

## Reduction of Intraepidermal Nerve Fiber Density (IENFD) in the skin biopsies of patients with fibromyalgia: A controlled study<sup>☆</sup>



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### ABSTRACT

**Objectives:** Fibromyalgia (FM) is one of the most common chronic pain syndromes. Various pathogenetic mechanisms have been implicated but none is proven. Our scope was to determine if Intraepidermal Nerve Fiber Density (IENFD) is reduced in the skin of FM patients, as observed in patients with painful small fiber sensory neuropathy (SFSN).

**Design, setting and participants:** We prospectively studied 46 FM patients (5 men and 41 women), aged 29 to 76 (mean: 52.5) years, diagnosed according to the ACR 2010 criteria, and 34 controls (18 women and 16 men) aged 19 to 84 (mean: 31.7) years. IENFD was measured using published guidelines and immune markers were sought immunocytochemically. In 30 FM patients, pain intensity was assessed with the Neuropathic Pain Symptom Inventory (NPSI), a scale validated for neuropathic pain.

**Results:** 15 of 46 (32.6%) FM patients had reduced IENFD [range: 0.6–12.5 fibers/mm (mean: 4.83 SD: 2.5)], compared to healthy controls [2.8–11.5 fibers/mm (mean: 7.35, SD: 1.85)] ( $p < 0.0001$ ). No significant correlation was noticed between NPSI scores and IENFD. No difference in the Langerhans cells, the major Antigen Presenting Cells (APCs) in the epidermis, or in IL-6 staining, was noted between FM and controls. IENFD was equally reduced in a subset of FM patients who also had another autoimmune disease.

**Conclusion:** This is one of the largest series of FM patients demonstrating a significant reduction of IENFD in their skin biopsies. The findings indicate that in a subset of FM patients, the pain syndrome is, at least partially, of neuropathic origin. Skin biopsy may prove a useful tool and a potential biomarker in future studies of FM patients.

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### 1. Introduction

Fibromyalgia (FM) is a syndrome of chronic widespread pain with concomitant depression, fatigue and sleep disturbances affecting 2% of the population in the United States [1]. Diagnostic criteria for FM, established by the American College of Rheumatology (ACR, 2010), require at least 3 months of symptom duration and no other disorder that would otherwise explain the pain. The clinical phenotype of FM dictates a widespread, deep musculoskeletal pain with tender points in the shoulder girdle, torso, hips and extremities; cognitive disturbances;

depression; and various somatic symptoms such as headaches, dizziness and irritable bowel syndrome [2].

The cause of FM is unknown but it has been hypothesized that it represents a central sensitization process with defects in sensory processing and impairment of descending pain inhibitory network [3]. Studies with fMRI have shown that in approximately 50% of FM patients, a lower stimulus intensity is needed to evoke a pain response compared to controls [4]. A growing body of evidence also suggests that immune factors such as cytokines may play a role either by acting systemically or locally at the pain receptors in the skin causing chronic pain. Among the pro-inflammatory cytokines, those predominantly associated with pain mechanisms are IL-1 receptor antagonist, IL-6, and IL-8, all of which were found increased in the serum in a meta-analysis of 1255 FM patients compared to 800 healthy controls [5]. Over-proliferation of mastocytes, potentially antigen presenting cells (APCs), and high values of IgG deposits have been also reported in the papillary dermis of FM patients compared to controls [6]. Although not studied in FM, Langerhans cells (LCs), the main APCs in human epidermis, were increased in the epidermis of patients with painful diabetic neuropathy [7,8].

**Abbreviations:** FM, Fibromyalgia; IENFD, Intraepidermal Nerve Fiber Density; SFSN, Small Fiber Neuropathy; IIF, Indirect Immunofluorescence; LCs, Langerhans cells; NPSI, Neuropathic Pain Symptom Inventory.

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FM patients have normal clinical examination besides tenderness to palpation and notoriously negative laboratory work up. Skin biopsies from recent studies showed a reduction of IENFD, as seen in small fiber sensory neuropathy (SFSN) [9–13]. In one cohort of 27 FM patients including children, 41% had reduced IENFD [13]; in another study of 25 adult FM patients a reduction of the median IENFD was noted compared to controls (5 fibers/mm vs. 9.5 fibers/mm) [12].

