=0.008). (B) Correlation between the large (blue) and small (red) myelinated fibers densities, and the time to symptoms onset in ATTR-FAP patients who developed early form of disease. A positive correlation was observed only between the large myelinated fibers density and the time to disease onset (r=0.52;p=0.008).
fibers density \((r= -0.370, p<0.001)\) and there was no correlation with small MF densities. We also found a weak positive correlation between MF densities (total and large MF) and disease duration (time to death) after nerve biopsy \((r=0.270, r=0.319; p<0.05;\) respectively).

Considering the amyloid deposition, all 41 patients in clinical stage II of PND score exhibited amyloid in their nerve biopsy \textit{versus} 35 of 45 patients in clinical stage I of PND score \((p=0.001)\). Comparing the MF density of ATTR-FAP patients according to the presence or absence of amyloid deposition \((10\) without amyloid \textit{versus} 76 with amyloid), the group with no amyloid showed higher total \((Z= -3.44, p=0.001)\), large \((Z= -3.42, p=0.001)\) and small \((Z= -3.46, p=0.001)\) MF densities than those with positive amyloid (table III). Disease duration and age at biopsy were similar in both groups. There was a tendency for age at disease onset to be lower in the group with amyloid \((p=0.051)\).

\textit{Early- versus late-onset cases (MF density and clinical stages)}

In the early-onset cases, the mean density of total MF was 1983 ± 2128 fibers/mm\(^2\) \((21\%\) of age-matched control density; \(p<0.001)\). Their mean density of large MF was 1262 ± 1277 fibers/mm\(^2\) \((29\%\) of age-matched control density; \(p<0.001)\) and that of small MF was 720 ± 947 fibers/mm\(^2\) \((14\%\) of age-matched control density; \(p<0.001)\). In late-onset cases, the mean density of total MF was 1361 ± 2203 fibers/mm\(^2\) \((17\%\) of age-matched control density; \(p<0.001)\), the mean density of large MF was 741 ± 1035 fibers/mm\(^2\) \((22\%\) of age-matched control density; \(p<0.001)\) and that of small MF was 632 ± 1256 fibers/mm\(^2\) \((14\%\) of age-matched control density; \(p<0.001)\).

The distribution of PND stages differed between these two groups; 61.5\% of the early-onset patients were at stage I at time of nerve biopsy against 38.5\% of the late-onset cases \((p=0.034)\), who were predominantly in advanced clinical stages. The late-onset cases presented a higher mean time gap from symptoms to nerve biopsy than did early-onset patients \((4.0±3.2 \textit{versus} 3.0±3.0\) years), however this difference did not reach statistical significance. In early-onset cases, a negative correlation was found between the time from onset of symptoms to nerve biopsy and the total and large MF densities \((r= -0.361, p=0.005; r= -0.439, p=0.001;\) respectively). This correlation was not found for the late-onset group.

Despite more frequent in the early onset group, no statistically significant differences were observed regarding the presence of amyloid deposition between the two groups \((92\% \textit{in early-} \textit{versus} 82\% \textit{in late-onset cases})\).

A positive family history was found in a higher proportion of early-onset cases than of late-onset cases \((82\% \textit{versus} 44\%,\) respectively; \(p<0.001)\).
Table II – Clinical stages of ATTR-FAP patients according to PND score

<table>
<thead>
<tr>
<th>PND score, % (n)</th>
<th>All patients (n=92)</th>
<th>Early-onset cases (n=55)</th>
<th>Late-onset cases (n=37)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage I</td>
<td>48.9 (45)</td>
<td>58.2 (32)</td>
<td>35.1 (13)</td>
</tr>
<tr>
<td>Stage II</td>
<td>44.6 (41)</td>
<td>36.4 (20)</td>
<td>58.8 (21)</td>
</tr>
<tr>
<td>Stage IIIa</td>
<td>3.3 (3)</td>
<td>1.8 (1)</td>
<td>5.4 (2)</td>
</tr>
<tr>
<td>Stage IIib</td>
<td>2.2 (2)</td>
<td>1.8 (1)</td>
<td>2.7 (1)</td>
</tr>
<tr>
<td>Stage IV</td>
<td>1.1 (1)</td>
<td>1.8 (1)</td>
<td>0</td>
</tr>
</tbody>
</table>

ATTR-FAP – familial amyloid polyneuropathy with transthyretin mutation; PND – Polyneuropathy Disability

Table III – Myelinated fiber densities of ATTR-FAP in PND stage ≤ II according to the presence or absence of amyloid deposits in sural nerve biopsy

