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Estudo Exploratório da Excitabilidade Axonal com a "Técnica Threshold Tracking": controlos saudáveis e doentes com polineuropatia amilóide familiar

Exploratory Study on axonal excitability with threshold tracking technique: in healthy controls and patients with familial amyloid polyneuropathy

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'Ion channels are involved in every thought,
every perception, every movement, every heartbeat.'

Clay M. Armstrong, 1999

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Abstract

Clinical neurophysiology provides quantitative measurements of peripheral nerve function. However, limits for measurements evaluating peripheral nerve function are difficult to establish as they depend on temperature, age, body mass index and, possibly, gender.

 Recently, nerve excitability testing using threshold tracking has been developed as a method to complement nerve conduction studies. We used this technique to study Portuguese population.

The technique of threshold tracking to test axonal excitability gives information about nodal and intermodal ion channel function.

We aimed to investigate variability of the motor excitability measurements in healthy controls, taking into account age, gender, body mass index (BMI) and of small changes in skin temperature and to in early affected patients with transthyretin (TTR)-type familial amyloid polyneuropathy (TTR-FAP) before and during tafamidis treatment.

The changes related to demographic features on TRONDE protocol parameters are small and less important than in conventional nerve conduction studies. Nonetheless, our results underscore the relevance of a careful temperature control, and indicate that interpretation of stimulus-response slope and accommodation half-time should take into account

age and BMI. In contrast, gender is not of major relevance to appreciate axonal threshold findings in motor nerves.

Threshold-tracking of motor fibers of the median nerve is not a sensitive test to support early diagnosis of TTR-FAP patients. Tafamidis was well tolerated. We observed a significant reduction of nerve excitability, not explained by channel function changes or overlying tissue edema.

Threshold tracking can contribute to detail the action of new drugs to treat neuropathies. Tafamidis can change nerve electrical properties by reducing amyloid burden.

Keywords: Age; body mass index, nerve excitability, gender, temperature, familial amyloid polyneuropathy, tafamidis.

Resumo

A neurofisiologia clínica permite avaliar quantitativamente a função dos nervos periféricos. No entanto, é difícil estabelecer valores limite para a função do nervo periférico sendo que este depende da temperatura, idade, índice de massa corporal e possivelmente, do género.

Recentemente, tem sido utilizada uma nova metodologia – tracking threshold – que estuda vários parâmetros da excitabilidade do nervo e complementa os estudos de condução do nervo. Nós utilizámos esta técnica para estudar a população portuguesa.

A técnica tracking threshold avalia a excitabilidade do axónio e fornece informação sobre a funcionalidade dos canais iónicos ao longo de toda a membrana axonal – nodal e internodal.

O nosso objetivo foi investigar a variabilidade dos parâmetros de excitabilidade do nervo motor em controlos saudáveis, analisando a idade, género, índice de massa corporal e pequenas variações de temperatura superficial, e nos indivíduos com polineuropatia amiloide familiar associada à transtirretina (TTR-FAP) na fase inicial da doença, antes e durante o tratamento com tafamidis.

As variações relacionadas com as características demográficas, analisadas com o protocolo TRONDE, são pequenas e de menor

importância em relação aos estudos de condução do nervo convencionais. No entanto, os nossos resultados realçam a importância de manter um controlo rigoroso da temperatura, e que nos parâmetros *stimulus-response slope* e *accomodation half time, a* idade e índice de massa corporal parecem ter influência. Em relação ao género, não é um fator relevante quando se analisam os parâmetros de excitabilidade com a técnica tracking threshold no nervo motor.

A utilização de tracking threshold nas fibras motores do nervo mediano não é um teste sensível para apoiar o diagnóstico precoce nos doentes TTR-FAP. O tratamento com Tafamidis foi bem tolerado. Observámos uma redução significativa de excitabilidade do nervo, que não se explica pelas alterações dos canais iónicos ou pelo edema de tecido sobrejacente.

A técnica tracking threshold pode contribuir para compreender e identificar o mecanismo de acção de novos fármacos no tratamento das neuropatias. Tafamidis pode alterar as propriedades elétricas do nervo ao reduzir o peso da substância amiloide.

Palavras-chave: Idade, índice de massa corporal, excitabilidade do nervo, género, temperatura, polineuropatia amiloide familiar

Abbreviations List

ATPase- Adenylpyrophosphatase

BMI – Body Mass Index

Ca^{2+} - Calcium

Cl – Cloro

CMAP – Compound Muscle Action potential

EMG - Electromyography

IQR – Interquartile range

IV – Current-threshold relationship

K^+ - Potassium

Kg - Kilogram

Mg^{2+} - Magnesium

Mg/d – milligram per day

Mm – millimeter

Ms - millisecond

MEM – Multiple excitability measures

MEF –Multiple excitability file

MoA – Move of Action

MV - milivolts

Na^+ - Sodium

PAF – Polineuropatia amiloide familiar

RC – Recovery cycle

SD – Standard deviation

SDTC – Strenght duration time constant

SNAP – Sensory action nerve potential

TE – Threshold Electrotonus

TEd – Threshold Electrotonus depolarization

TEh – Threshold Electrotonus hiperpolarization

TTR-FAP – Transthyretin type familial amyloid polyneuropathy

TTR – Transthyretin type

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1. Introduction

1.1. Axonal Excitability

In 1952, Hodgkin & Huxley were the pioneers in describing the functionality of ion channels and its relationship with membrane action potential through the surface membrane of a giant nerve axon, *Goligo pealei*, which represented a landmark in human physiology. These findings enabled to establish that membrane action potential is generated by two currents: *inward* and *outward*, and that the permeability of the axonal membrane increases with action potential (Hodgkin et al., 1952a; Hodgkin et al., 1952b). After this finding, two new different techniques as, patch clamping (Neher and Sakmann, 1976) and site-directed mutagenesis (Hutchison et al., 1978), have contributed to the characterization of axons ion channels and its functionality.

Since then, the scientific community have demonstrated an increasing interest in studying the biophysical properties of the human peripheral nerves.

The functionality of the axon, depends on its electrical excitability, that is defined by the threshold current that excites it (Burke et al., 2001), and if this process does not happen properly, it will result in several neurological diseases. Understanding the biophysical properties of human peripheral nerve, the axonal ion channel function, it becomes very important to study

neurological dysfunctions and inherent processes (Bostock, 1985; Burke et al., 1997; Inglis et al., 1998; Cappelen-Smith et al., 2000; Kaji et al., 2000).

The axonal membrane has a very complex molecular structure and contains a multiplicity of ion channels, pumps and exchangers responsible for determining axonal excitability through the maintenance of electrochemical gradients for the major cations (Na^+, K^+, Ca^{2+}, Mg^{2+}) and anions (Cl^-, HCO_3^-).