Our study is aimed to confirm and to expand on these findings with a large number of well-selected FM patients and controls including patients with rheumatic diseases. We also investigated whether autoimmune mechanisms play a role searching for relevant immunological markers on the skin biopsies because: a) certain cytokines are thought to be responsible for inducing painful syndromes; b) FM is also seen in rheumatic diseases; c) some FM patients respond to immunotherapies; and d) SFN is now recognized as the most common neuropathy in Sjögren's syndrome [14,15].

## 2. Patients & methods

### 2.1. Patients

46 patients (5 men and 41 women) aged 29–76 years (mean: 52.5) with the diagnosis of FM according to the ACR 2010 established criteria and 34 healthy controls were enrolled into the study between January 2012 and May 2013. Patients were referred from the Rheumatology outpatient clinic of “Laiko” University Hospital. All of them were examined from at least 2 experienced rheumatologists and one neurologist. Among the 46 patients, 30 had FM without any other known disease, while the remaining 16 had FM in a setting of a known autoimmune disorder including Sjögren's Syndrome (4 patients), Systemic Lupus Erythematosus (3), Rheumatoid Arthritis (4), and one each with Discoid lupus, Psoriasis, Mixed Connective Tissue Disorder, Crohn's disease and Myasthenia. In all of them, the FM symptoms preceded the use of immunomodulating drugs. Two patients, retrospectively discovered to have Type-2 diabetes, were excluded from the analysis. In all patients with autoimmune co-morbidities, there was no evidence, of peripheral neuropathy and no clinical symptoms consistent with small fiber sensory neuropathy, based on clinical history and a thorough clinical neurological examination, paying attention to changes in light touch, pinprick and vibration sensation, tendon reflexes and motor strength. 18 from the 46 FM patients had not received any medications at the time of skin biopsy; 34 had received antidepressants or anti-epileptics as treatment for FM and 13 had received immunosuppressants-immunomodulators for the underlying rheumatologic disorder, as noted above. All known causes of central or peripheral sensory symptoms or pain were excluded following a careful clinical evaluation and extensive laboratory work-up including brain and spine MRI's, nerve conduction studies and electromyography in almost all the patients studied, along with complete biochemical hematological and immunological studies. The study protocol was approved by the Ethics Committee of the Faculty of Medicine, National and Kapodistrian University of Athens and all patients and healthy volunteers signed an informed consent prior to entering the study.

### 2.2. Intraepidermal Nerve Fiber Density (IENFD)

5 mm punch biopsies were taken under sterile conditions using local anesthesia, 10 cm above the right lateral malleolus in accordance with the EFNS/PNS 2010 guidelines [16]. Tissues were fixed for 2–4 h in 2% PFA in 0.1 M Phosphate Buffer and stored in 10% Sucrose in 0.1 M Phosphate Buffer for at least 24 h at 4 °C. Samples were then snapped-frozen in isopentane. At least four 40 µm cryostat sections from each sample, oriented perpendicularly to the skin surface, were incubated in a wet chamber with a blocking solution of 10% BSA in PBS for 30 min. Sections were subsequently incubated overnight at room temperature with a polyclonal rabbit anti-human antibody to PGP 9.5

(1/400, Ultraclone, UK) in 1% BSA in PBS containing 0.3% Triton-X. Sections were then thoroughly washed with PBS, incubated with the Alexa 594 goat anti-rabbit IgG secondary antibody (Invitrogen) at 1/100 dilution in 1% BSA in PBS for 2 h and mounted with fluorescence mounting medium (Dako).

Nerve fiber counting was performed in 4 sections at 40× magnification by two independent observers (MLK and IM) according to established guidelines [16]. Samples were photographed at 5× magnification using a Leica digital microscope camera. Skin length from each sample was calculated using the Image J 1.45s software analysis program (National Institutes of Health, Bethesda, MD, USA).

### 2.3. Double immunostaining for LCs and epidermal nerve fibers

5 controls and 5 samples from FM patients, prepared as previously described, were incubated with polyclonal rabbit anti-human antibody to PGP 9.5 (1/400, Cedarlane, USA) and a monoclonal mouse anti-Langerin antibody 1/200 (Abcam) in 1% BSA/PBS with 0.3% Triton X. Langerin is a C-type lectin that forms Birbeck granules and represents the structural hallmark of LC's [7]. Samples were then washed 3 times with PBS and incubated with the secondary antibodies Alexa 594 goat anti-rabbit 1/100 and Alexa 488 fluor goat anti-mouse 1/100 (Invitrogen) for 2 h. LCs were manually counted by a single observer (MLK) at 40× magnification.