<table>
<thead>
<tr>
<th>Pathology of the sural nervea</th>
<th>ATTR-FAP patients without amyloid deposits (n=10)</th>
<th>ATTR-FAP patients with amyloid deposits (n=76)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total myelinated fiber density, n/mm²</td>
<td>5244 ± 3660</td>
<td>1407 ± 1592</td>
<td>0.001</td>
</tr>
<tr>
<td>Large myelinated fiber density, n/mm²</td>
<td>2739 ± 1802</td>
<td>926 ± 1022</td>
<td>0.001</td>
</tr>
<tr>
<td>Small myelinated fiber density, n/mm²</td>
<td>2506 ± 1989</td>
<td>486 ± 696</td>
<td>0.001</td>
</tr>
</tbody>
</table>

ATTR-FAP – familial amyloid polyneuropathy with transthyretin mutation

Values are expressed as mean ± SD; Statistical analyses were performed using the Mann-Whitney U test.

2.3 De novo ATTR amyloidosis patients

Due to the advanced age at biopsy in de novo ATTR amyloidosis patients, clinical features and morphometric studies were compared with control and ATTR-FAP patients who had undergone nerve biopsy within the same age range than of de novo ATTR amyloidosis patients (58 to 69 years). The mean time from onset of neuropathy to nerve biopsy was shorter in de novo ATTR amyloidosis patients compared to ATTR-FAP patients (1.1 ± 1.3 years vs 5.3 ± 3.9 years; Z = 12.73, p<0.001), and the mean time between onset of symptoms and patients’ death was considerably lower in de novo ATTR amyloidosis patients (3.8 ± 1.7 years) when compared to the one in ATTR-FAP patients in the same preceding conditions (8.8 ± 2.7; Z= 6.951, p = 0.008).

The PND score at time of nerve biopsy was statistically different between the ATTR-FAP and the de novo ATTR amyloidosis patients, as the most of the latter were at stage I of PND score when compared to ATTR-FAP population (90% vs 33%, respectively; p=0.028). Due to this asymmetric distribution between the two patients’ group according to the clinical stages, and the absence of de novo ATTR amyloidosis patients in PND stages greater than II, we considered only stage I and II patients for morphometric studies.

The de novo ATTR amyloidosis population exhibited higher large (Z= −3.47; p =
0.002), small (Z= –2.98; p = 0.009), and therefore higher total (Z= –3.31; p = 0.003) MF densities than did ATTR-FAP patients. Despite their lower total, large and small MF densities, no statistically significant differences were observed in these parameters between de novo ATTR amyloidosis patients and controls. However, the de novo ATTR amyloidosis patients showed lower ratio of small to large MF densities than did controls (Z= –2.50; p = 0.037).

Considering the same time gap from onset of symptoms to nerve biopsy in the same clinical stages, de novo ATTR amyloidosis patients continued to show higher total (Z= –3.15; p = 0.005), large (Z= –3.26; p = 0.003), and small (Z= –2.95; p = 0.009) MF densities than of TTR-FAP patients.

Seven out of ten patients with acquired ATTR exhibited amyloid deposits in sural nerve biopsy, as well as 23 out of 24 TTR-FAP patients in the same clinical conditions (p>0.05)
DISCUSSION