The Figure 1, represents a myelinated axon with the main intervenient in excitability and resting membrane potential.
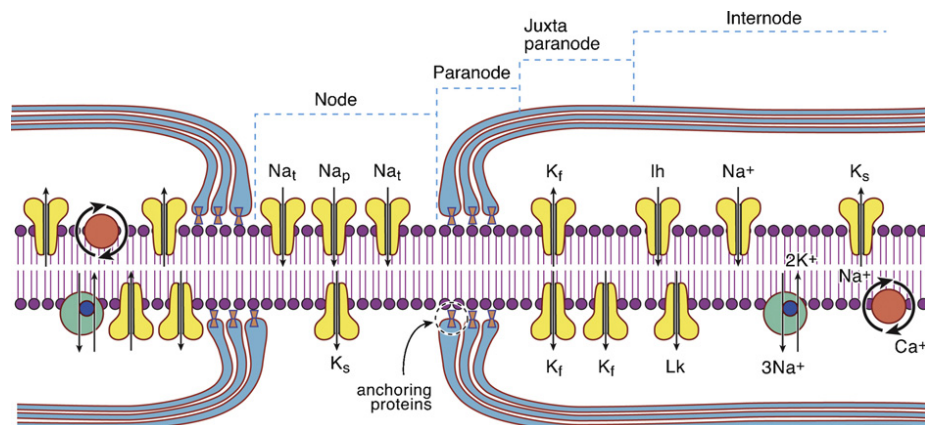

Figure 1. Molecular structure of myelinated axon illustrating the ion channels, pumps and exchangers responsible for determining axonal excitability. Ion channels are represented in yellow, ion exchangers in orange and energy-dependent pumps in green. In the node of Ranvier, transient Na^+ channels (Na_t^+) are clustered at high density, with persistent Na^+ channels (Na_p^+) and slow K^+ channels (K_s^+) also contributing to excitability and resting membrane potential. Fast K^+ channels (K_f^+) are located at highest density in the juxtaparanode, acting to limit re-excitation of the node following an action potential. Internodal conductance include voltage-independent ´leak´

conductance (Lk) and the hyperpolarization-activated cation conductance (I_H). The Na^+-K^+ pump (Na^+/K^+-ATPase) utilises energy to maintain the electrochemical gradient necessary for impulse conduction by removing 3 Na^+ ions for every 2 K^+ ions pumped into the axon. The Na^+-Ca^{2+} exchanger exports Ca^{2+} ions and imports Na^+, driven by the electrochemical Na^+ gradient. In the paranodal region, paranodal myelin terminal loops are depicted with anchoring proteins to form paranodal junctions (Krishnan et al., 2009[5]).

Action potential generation is highly determined by the activation of nodal voltage-gated Na^+ channels, located in the node of Ranvier, where the density is higher. The passage of Na^+ ions into the axon is a regenerative process: it increases with depolarization, which in turn leads to further depolarization of the node of Ranvier (Krishnan et al., 2009).

The distribution of Na^+ channels is different between nonmyelinated and myelinated axons. In a nonmyelinated axon, the Na^+ distribution is uniform and has a low density, instead of the Na^+ channels distribution in a myelinated healthy axon that have a heterogeneous distribution, with higher density in the nodes of Ranvier (density of 1000 um^{-2}) (Krishnan et al., 2009; Ritchie et al., 1977). The mechanism of action potential propagation is also different between unmyelinated and myelinated nerves fibers, reflecting channel distribution and the role of myelin sheath. In unmyelinated nerve fibers, the reversal of polarity cause by the local action potential leads to a local current flow, from the positive to the negative that depolarizes contiguous regions of the nerve causing a new action potential and so, until the action potential has moved along the full length of the stimulated axon. In myelinated fibers, the myelin sheath acts as insulator

that do not block transmembrane current flow in the internodes. The ion exchange occurs only at the nodes of Ranvier, it originates that the impulse step in from one node to the next, where a new action potential is generated – *saltatory conduction*. Nerve conduction in myelinated nerve fibers is faster and spends less energy.

Previous studies, confirmed that when axons are damaged or diseased, disturbed axonal excitability might affect the normal conduction of a meaningful impulse train, which could underlie the development of different peripheral nerve conditions (Krishnan et al., 2009; Bostock, 1985; Burke et al., 1997; Inglis et al., 1998; Cappelen-Smith et al., 2000; Kaji et al., 2000; Burke et al., 2001).

Investigation of nerve excitability dysfunction is critical to understand a large number of different neurological disorders, in particular those affecting ion channel function - *channelopathies*. Is important to note, that it is not possible to access membrane potential in intact human axons, but it is possible to obtain indirect evidence of membrane potential by studying excitability indices, that are dependent on membrane potential (Burke et al., 2001). This is a key neurophysiological investigation and will constitute the core of this thesis.

1.2 Excitability Studies

Many electrophysiological techniques have been developed to diagnose demyelization, axonal loss, conduction block or abnormal neuromuscular transmission. They focus on the number of conducting fibres and conduction velocity due to its sensitivity to the integrity of myelin sheath (Bostock et al., 1998). Conventional electrophysiological studies, like nerve conduction studies, have contributed to the understanding of the mechanisms of nerve lesion, to the clinical diagnosis and management of peripheral nerve diseases. On the other hand, they provide little insights into the excitability of the axonal membrane, which represents a significant information gap in neurophysiology field (Maurer, 2007).

More than 40 years ago, Joseph Bergmans, had made a remarkable scientific discovery, in demonstrating that single human motor axons could often be studied, non-invasively, by selective electrical stimulation (Bergmans, 1970). He defined the threshold of an axon as the lowest stimulus that would excite it reliably, and found that considerable and relevant information about the axonal membrane could be inferred from the measurements of the threshold changes when activated with an electric impulse or by artificial polarization. The great value of the threshold changes measurements is because it can provide an accurate indication of excitability changes in membrane potential (Bergmans, 1970). But, although Bergman's work achievements were remarkable, the technique was difficult to execute (*Bergmans, 1970; Bostock and Baker,*

1988). To measure the threshold changes it would be necessary to isolate a single unit with surface electrodes, and then determining its threshold manually. This could be the main reason for the investigation on this matter did not move forward for many years (Bostock et al., 1998).

The difficulty to isolate a single motor unit can be avoided by tracking the "threshold" of a compound action potential, it means, the required stimulus to produce a compound action potential of a specific size (Raymond, 1979). A compound action potential threshold, behaves the same way as the threshold of a single unit (Franz et al., 1988; Weigl et al., 1989). Since then, the compound action potential has been preferentially used in excitability studies.

These non-invasive techniques, which enable the recording of compound motor response or the sensory nerve action potential, are an accessible method to provide insights into axonal membrane excitability and ion channel function. These techniques gave the possibility to assess axonal membrane function *in vivo* in a clinical setting, providing meaningful information of the biophysical properties of human peripheral nerves, into both normal nerve function and in disorders affecting nerve excitability (Krishnan et al, 2009).

Threshold measurements are essentially complementary to conventional nerve tests. They are insensitive to the number of conducting fibres and its conduction velocity; they test the nerve at a point rather than over a

length; and test the excitability properties of the axonal membrane rather than the integrity of the myelin sheath (Bostock 1998). The threshold changes, when are expressed in percentage, can be compared directly between other isolated or multi-fibres, different stimulation sites in the nerve, and different subjects.

1.3. Threshold Tracking technique

The technique of threshold tracking has been developed to provide information about nodal and internodal ion functionality, energy-dependent pumps and ion exchange processes activated by electric impulses in the human peripheral nerve axons. With the technique of threshold tracking we can investigate the response obtained either from the compound motor action potential amplitude over muscle when stimulating the motor fibres, and the sensory action potential when stimulating sensory fibres. The stimulus current is varied to produce a target potential of fixed size. Commonly, is used the target size of 40%, because these sizes fall on the steeply rising phase of the stimulus response curve (Kiernan et al., 2000, 2001). But if a maneuver changes the size of the maximal response potential, it will be necessary to measure the maximal response in a regular basis to change the size of the target potential tracked by the computer accordingly. Then, membrane potential is disturbed by subthreshold conditioning currents or by suprathreshold stimuli that cause axons to discharge. The proportional change in current required to illicit the target potential in response to these conditioning stimuli is measured.