### 2.4. IL-6 staining procedure

We followed the same protocol as described in B but used as primary antibody rabbit anti human IL-6 at a dilution of 1/200 (Serotec) and as secondary antibody Alexa 594 goat anti-rabbit 1/100 (Invitrogen).

### 2.5. Neuropathic pain scale inventory (NPSI)

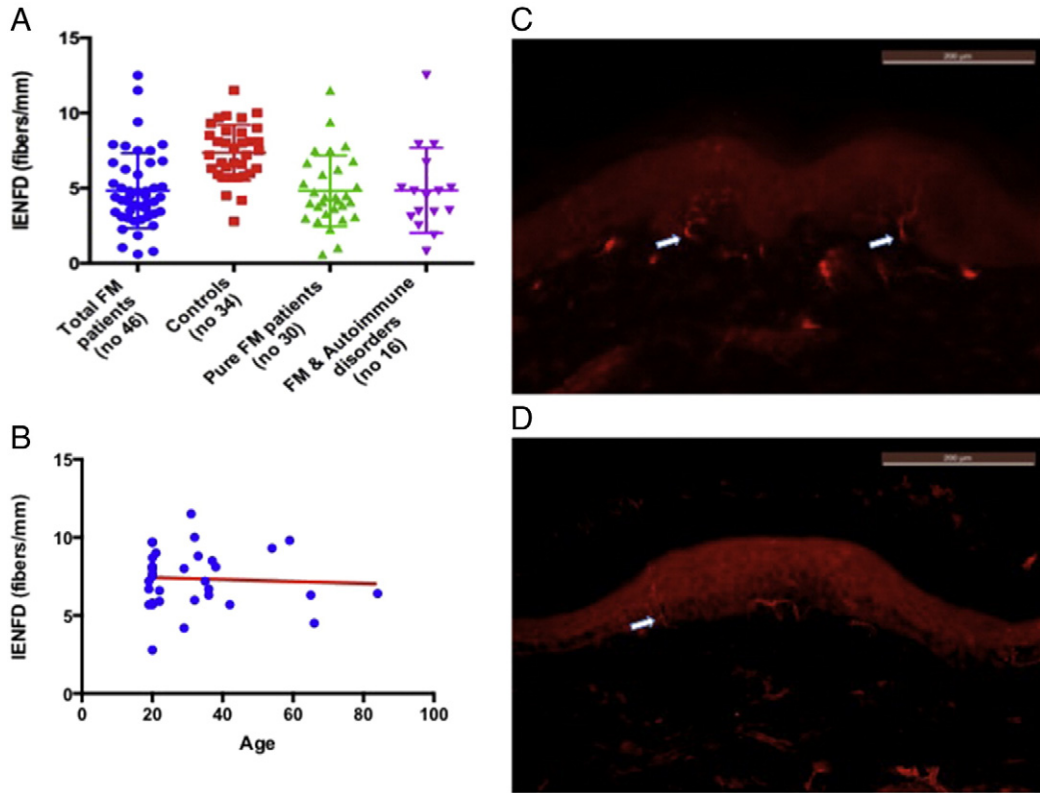
30 of the participating FM patients, were contacted either after the skin biopsy or at the time of the biopsy to respond to a Neuropathic Pain Symptom Inventory (NPSI) [17], a validated self-questionnaire consisting of 10 items assessing 5 different qualities of neuropathic pain, such as spontaneous pain (mainly causalgias), deep neuromuscular pain, paroxysmal pain, evoked pain and paresthesias-dysesthesias. Each question asks from the patient to describe his/her symptoms and quantify it using a scale from 0 (no symptom) to 10 (the worst imaginable intensity and severity).

### 2.6. Study end points and statistical analysis

The primary study end-point was the difference in IENFDs between pure FM, FM plus autoimmune disorders and healthy controls. We used one-way ANOVA to compare the 3 groups and an unpaired *t*-test with Welch's correction to examine an interrelationship between NPSI score and IENFD in FM patients. The unpaired *t*-test was also used to examine the difference in the number of LCs between FM and controls.