In the current study, we established the morphometric pattern of peripheral neuropathy in ATTR-FAP and de novo ATTR amyloidosis. The earliest lesions found in the peripheral nerve in ATTR-FAP are degeneration of unmyelinated and small myelinated fibers, simultaneously with, although in a somewhat erratic manner, amyloid deposition. Several authors have previously reported a size-dependent fiber loss in ATTR-FAP, but the precise mechanism of nerve fiber depletion has not yet been fully clarified. Ischemia from obliteration or dysfunction of small vessels, mechanical compression or infiltration by the amyloid deposits, and their metabolic and toxic effects are some of the adduced pathomechanisms. As expected, our results revealed that ATTR-FAP patients have loss of all fiber classes unlike the ones of the asymptomatic mutation carriers and controls, which are in agreement with the clinical manifestation of sensorimotor polyneuropathy experimented by the ATTR-FAP patients in the disease course. Furthermore, using a larger sample, we also showed that the loss of MF increases with disease progression (PND I versus PND II), and that patients in more advanced clinical stage showed more frequent amyloid deposition in the nerve. A $g$-ratio value between 0.5 and 0.7 reflects a theoretical optimal value for the conduction velocity of nerve impulses. Values lower than 0.5 may indicate the presence of degenerated nerve fibers with abnormal thickening of the myelin sheath, whereas values higher than 0.7 denote the presence of demyelinated nerve fibers, or regenerated fibers with thinner myelin sheath. We found that ATTR-FAP patients had lower mean $g$-ratio than controls, which supports axonal degenerative changes of MF. Despite lower numbers of ATTR-FAP patients with no amyloid in the sural biopsy, there was a significant higher MF density in this group compared to the amyloid positive group. A previous study did not find a clear relationship between the amount of endoneurial amyloid deposition and the degree of fiber loss. In the present study, we did not performed quantitative measures for amyloid deposition and a different clinical scale was used. For analysis purposes, this study took in consideration the presence or absence of amyloid, and only the earlier clinical stages were considered. Nevertheless, our findings suggest a possible direct role of amyloid deposition in disease pathogenesis, at least at this stage of the disease. Recently, direct insult of Schwann cells by amyloid fibrils has been suggested, particularly in early-onset cases. Alternatively, amyloid deposition can represent a marker of disease progression, appearing as a consequence of an amyloidogenic mechanism but not obligatory as the causative insult. In the previous study, considering 18 patients in the same disease stage, no correlation between fiber loss and time from symptoms onset to biopsy was found. In our study, with a larger number of patients, despite weak, there was a negative correlation for this analysis. This finding seems to be
associated with only large myelinated fibers and only present in the early-onset form of the disease. Several studies have reinforced the idea that the differences in clinical features between early- and late-onset ATTR-FAP patients correlate well with their pathological differences.\textsuperscript{9, 29} In this larger sample, and in line with other publications\textsuperscript{11, 29}, our findings revealed that late-onset patients have more reduced MF densities, particularly the large myelinated fibers, than the early-onset ones. These findings corroborate with the impairment of all sensory modalities in late-onset cases when compared to the sensory dissociation found in early-onset cases.\textsuperscript{41, 42} As previously described, family history was positive in a lower proportion of the late-onset patients, a fact which may lead to a delay in diagnosis and poorer patient outcomes at diagnosis and time of nerve biopsy.\textsuperscript{43-46} In our sample, despite similar time gap between disease onset and nerve biopsy, the late-onset group was associated with severe PND score at nerve biopsy. Different and additional mechanisms must be present to explain this clinical difference. In contrast to various descriptions\textsuperscript{9, 29}, we just found slight differences regarding the presence of amyloid deposition between both early- and late-onset cases. The amyloid was heavily deposited in the two groups, with a discreet predominance in the early-onset patients. An early Portuguese report\textsuperscript{26} unveiled similar findings by opposition to Japanese results\textsuperscript{29}, which have revealed that there were abundant amyloid deposits in the sural nerve from early-onset cases in relation to scarce deposition in the late-onset cases. Additionally, differences concerning amyloid composition, congophilic and morphological features have been already described between these two groups.\textsuperscript{7, 47}

An early description of pathological features in asymptomatic carriers has already disclosed abnormal findings in more than 50% of this population.\textsuperscript{29} In the present study, we extended the number of studied asymptomatic carriers studied and clearly demonstrated the loss of small MF, just as the early depletion of unmyelinated fibers, already exists in these subjects several years before symptom onset. The presence of greater MF diameters in the symptomatic and asymptomatic ATTR-FAP subjects, together with the lower ratio of small to large MF in these groups in comparison to controls, reinforces the fact that the small MF are early and preferentially involved in this neuropathy. Our findings support the recent reported changes in imaging studies with magnetic resonance neurography and peripheral nerve cross sectional areas assessed through ultrasonography in asymptomatic ATTR-FAP subjects, and confirms that the pathological mechanisms starts in a pre-symptomatic stage.\textsuperscript{46, 49} Approximately only one third of the asymptomatic mutation carriers showed amyloid deposits in their sural nerve biopsies. Contrarily to the symptomatic FAP patients group, we did not find differences in MF densities between the subjects with and without amyloid deposits in the sural nerve. This goes in agreement with previous reported data on asymptomatic gene carriers.\textsuperscript{50}
Together with evidence of axonal degeneration already there, this finding suggests the importance of other factors in this disorder and/or of toxicity of non-fibrillar ATTR aggregates at this stage.\textsuperscript{50-52} Considering a biological continuum, fibrillar amyloid seems to add pathological insult in a second phase of the disease.