When the membrane depolarizes, the threshold current required to produce the target potential decreases, and the opposite occurs with membrane hyperpolarization. As a result, threshold is often used as a surrogate marker of membrane potential. There is, however, considerable variation in threshold between subjects due to geometric factors; therefore, resting threshold on its own provides little information (Krishnan et al, 2008). Such relative changes in threshold are more useful than absolute changes that can be influenced by current access to the nerve. These threshold changes are monitored during various stimulation protocols: during changes of stimulus-duration (strength-duration relationship); during long-lasting subthreshold polarizing current pulses (threshold-electrotonus, current-threshold relationship); or following a single supramaximal stimulus (recovery cycle) (Burke et al., 2001).

A battery of excitability tests is most efficiently performed now with software ('QTracs') developed by Professor Hugh Bostock at the Institute of Neurology in London. The development of a semi-automated computer controlled protocol to perform threshold tracking, known as TROND (originally developed as part of a 3-day training symposium that was held in 1999 at Trondheim, 918 Norway), has enabled the use of the technique in many neuromuscular and nerve disorders (Kiernan et al., 2000, 2001). The study usually takes 10 minutes for motor nerve, and 15-20 minutes for sensory studies.

Multiple excitability variables are recorded using the following measures (Kiernan et al., 2000):

Stimulus-response curve

The stimulus-response curves were first recorded separately for test stimuli of duration 0.2 to 1 ms. The stimulus response data were used for several purposes. First, the 1 ms peak response was used to set the target submaximal response (40% of peak), for threshold tracking. Secondly, the slope 1ms stimulus-response curve was used with the tracking error (deviation from the target) to optimize the subsequent threshold tracking. Finally, the ratio between the 0.2 ms and 1 ms stimuli required to evoke the same responses were used to estimate the strength-duration time constants and rheobase of the stimulated axons (Kiernan et al., 2001).

Strength-duration time constant (SDTC)

Is a measure of the rate at which the threshold current for a target potential declines as stimulus duration is increased, and reflects resting nodal sodium current. In the formulation of Weiss (1901), it equates chronaxie.

Rheobase

Is the threshold current stimulus that can be infinitively long, and is related to SDTC by Weiss's law, and reflects also the nodal persistent sodium ion conductances (Bostock et al. 1998; Burke et al., 2001; Lin et al., 2011).

SDTC and rheobase are calculated by measuring threshold for stimuli from 0.2 to 1 ms and plotting stimulus charge versus duration.

Threshold Electrotonus (TE)

TE measures the threshold changes produced by prolonged depolarizing and hyperpolarizing currents, which are too weak to trigger action potentials (subthreshold currents). The changes in threshold are produced by 100-millisecond polarizing currents with intensities of +-40% and +-20%, of the unconditioned threshold using 1 ms test pulses, the threshold is tested at different time points during and after the 100-millisecond polarizing currents. TE is the selected method to examine the behavior of internodal conductances in human axons (Bostock, 1998; Bostock 1988). Threshold electrotonus provides insight into how axons accommodate long-lasting changes in membrane potential.

Current-threshold relationship (IV)

Describes the maximal extent of threshold changes, resulting from long duration (200ms) polarizing currents, with strength from +50 to - 100% of the resting threshold current. Threshold changes are tested with 1 ms pulses at the end of subthreshold polarizing currents lasting 200ms.

Recovery Cycle (RC)

The RC is investigated by double stimulation technique in which a supramaximal conditioning stimulus is given followed by the submaximal test stimulus, separated by variable interval (2 to 200 ms), to evaluate the

refractory, supernormal and late subnormal periods. The interstimulus interval is decreased in an irregular, roughly logarithmic sequence. Several periods can be identified and analyzed that represent the axon physiology. Following axonal depolarization there is a period in which is impossible to depolarize the axon – absolute refractory period. Following this phase, axons are relatively refractory for up to 5 ms, during which greater current is requires do generate an action potential. This period depends on the temperature, a cooler axon has a longer refractory period. This later period is followed by a superexcitable (or supernormal) phase, characterized by a reduction in threshold occurring over a 10-15 ms interval. Finally, there is a period of phase of late subexcitability (or late subnormality) ending at about 100ms.

1.4 . Studies in neurological disorders

Modelling of excitability parameters has allowed new insights into peripheral nerve pathophysiology (Karup et al., 2005).

During this last 5 decades, several studies were already published in peripheral nerve function and in some pathologies.

In table 1, it can be found a brief summary in several pathologies where tracking threshold technique was already applied.

Table 1 – - Summary of the results using tracking threshold technique in different disorders.

Neurological disorder	SD	Threshold electrotonus	Recovery cycle	Interpretation
Primary Amyloidotic neuropathy (Hafner J. et al., 2015)	-	-	-	Amyloid-related neuropathy does not involve a change in membrane potential as either a primary or secondary event.
Multiple myeloma (Nasu S. et al., 2014)		Decreased depolarizing	Decreased Superexcitability	Bortezomib induces a depolarizing shift in resting membrane potential prior to the development of neuropathy
Amyotrophic lateral sclerosis (Vucic, S., 2013)	-	-	Reduction in Superexcitability and Refractoriness	Riluzole exerts effect in peripheral nerve function: reduction of transient Na+ conductances
Myotonic dystrophy type 1 (Bae JS., et al., 2011)	-	Depolarizing and Hyperpolarizing reduced	Increased refractoriness	Peripheral neuropathy is a primary event of MyD1 rather than secondary complication of Diabetes mellitus
Episodic ataxia type 1 (Tomlinson SE. et al., 2010)		Increase in excitability due to depolarizing current	Elevated superexcitability	increased instability of the axonal membrane
Familial amyotrophic lateral sclerosis (Vucic S., 2010)	Increased	-	-	Persistent Na+ conductance is upregulated in familial ALS
Critical Ilness Myopathy (Z'Graggen et al., 2011)	-	-	↓ Supernormality ↑ Relative refractory period	Na$^+$ channel inactivation was increased

Disease (reference)		Greater threshold changes in depolarizing and hyperpolarizing (fanning out)	Progressive decline in refractoriness	
Sensory Neuropathy (Park et al., 2009; Krishnan et al, 2009)			Progressive decline in refractoriness	Sensory abnormalities occur prior to significant changes
Relapsing-Remitting Multiple Sclerosis (Misawa et al., 2008)	-	N	N Supernormality	MS patients do not generally have peripheral nerve demyelination
Episodic ataxia type 2 (Krishnan et al., 2008)	N	Early TE	↑ Refratoriness ↓ Late subexcitability	Indirect effects on KCNQ channels.
Liver disease (Karl et al.,2007)	N	↓ Reduced depolarizing	Reduxed Superexcitability	Indicative of mild depolarization probably due to ischemia resulting from poor perfusion
Hepatic failure (Ng et al., 2007)	N	Fanning in	↓ Superexcitability	Membrane despolarization
Hemifacial spasm (Krishnan et al., 2007)	N	N	N	Normal facial nerve excitability
Kennedy's disease (Vucic and Kiernan, 2007)		TEd		Membrane Hyperpolarization
Spinal cord injury (Lin et al., 2007)	↓	Fanning in	↓ Late subexcitability	Decentralization and consequent inactivity
ALS (Kanai at al., 2006; Vucic and Kiernan, 2006a, 2006b)	↑	Fanning out	↑ Superexcitability	
	N			