## 3. Results

Eight of the 30 (26.6%) FM patients from the first group and 7 of the 16 (43.75%) FM patients with another autoimmune disorder had IENFD lower than our cut-off level of 2 SD below the mean. The IENFD in the 34 healthy controls was between 2.8 and 11.5 fibers/mm (mean: 7.35; ± 1.85), with the lower cut-off of 2 SD below the mean at 3.65 fibers/mm. In 32.6% of the total number of FM patients the IENFD was 2 SD below our cut-off level ( $p < 0.0001$ ) (Fig. 1A). The IENFD in the 46 FM patients with symptom onset from 3 months to 30 years (mean: 7.14 years), ranged from 0.6 to 12.5 fibers/mm (mean: 4.83 ± 2.5). Using the Pearson's correlation analysis to determine whether there was any linear relationship between advancing age and IENFD, we found no significant correlation ( $r = -0.05$ ,  $p = 0.75$ ) [Fig. 1B].

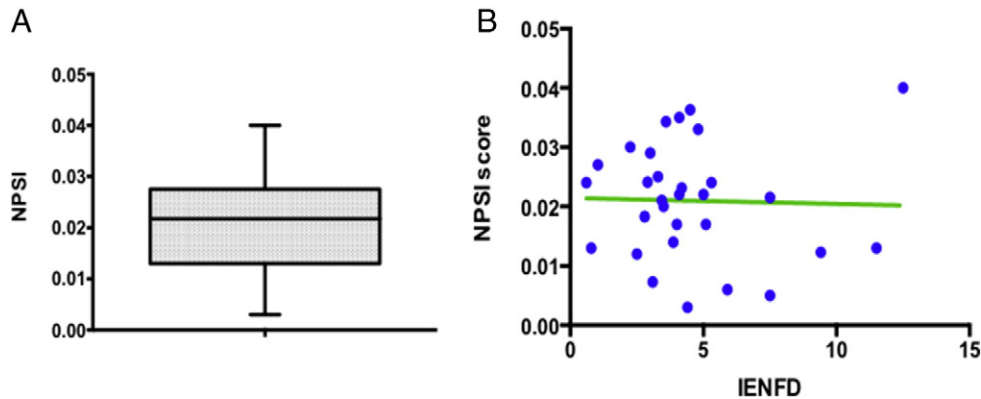


**Fig. 1.** A. IENFD's in 46FM patients are significantly lower compared to 34 healthy controls ( $p < 0.0001$ ) [Unpaired *t*-test with Welch's correction]. The same conclusion can be done for the two subgroups of 30 pure FM and 16 FM plus other autoimmune disorders ( $p < 0.0001$  and  $p = 0.0041$ ). B. Pearson's correlation analysis for the relationship of IENFD's with advancing age of 34 healthy controls shows no significant correlation ( $r = -0.05$ ,  $p = 0.75$ ). C. IIF in a skin biopsy from the distal leg in a healthy control (6.7 fibers/mm). D. A patient with FM (2.8 fibers/mm). Primary antibody: anti-PGP9.5, 1/400 (Ultraclone, UK), secondary antibody Alexa 594 goat anti-rabbit IgG 1/100 (Invitrogen). Arrows indicate nerve fibers that clearly cross the basal lamina.