Despite extensive knowledge of epidemiological and genetic factors, it remains impossible to predict the age at onset of clinical symptoms in asymptomatic Mutation gene carriers.\textsuperscript{12} Furthermore, in this population, some subjective complaints related to anxiety due to the knowledge of developing such devastating disease at unknown time in the future are difficult to differentiate from true disease onset. For the first time we described a longitudinal follow-up study that showed a positive correlation between the MF density and the disease onset in the asymptomatic mutated gene carriers that developed early-onset form of ATTR-FAP. Surprisingly, taking into account that small MF loss are initially affected in the disease process, our results suggests that the loss of small MF occurs in an independent way of the time for disease progression, and that the turning point to become symptomatic seems to be the loss of large MF. Interestingly, the negative correlation between time since symptoms onset and myelinated fiber densities in TTR-FAP patients was only observed in the large myelinated fibers. Moreover, it strengthens the fact that late-onset cases are more heterogeneous in terms of clinical presentation and pathological features\textsuperscript{9}, but also in terms of prediction for disease onset regarding nerve fiber loss. Despite the absence of association between the presence of amyloid and MF loss, the presence of amyloid in the biopsy was associated to a short period to disease onset. The statistical model also revealed that these associations (MF loss and amyloid presence) were stronger for the female subjects.

\textit{De novo} ATTR amyloidosis is a well-established complication in DLT recipients from TTR-FAP patients. Different authors have described similar pathological\textsuperscript{53, 54} and clinical features between ATTR-FAP and de novo ATTR amyloidosis patients.\textsuperscript{17, 55} They unveiled an asymmetric depletion of nerve fibers, with predominant loss of the small ones associated with initial symptoms of length dependent small fiber polyneuropathy, affecting pain and thermal sensory modalities and autonomic function.\textsuperscript{55} In this study, we noticed that de novo ATTR amyloidosis patients had an intermediate density of myelinated fibers, having densities ranging between the hereditary FAP patients and controls MF density values. Additionally, we found a lower ratio of small to large MF compared to controls, confirming a higher reduction of small MF. A simplistic explanation for patients with de novo ATTR amyloidosis presenting higher densities of all fiber modalities than did hereditary patients, matched for the same clinical stage at nerve biopsy, could be the shorter time gap between symptoms onset and biopsy in the former. However, even when considering the same time gap from onset of symptoms to nerve biopsy for both groups,
*de novo* ATTR amyloidosis cases maintained higher values of MF densities when compared to ATTR-FAP patients. *De novo* amyloidosis is similar to inherited ATTR-FAP in terms of the frequency of found amyloid deposition and the predominant involvement of small fibers. The reason for lesser magnitude of fiber loss, together with a shorter period to disease onset to what was theoretical conceivable, taking into account the natural history of the inherited FAP, remains to be explained. Age, previous incipient neuropathy of other origin, and contemporary immunosuppressive drugs are factors that can promote amyloidogenesis and accelerate onset of neuropathy in these patients. Further studies will be necessary to identify such factors and explain their role in the pathophysiology of the disease.

This study has limitations. Despite detailed clinical records from the Corino de Andrade Unit, this is a retrospective study. Also, we only assessed the morphometric features of this polyneuropathy with respect to the myelinated fibers, wherefore, for a better understanding of the disease’s pathogenesis, it would therefore be important to evaluate other pathological features such as amyloid composition and ultrastructure findings.
CONCLUSIONS

In this study, we performed detailed morphometric analysis in a large ATTR-FAP sample including asymptomatic mutation gene carriers, patients in different disease stages and acquired ATTR amyloidosis post liver transplant. We were able to demonstrate the initial involvement of small fibers that started in pre-symptomatic stage, the correlation of the fiber loss with the disease stage in the earlier stages and the axonal nature of the neuropathy. We hypothesized that amyloid deposition can have a direct role in disease mechanism in the earlier symptomatic stages. For the first time, we performed this analysis with such extended clinical follow up in a group of asymptomatic mutation carriers that have undergone sural nerve biopsy. We found that large myelinated fiber loss and amyloid deposition are pathological features that correlated independently to a shorter period to symptoms onset. These associations were only observed for the early-onset form of the disease and stronger for the female subjects. Our data supports the idea that early-onset form of the disease may follow a biological disease model more linearly associated to amyloid deposition and degree of fiber loss, however, with these two variables not correlated at this stage. Unmyelinated and small myelinated fiber loss start independently of fibrillar amyloid deposition, with the latter probably adding direct insult to an ongoing process, accelerating disease onset and progression. At this stage large myelinated fibers started to become involved. Finally, we concluded that de novo ATTR amyloidosis have similar pathological findings to their inherited counterparts, but with lesser degrees of fiber loss for the same disease clinical stage.

We believe this description will be therapeutically relevant, as it would allow for a better interpretation of the role of disease-modifying agents in ATTR amyloidosis.
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