Myotonic dystrophy (Krishnan and Kiernan, 2006)		Fanning in	↑ Refratoriness	Membrane depolarization
Critical Illness neuropathy (Z'Graggen et al., 2006)	N	Fanning in	↑ Refratoriness, ↓ Superexcitability, ↑ Late subexcitability	Membrane depolarization
Amyotrophic lateral sclerosis (Vucic S., 2006)	Increased	Greater threshold change to both depolarizing and hyperpolarizing	Increased superexcitability	Increased persistent Na+ channel conduction, abnormalities of fast paranodal K+ and internodal slow K+ channel function
Diabetic Neuropathy (Krishnan et al., 2005)	↓		↓ Refratoriness	Na^+ channel conductance decrease
Uremic neuropathy (Krishnan et al., 2005a)	↑	Fanning in	↑ Refratoriness, ↓ Superexcitability, ↑ Late subexcitability	Membrane depolarization
Tetrodotoxin poisoning (Kiernan et al., 2005b)	↓	↓ TEd ↑ TEh	↓ In all RC parameters	Na^+ Channel blockade
Oxaliplatin-induced neuropathy (Krishnan et al., 2005c)	N	N	↑ Refratoriness	Involvement of nodal transient Na^+ channels
SCN 1 (beta) mutation – GEFS + (Kiernan et al., 2005b)	↓	↑ TEh	↓ Refratoriness	↓ Nodal Na^+ conductances

Disease				
Fabry's Disease (Tan et al. 2005)	-	Fanning in	↓ Superexcitability	Membrane depolarization
Diabetic neuropathy (Kitano et al, 2004; Krishnan and Kiernan, 2005)	↓	Fanning in	↓ In all RC parameters	↓ Na⁺ conductances, Na⁺/K⁺ pump dysfunction
CMT 1A (Nodera et al, 2004)	N	Fanning out	↓ Refratoriness ↓ Superexcability	↑ Fast K⁺ conductance
Machado-Joseph Diesease (Kanai et al., 2003)	↑	N	N	↑ Persistent Na⁺ conductance
AIDP (Kuwabara et al., 2002C)	N	N	N	Normal axonal excitability
AMAN (Kuwabara et al., 2002C)	N	N	↑ Refratoriness	Distal conduction failure
MMNCB (Kierman el al., 2002a)	N	Fanning out	↑ Superexcitabity	Membrane hyperpolarization
Hypokalemia (Kuwabara et al., 2002b)	↓	Fanning out		
Neuromyotonia (Kiernan et al., 2001c)	N	↑ Excitability overshoots	↑ Late Subexcitability	Upregulation of slow K⁺ condutance

CIDP (Cappelen-Smith et al., 2001)	↓	N	↓	In all RC parameters	Morphological changes

The advent of dedicated tools for the assessment of axonal excitability has promoted their use to detect early changes of nerve function, to investigate the pathophysiology of nerve lesion and to follow improvement of axonal membrane function following treatment (Bostock et al., 1998; Krishnan et al., 2009).

The sensory neuropathy associated with chronic oxaliplatin treatment shows higher threshold changes in both depolarizing and hyperpolarizing threshold electrotonus ("fanning out") and a progressive decline in refractoriness in axons (Park, et al, 2009; Krishnan et al., 2009), which has been identified as a hallmark of axonal hyperpolarization in previous human studies (Bostock et al., 1998; Kiernan et al., 2000). Sensory abnormalities occurred prior to significant reduction in the sensory amplitude, showing that the excitability changes are warning early findings.

Diabetic neuropathy is related to reduction of strength duration time constant and refractoriness, both derived from Na+/K+ pump dysfunction combined with a lower in Na+ conductance (Krishnan et al., 2008; Krishnan et al., 2009. In the end-stage kidney disease neuropathy, excitability recordings demonstrated significant changes in axonal excitability in patients immediately prior to a dialysis session; in particular, investigators have observed 'fanning-in' of threshold electrotonus curves consistent with axonal depolarization, associated with increased refractoriness and reduced superexcitability (Krishnan et al., 2006; Krishnan et al., 2009). Sequential studies performed during and after a

session of hemodialysis demonstrated that hemodialysis leads to normalization of excitability (Krishnan et al., 2006; Krishnan et al., 2009). Findings consistent with axonal membrane depolarization and increased axonal threshold were also found in porphyria associated neuropathy (Lin et al., 2008).

Excitability studies in multifocal motor neuropathies distal to conduction blocks shows a reduction of threshold electrotonus and increased superexcitability that is consistent with axonal depolarization (Kiernan et al., 2002a). In demyelinating neuropathies it has been observed threshold increase as well as reduced accommodation in early threshold electrotonus, probably derived from increased access of currents to the internodal compartment of the axon, due to demyelination (Krishnan et al., 2009).

1.4.1 TTR-FAP

Transthyretin (TTR)-type familial amyloid polyneuropathy (TTR-FAP) is an autosomal dominant disease caused by amyloidogenic genetic variants of TTR, which is characterized by progressive polyneuropathy associated with sensory loss, motor weakness, and autonomic dysfunction. Typically, small fibers are affected first, but shortly thereafter large fibers become progressively involved. Andrade was the first to identify the commonest form of TTR-FAP, TTR V30M, in which methionine replaces valine in position 30. Portugal is the country with the largest focus of the disease,

with over 1000 kindreds. The outcome of TTR V30M is always fatal, in general over 10-15 years from the initial symptoms. In the final stages, cachectic and incontinent patients die from renal insufficiency, metabolic imbalance, arrhythmia or infections (Coelho, 1996; de Carvalho, 2012). A recently completed phase II/III, randomized, double-blind, placebo-controlled, multicenter international, 18-month study (Fx-005) demonstrated that tafamidis 20 mg/d provided Class II evidence of efficacy (Coelho et al., 2012; de Carvalho, 2012). Based on a number of positive results and complementary statistical tests, the European Medicine Agency approved this new drug for use by patients in phase 1 - able to walk independently without assistance (de Carvalho, 2012). Early affected patients are the best candidates to benefit from the available treatments (tafamidis and liver transplantation) and to be included in future clinical trials for testing new compounds. The available methods to confirm early signs of neuropathy show low sensitivity (Conceição et al., 2008; Conceição et al., 2013). This is the first study known in patients with TTR-FAP and was undertaken to identify potential changes of axonal excitability in early affected patients with TTR-FAP and to test changes associated with tafamidis treatment.

2. Objectives

One of the two main objectives of this study was to apply the standardized protocol for multiple measures of nerve excitability in healthy controls and create a new and large reference database of excitability measures in

healthy control subjects and examine the effects of demographic characteristics, as age, BMI and sex.

The second main objective was to assess the effect of Vyndaqel® (tafamidis), indicated for the treatment of transthyretin amyloidosis in adult patients with stage 1 symptomatic polyneuropathy, in the excitability measures evaluated by the same standardized protocol.