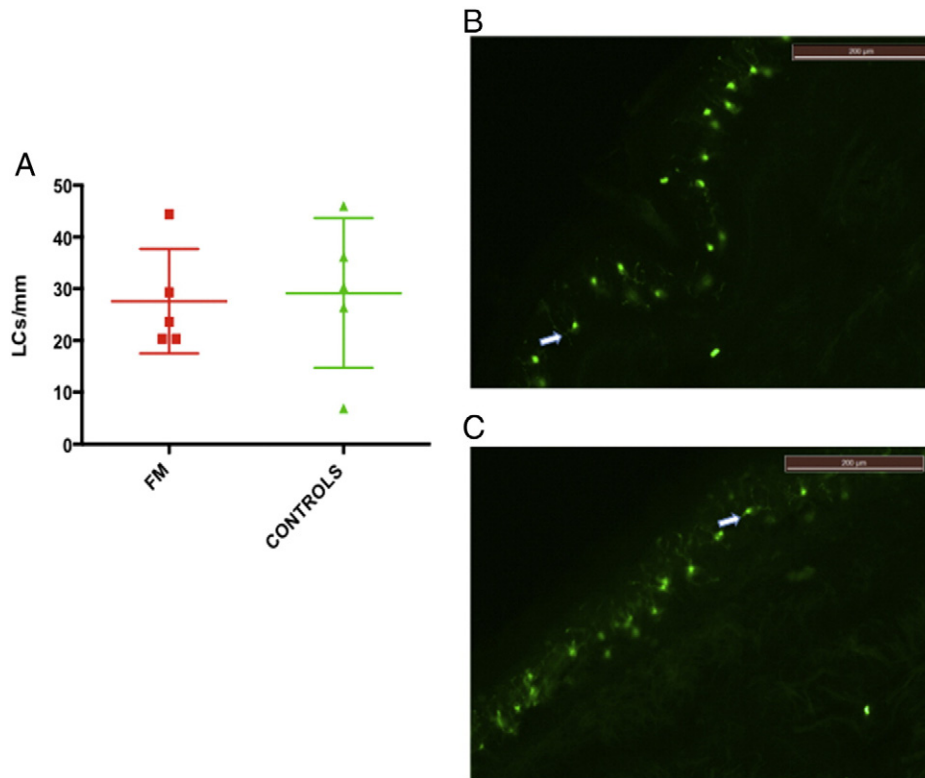
Although our control subjects were not strictly age-matched with the FM cohort, this does not affect the conclusions because the IENFD in our controls did not correlate with advancing age. Further, when the very low and the very high ages were removed to artificially create age-matched groups, the difference remained statistically significant.

Among the 46 study patients, we included a group of 16 patients with another autoimmune rheumatic disorder, but without any clinical symptoms of small fiber neuropathy or history of neurotoxicity, to assess whether FM is more common or more severe in this group which has an overt immune dysregulation. Both groups had an equally statistically significant reduction of IENFD compared to controls ( $p < 0.001$  and  $p = 0.0041$  respectively).

In the group of 30 FM patients followed with the NPSI scale, no statistically significant correlation was observed between NPSI scores and IENFD; however, 12 patients out of 30 (40%) with the highest NPSI scores ( $>0.02$ ) exhibited fiber densities below 5 fibers/mm. Also, based on clinical correlations, 10 of 13 (76.9%), patients diagnosed as FM, with IENFD below our cut-off level, reported causalgias in many areas with severity close to 10 (representing cumulative symptoms of maximum intensity) [Fig. 2]. The number of LCs and their staining pattern was not significantly different between controls (6.9–46 cells/mm, mean: 29.16, SD: 14.4) and FM patients (20.3–44.4 cells/mm, mean: 27.5, SD: 10.1) [Fig. 3]. There was also no difference in the IL-6 staining pattern between the controls and the FM patients (data not shown).



**Fig. 2.** A. Neuropathic Pain Symptom Inventory (NPSI) scores in 30 fibromyalgia patients. B. Pearson's correlation analysis shows no significant relationship between NPSI and IENFD ( $r = -0.02$ ,  $p = 0.87$ ).



**Fig. 3.** A. Numbers of LCs/mm are not significantly different in skin biopsies of 5 patients with FM and 5 healthy controls. B. IIF for LC's in a healthy control [primary antibody: monoclonal mouse anti-Langerin 1/200 (Abcam), secondary antibody: Alexa 488, goat anti-mouse 1/100 (Invitrogen)]. C. The same procedure in FM patient shows no statistically significant differences in the number and staining pattern of LC's. Arrows show LC bodies with their dendrites.

#### 4. Discussion

In our cohort of 46 FM patients prospectively studied with skin biopsy, 16 (34.7%) had significantly reduced IENFD compared to controls, similar to the pattern seen in small fiber sensory neuropathy, suggesting that a component of their symptoms may have neuropathic origin related to loss of small epidermal nerve fibers. Our results, based on a large number of patients, confirm recent observations from other studies [9–13] and provide credence to the view that reduced IENFD occurs in a subset of FM patients offering new insights into the pathomechanism of FM.