3. Methodology

3.1. The technique.

Excitability studies were performed by stimulating the left median nerve at wrist in all subjects, while seated in a relaxed position. The left arm was resting on a pillow kept warm by a heater device. Before starting the study, the left hand of the subject was immersed in warm water, or heated on the pillow, as necessary to reach a minimum skin temperature of 31ºC. A thermistor was used to measure skin temperature at the beginning of the study, after each test over the course of the investigation protocol, (Tomlinson et al., 2010) and at the end of the study. The study was interrupted and the hand warmed every time the measured skin temperature fell below 31ºC. Excitability measurements were performed using the TRONDE protocol of the QTRAC program (Professor Hugh Bostock, Institute of Neurology, Queen Square, London, UK), which compares threshold, defined as the stimulus current required to achieve a

target response under a variety of conditions (Bostock et al., 1998; Jankelowitz et al., 2007). The EMG signal was recorded through a NL844 - Four Channel AC Preamplifier (Digitimer, Welwyn Garden City, UK) connected to a NeuroLog System (Digitimer, Welwyn Garden City, UK) and filtered between 2Hz and 10kHz. The active electrode was placed overlying the motor point of the abductor pollicis brevis and the reference on the proximal phalanx (20 mm diameter disk, E.K50430-001, Digitimer, Welwyn Garden City, UK). Stimulus waveforms were generated by the test computer and converted to current by a DS-5 isolated linear bipolar constant-current source (Digitimer, Welwyn Garden City, UK) with a maximal output ±50 mA. The stimulus currents were applied via non-polarizable electrodes (20 mm diameter disk, E.K50430-001, Digitimer, Welwyn Garden City, UK) with the active electrode over the nerve at the wrist and the reference electrode ~10 cm proximal at the lateral region of the forearm. Test current pulses of 0.2 or 1 ms were applied regularly at 0.8 s intervals and were combined with suprathreshold conditioning stimuli or subthreshold polarizing currents as required. The amplitude of the compound muscle action potential (CMAP) was measured from baseline to negative peak. For all tracking studies, the target CMAP was set to 40% of the peak response.

The threshold-tracking technique uses a simple experiment that is illustrated schematically in Figure 2 as the equipment necessary to apply the technique on Figure 3.
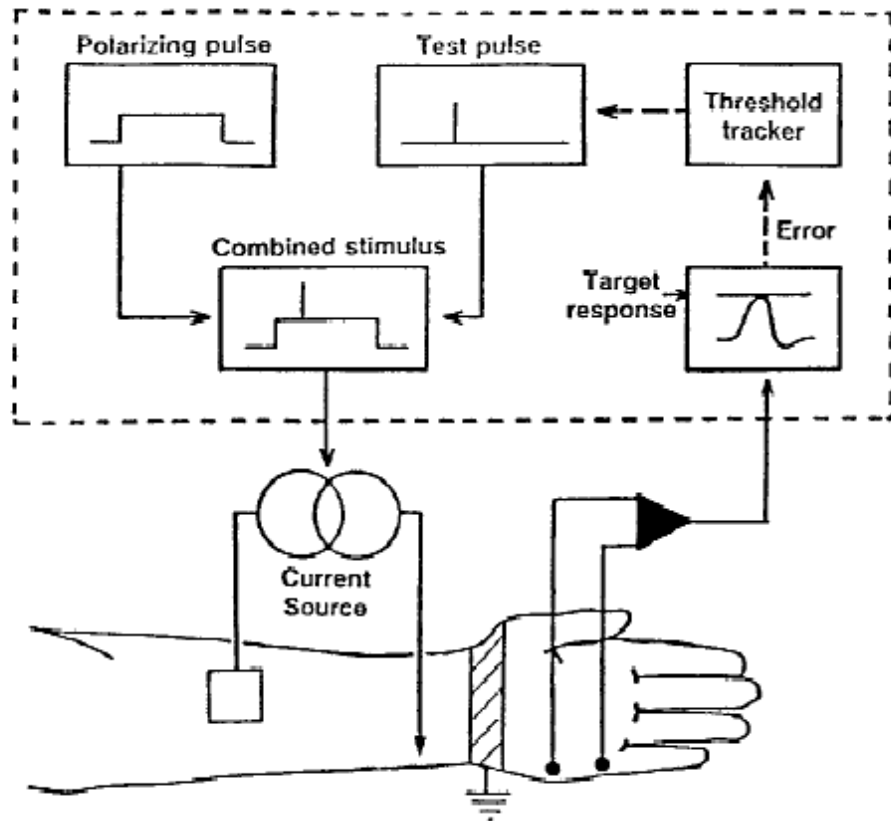
Figure 2 – Schematic diagram of typical threshold tracking experiment. Functions within broken box are carried out by personal computer. Compound muscle action potential (CMAP) recorded from hypothenar muscle is compared with target response (typically 30-40% of maximal response) and error signal is used to alter amplitude of test stimulus pulse, which is applied to the ulnar nerve at the wrist via an isolated voltage-to-current converter.
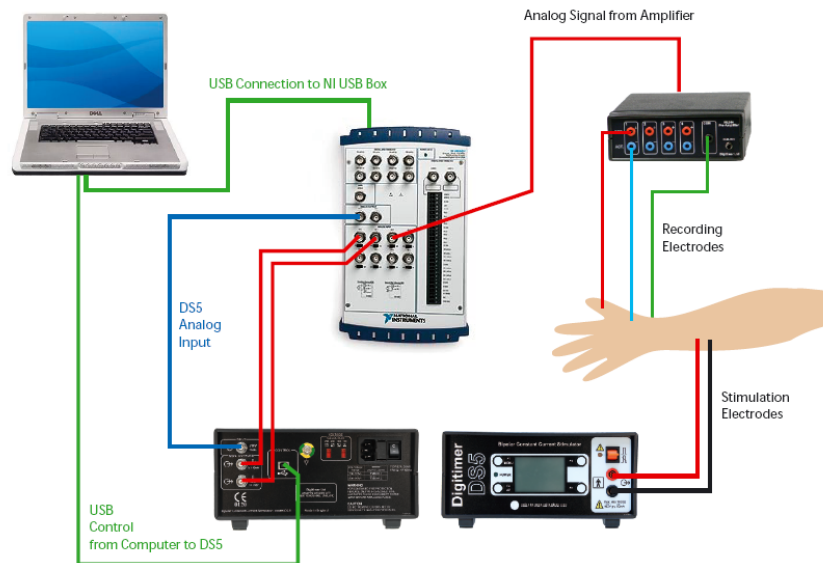
Figure 3 - All equipments necessary to apply tracking threshold technique. Including software and computer (top left), A-D convertor (top middle), amplifier (top right), stimulator (bottom, back on the right and front on the left).

Data analysis

The TROND recordings were analyzed and plotted with QTRAC. This program enables automated analysis of multiple excitability parameters. First the raw stimulus and response values for each stimulus condition were used to estimate the threshold stimulus required to elicit the target response. These data were then used for statistical analysis performed by QTRAC-P. One MEM (multiple excitability measures) file (which corresponds to an individual test) was generated from one TROND recording and contained a list of 32 derived excitability parameters that could be used for statistical analysis. Several MEM files were indexed in a MEF file, and QTRAC-P also provides for statistical comparisons between groups of recordings specified by the MEF files.

Mean parameter values were compared between groups using parametric tests for normally distributed and nonparametric tests for non-normally distributed data. Parameter variability is indicated by the standard deviation (SD) on interquartile range (IQR).

3.2 Subjects

3.2.1 Healthy controls

To investigate the influence of age, body mass index (BMI), gender and temperature on the excitability measures we included 47 healthy volunteers of both sexes, working in our institution or acquainted retired people. We excluded those with any medical condition that could affect the peripheral nerve function, those taking drugs that could change nerve excitability, and those with any signs or symptoms suggestive of carpal tunnel syndrome or peripheral neuropathy. Conventional nerve conduction studies excluded median nerve lesion in controls. Height and weight were recorded and BMI calculated in all cases (BMI = mass (Kg)/(height(m)^2). To illustrate the high reproducibility of this method, one subject was investigated four times every three months and his results included in this thesis.

The differences between genders were analyzed by unpaired t-test. The relation between results and age, temperature (assessed at the study end) and BMI was approached by multiple regression analysis (backward

elimination). Due to the multiple comparisons we set a p value < 0.01, as significant.

This protocol was approved by the local Ethics Committee and all subjects gave informed consent before study entry.

3.2.2 FAP patients

To compare motor axon excitability measures between controls and early affected TTR-FAP patients, and to test the influence of tafamidis treatment on those parameters we included ten TTR-FAP (TTR V30M) patients of both sexes, 5 men and 5 women, with a median age of 34 years (range, 29 to 43) and a median BMI of 22 (range, 18.6 to 26.6). In all patients the diagnosis was confirmed by genetic testing and by identification of amyloid deposit in the salivary gland (Do Amaral et al., 2009). Median disease duration was 1.5 year (range, 0.5 to 6 years), all patients had 3 or less years of disease duration, the only case with longer duration (6 years) was a 56-year old woman in whom a pacemaker was implanted due to arrhythmia.

Symptoms at disease onset were diarrhea (5 cases), distal lower limb parenthesis (5), erectile dysfunction (3), bladder paresis (2), weight loss (2) and pacemaker implantation (2). No patient had symptoms involving upper limbs and carpal tunnel syndrome was excluded by conventional conduction velocity studies. Patents with median nerve motor responses lower than 3 mV (peak-to-peak) were excluded. All TTR-FAP patients were early affected, a necessary condition to start tafamidis treatment.

To compare with FAP patients, a control population of 21 healthy controls matched for age (median age 31, from 21 to 45), gender (9 males, 43%) and body-mass index (median 22, from 17.6 to 26.8) was investigated. This population was extracted from the previously described larger population of healthy controls.

TTR-FAP patients were tested on the day before the tafamidis treatment onset (time 0), as well as 3 months (time 1, T1) and 6 months (time 2, T2) after. The differences between TTR-FAP at and controls were explored by the unpaired-t test. Longitudinal changes in the excitability study measurements in TTR-FAP patients were analyzed by applying the paired t-test. To correct for multiple comparison a $p \leq 0.001$ was considered as significant and a $p \leq 0.01$ as a trend. Table 4 specifies the parameter considered for statistical analysis (Annex I)

This protocol was approved by the local Ethics Committee and all subjects gave informed consent before study entry.

4. Results

<u>Reproducibility</u>

This procedure confirmed that excitability parameters were highly repeatable, with minor variability between these multiple sessions over 12 months (figure 4).

4.1 Healthy controls

The group of 47 healthy controls consisted of 18 men and 29 women, with a median age of 47 years (mean age 45.9, SD 17.7, ranging from 21 to 84 years), with a median BMI of 23.8 (mean value of 24.0, SD 3.92, from 17.5 to 32.5) and a median temperature of 33ºC at the study end (mean value 32.6, SD 1.5, from 31 to 35). No subject had any side effect.

Nerve excitability parameters derived from CMAP recording of the left median are summarized in Table 2. The mean of the excitability data for all healthy subjects are expressed in figure 5.