FM remains a complex disorder with diffuse pain symptoms and trigger points not limited to the feet. The noted reduction of IENFD in the distal feet alone, cannot arguably explain the FM's clinical phenotype of diffuse pain syndrome. SFN is in most cases a length-dependent neuropathic process, which involves peripheral A $\delta$  and C fibers, presumably due to small ganglionic neuronal dysfunction, characterized by distal acral pain and dysesthesias, while FM is characterized by a deep generalized pain located both in the torso and the extremities. Whether in FM patients changes similar to those observed in the feet also occur in the trunk and proximal extremities, reflecting a length-dependent process due to dysfunction of the respective small ganglionic neurons, remains to be determined. It remains also unexplained whether the reduced IENFD in FM is the cause of pain, especially since there was no correlation with NPSI. It has been emphasized however that the painful syndromes may not be due to lack of small sensory fibers per se but rather due to excessive firing of the normal-appearing fibers or those undergoing degeneration [18]. Other methods, albeit with questionable reliability, used for studying SFN include quantitative thermal sensory testing (QST) and quantitative sudomotor axon reflex testing (QSART) [19]; whether these tests will supplement the skin biopsy data to better understand the mechanism of FM remains to be determined.

The strength of our study is the large number of FM patients and controls (the largest to our knowledge) studied concurrently, both clinically and with skin biopsy. A concern, not only in our study but also in previous studies, is that only a portion of FM patients (a third in our study) had abnormal IENFD. It is possible that this may reflect a threshold phenomenon, or more likely a widespread dynamic process that is not captured by one skin biopsy performed once from one small area. Others also support this hypothesis [20]. Perhaps examining multiple areas, especially the painful spots longitudinally, may be more informative. Other factors such as polymorphisms in genes associated with pain may also play a role [21]. A possible factor that might be perceived as weakness in our study is the examination only of a distal leg with a single skin biopsy and not concurrently with a second biopsy from the proximal thigh, as done by others [9,11,12]. Our approach—used also by others—is justified because dysfunction of the small fibers was expected to be more prominent in the distal parts. Further, our conclusions were based on robust comparisons (2 SD) with a large number of normal controls. It is likely that the IENFD may be also abnormal in the thigh, as recently shown in some FM patients. Future studies may be more informative if skin biopsy samples from the proximal and distal trunk areas are also examined, especially from areas corresponding to deep pain. Collectively, the overlapping IENFD pathology between FM subsets and patients with SFN raises interesting questions regarding the need to further define FM in large-scale studies.

A question of critical importance is what causes the noted reduction of IENFD in FM patients. A plausible scenario would be an “autoimmune process” that diffusely affects small ganglionic neurons resulting in distal nerve fiber degeneration in the epidermis and deep skin owing to a noxious effect of pain-related cytokines or other immune mediators [5,6]. This hypothesis was also supported by the noted improvement of some FM patients with IVIg or other immunotherapies. Of interest, 16 patients in our FM cohort with reduced IENFD also had an accompanying autoimmune disorder such as Rheumatoid arthritis, Sjögren's

syndrome or SLE. Our limited search for an immune dysfunction however was negative. Alternatively, the main process in FM may be degenerative, owing to a small ganglionic neuronal degeneration, necessitating the need to also explore neuroinflammatory mediators in the skin specimens.

Our findings have significant implications in the effort to identify an objective biomarker for FM. These patients are often labeled as having a psychiatric disease, even though they do not fit into a true primary psychiatric disorder, nor do they have history of stressful or depressive events preceding the onset of their painful syndromes. Rheumatologists or neurologists who see FM patients base their diagnosis on clinical consensus criteria, which, according to the skin biopsy findings, may need to be revised. It is also likely that some SFN patients may have overlapping features with FM and vice versa. Finding an objective biomarker, at least in a subset of these patients, is a step forward. Exploring further the powerful method of skin biopsies may also help us understand the various causes responsible for such a diffuse pain syndrome.

### Contributorship statement

Collection of patients: Dr Kosmidis, Dr Koutsogeorgopoulou.

Biopsy performance and laboratory experiments: Dr Kosmidis, Dr Mamali.

Manuscript editing: Dr Kosmidis, Dr Alexopoulos, and Professor Dalakas.

Intellectual contribution: Professor Tzioufas, Professor Moutsopoulos, Professor Vlachoyiannopoulos, Dr Voulgarelis, Dr Alexopoulos, Professor Dalakas.

Study supervision: Professor Dalakas.

### Financial disclosure

The authors have no financial interests related to this study.

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