| Excitability parameters | Sex | | BMI | | Age (years) | | | | | |
| | | | | | Young | | | Old | | |
	Females	Males	<mean	>mean	BMI <mean	BMI >mean	Total	BMI <mean	BMI >mean	Total
1. Peak CMAP (mV)	7.45 (1.07)	8.47 (1.06)	8.32 (1.07)	7.11 (1.07)	8.85 (1.08)	8.24 (1.11)	* 8.67 (1.06)	7.46 (1.13)	6.63 (1.09)	* 6.93 (1.07)
2. Stimulus for 50% CMAP (mA)	4.39 (1.06)	4.80 (1.09)	4.76 (1.06)	4.26 (1.08)	4.49 (1.08)	3.68 (1.05)	4.27 (1.06)	5.06 (1.1)	4.56 (1.11)	4.74 (1.08)
3. Stimulus-response	4.81 (1.06)	4.56 (1.07)	* 5.15 (1.06)	* 4.21 (1.05)	5.30 (1.04)	4.77 (1.07)	* 5.15 (1.04)	4.83 (1.14)	3.97 (1.07)	* 4.27 (1.07)
4. Rheobase (mA)	2.89 (1.07)	3.11 (1.1)	3.18 (1.07)	2.74 (1.1)	* 3.03 (1.09)	* 2.29 (1.06)	2.85 (1.08)	3.20 (1.11)	2.98 (1.14)	3.06 (1.09)
5. Strenght duration time constant	0.50 (0.02)	0.49 (0.02)	0.47 (0.02)	0.52 (0.03)	0.45 (0.028)	0.54 (0.04)	0.48 (0.02)	0.52 (0.02)	0.51 (0.03)	0.51 (0.02)
I/V parameters										
6. Resting I/V slope	0.58 (0.02)	0.60 (0.01)	0.59 (0.01)	0.57(0.02)	0.60 (0.02)	0.59 (0.03)	0.59 (0.01)	0.59 (0.01)	0.57 (0.02)	0.58 (0.02)
7. Minimum I/V slope	0.24 (0.01)	0.24 (0.01)	* 0.25 (0.01)	* 0.23 (0.01)	* 0.25 (0.01)	* 0.20 (0.01)	0.24 (0.01)	0.26 (0.01)	0.24 (0.01)	0.25 (0.01)
8. Hyperpolarizing I/V slope	0.37 (0.01)	0.37 (0.02)	* 0.39 (0.02)	* 0.34 (0.01)	* 0.38 (0.02)	* 0.29 (0.01)	0.35 (0.02)	0.40 (0.03)	0.36 (0.01)	0.38 (0.01)
Threshold Electrotonus										
9. Accomodation half time	37.28 (1.02)	38.63 (1)	38.35 (1.08)	37.37 (0.97)	39.57 (1.02)	36.33 (0.94)	* 39.57 (1.02)	35.95 (2.09)	36.56 (0.91)	* 36.33 (0.94)
10. S2 accomodation	22.55 (1)	22.76 (0.72)	* 24.02 (0.82)	* 21.21 (0.84)	23.51 (0.96)	21.91 (0.78)	23.51 (0.96)	23.26 (1.51)	21.1 (0.85)	21.91 (0.78)
11. TEd peak	68.1 (1.1)	68.03 (1.0)	69.24 (0.82)	67.29 (1.21)	70.27 (0.95)	66.33 (1.26)	68.97 (0.81)	67.62 (1.52)	67.74 (1.7)	67.69 (1.18)
12. TEd20 (peak)	40.6 (0.98)	38.35 (0.77)	40.33 (0.79)	39.08 (1.01)	40.33 (0.85)	39.19 (0.94)	40.33 (0.85)	39.73 (1.5)	38.86 (1.24)	39.19 (0.94)
13. TEd40 (Accom)	23.76 (0.73)	22.99 (0.71)	** 24.75 (0.63)	** 22.05 (0.66)	25.21 (0.732)	22.94 (1.13)	* 24.59 (0.63)	23.74 (1.21)	21.63 (0.81)	* 22.42 (0.69)
14. TEd20 (10-20 ms)	* 38.02 (0.76)	* 35.69 (0.67)	37.67 (0.71)	36.63 (0.76)	37.31 (0.81)	36.16 (1.42)	37.04 (0.69)	38.03 (1.44)	36.85 (0.92)	37.3 (0.78)
15. TEh20 (10-20 ms)	-39.22 (0.75)	-39.67 (1.38)	-38.14 (0.79)	-40.28 (1.21)	-38.32 (0.78)	-39.19 (1.74)	-38.11 (0.88)	-39.03 (1.26)	-40.79 (1.6)	-40.13 (1.1)
16. TEd (10-20 ms)	68.75 (1.12)	68.59 (0.99)	69.77 (0.85)	68.07 (1.24)	69.25 (0.89)	68.68 (1.18)	69.25 (0.89)	68.68 (1.48)	68.68 (1.71)	68.68 (1.18)
17. TEd (90-100 ms)	45.55 (0.85)	45.27 (0.73)	45.21 (0.62)	46.08 (1.06)	45.46 (0.83)	45.78 (0.87)	45.46 (0.83)	44.36 (0.94)	46.64 (1.25)	45.78 (0.87)
18. TEd (undershoot)	-21.24 (0.76)	-19.08 (0.89)	* -21.48 (0.73)	* -19.01 (0.82)	-21.05 (0.69)	-19.6 (0.89)	-21.05 (0.69)	-21.51 (1.38)	-18.46 (1.08)	-19.6 (0.89)
19. TEh (10-20 ms)	-79 (1.34)	-78.06 (1.86)	-76.94 (1.31)	-79.86 (1.63)	-76.76 (1.46)	-79.79 (1.46)	-76.76 (1.46)	-78.25 (2.38)	-80.71 (1.87)	-79.79 (1.46)
20. TEh (90-100 ms)	-128.8 (4.75)	-126.6 (4.06)	-126.2 (3.8)	-128.6 (4.97)	-129.7 (4.72)	-125.2 (4.02)	-129.7 (4.72)	-122.1 (4.92)	-127 (5.8)	-125.2 (4.02)
21. TEh (overshoot)	16.19 (1)	13.71 (0.84)	16.29 (1.04)	14.25 (0.77)	15.37 (0.81)	15.27 (1.04)	15.37 (0.84)	17.19 (2.12)	14.11 (1.02)	15.27 (1.04)
Recovery cycle parameters										
22. RRP (ms)	2.92 (1.03)	2.81 (1.03)	2.87 (1.03)	2.98 (1.03)	2.902 (1.03)	2.856 (1.03)	2.902 (1.03)	2.889 (1.06)	2.836 (1.05)	2.856 (1.03)
23. Refractoriness at 2 ms	72.71 (9.03)	57.5 (8.11)	69 (8.97)	69.92 (9.3)	71.26 (8.83)	67.74 (9.37)	71.26 (8.83)	67.86 (12.1)	67.57 (15.6)	67.74 (9.37)
24. Refractoriness at 2.5 ms	19.43 (4.38)	14.41 (4.4)	18.7 (4.45)	16.8 (4.35)	20.09 (4.1)	15.63 (4.65)	20.09 (4.1)	19.48 (9.31)	13.16 (4.96)	15.63 (4.65)
25. Superexcitability at 5 ms	-25.41 (1.31)	-25.54 (1.46)	-26.14 (1.21)	-23.87 (1.82)	-28.56 (1.31)	-25.37 (1.95)	-27.16 (1.11)	-23.04 (2.01)	-23.17 (2.53)	-23.12 (1.72)
26. Superexcitability at 7 ms	- 22.69 (1.29)	-22.05 (1.36)	-23.45 (1.21)	-20.68 (1.65)	-25.52 (1.32)	-23.2 (0.97)	* -24.63 (0.92)	-20.39 (2.19)	-19.5 (2.35)	* -19.83 (1.65)
27. Superexcitability (%)	-25.03 (1.18)	-24.56 (1.34)	-25.74 (1.07)	-23.09 (1.71)	-26.42 (0.99)	-22.69 (1.62)	- 26.42 (0.99)	-23.31 (1.69)	-22.31 (2.43)	-22.69 (1.62)
28. Subexcitability (%)	17.28 (0.93)	15.48 (0.95)	16.84 (0.83)	16.34 (1.08)	17.15 (0.90)	16.1 (0.99)	17.15 (0.90)	15.15 (1.16)	16.66 (1.44)	16.1 (0.993)

Table 2 – Summary of excitability differences between dichotomous groups for gender, age (young vs old) and BMI (slim vs fat). The cutoff for age and BMI were defined as above and below the mean value (age = 45.9; BMI = 24.0) for the whole group. * = $p < 0.05$ (trend) , ** = $p < 0.01$ (significant), Student's t-tests using QTRAC; BMI Mean (total): 24,0 Kg/m2; Age Mean: 45,9 years.
Data are given as mean (SEM): by convention, threshold electrotonus, refractoriness, super- and subexcitability are all expressed as % change in threshold; <45 years (n= 23); >= 45 years (n=24). Median age of 47 years (mean age 45.9, SD 17.7, ranging from 21 to 84 years), with a median BMI of 23.8 (mean value of 24.0, SD 3.92, from 17.5 to 32.5)
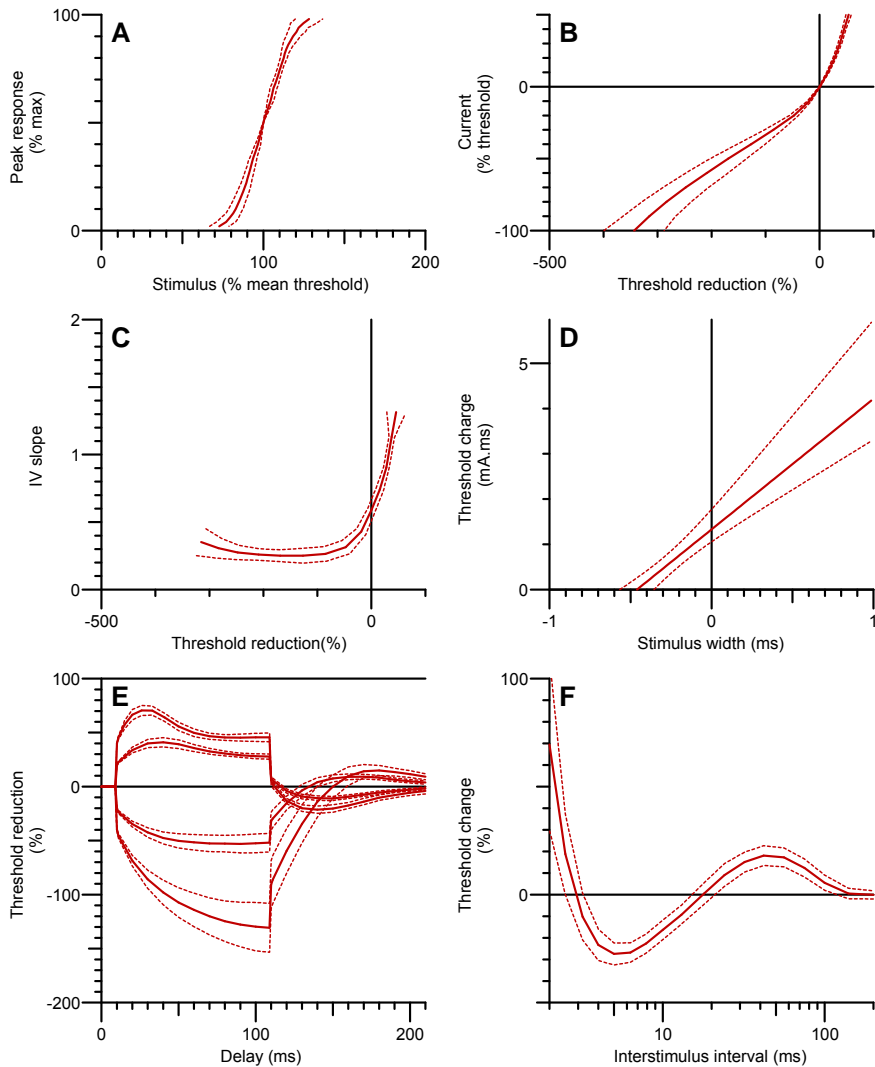
Figure 5: Multiple nerve excitability measures, recorded with a standard 10-minute protocol, in the population of healthy controls (n=47). Solid lines and broken lines represent means and 95% confidence limits, respectively. A – Normalized stimulus response curves; B – Current-threshold relationship ; C- Current-voltage relation; D- Threshold charge-stimulus duration relationship, E- Threshold-electrotonus; F – Recovery cycle.

Comparisons between genders revealed similar age (46.1 vs 45.9, p=0.19), BMI (25.1 vs 23.5, p=0.19) and temperature (32.8 vs 32.4, p=0.26). Moreover when comparing between gender all the parameters present in table 2, we did not find any significant difference (p >0.2 for all comparisons), only the TEd20 (10-20 ms) tended to be higher in females (p<0.05), but did not attain statistical significance.

Multiple regression analysis showed that CMAP amplitude decreases with age and temperature, stimulus-response slope decreases with age and BMI, and that accommodation half-time decrease with age and temperature (table 3). No other statistically significant relation was found (figure 6 and figure 7).

Measurements	Age (years)	BMI	Temperature (ºC)
CMAP peak-ampl	p=0.001 (r= -0.488)	NS	p= 0.002 (r= -0.4443)
Stimulus-Response Curve	p= 0.002 (r= -0.437)	p= 0.002 (r= -0.441)	NS
Accommodation half-time	p= 0.005 (r= -0.411)	NS	p< 0.001 (r= -0.614)

Table 3: Results of the multiple regression analysis. BMI – body mass index; NS – non-significant
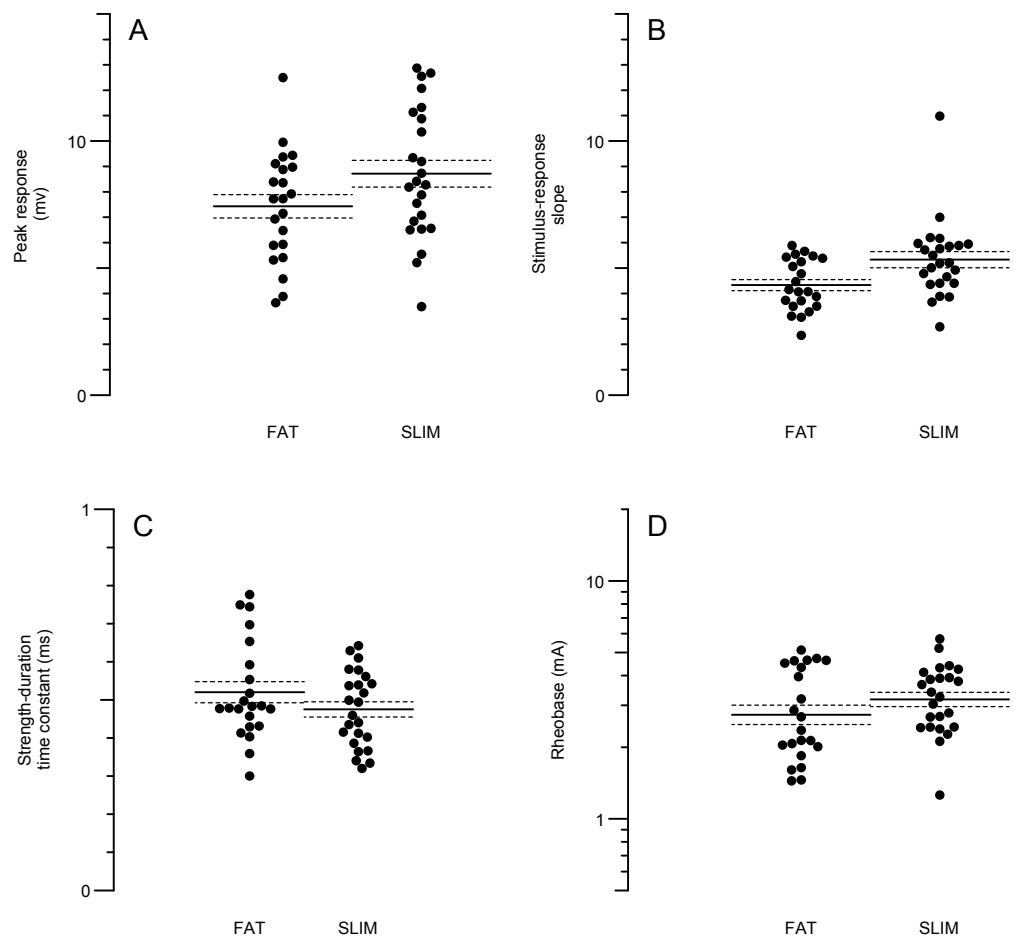
Figure 6 – Nerve excitability measures results on peak response amplitude (A), stimulus response slope (B), strength-duration time constant (C) and in rheobase (D) between slim and fat subjects. Mean and standard deviation values.
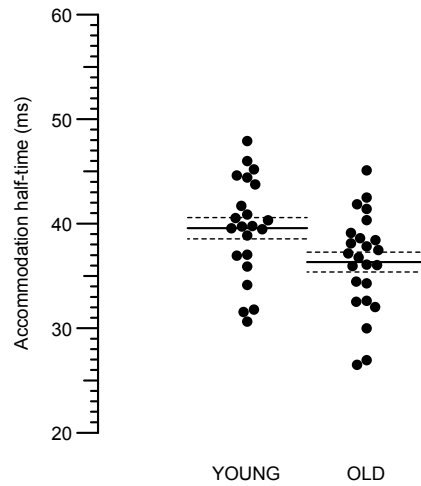
Figure 7 - Comparison between young and old subjects showing a much lower value of accommodation half-time in old people. Mean and standard deviation values.

4.2 TTR-FAP patients

4.2.1 Baseline

The individual values observed in the TTR-FAP population showed some variability (figure 8). The baseline comparison between TTR-FAP patients and controls gave non-significant differences for all comparisons (table 4 and figure 9).
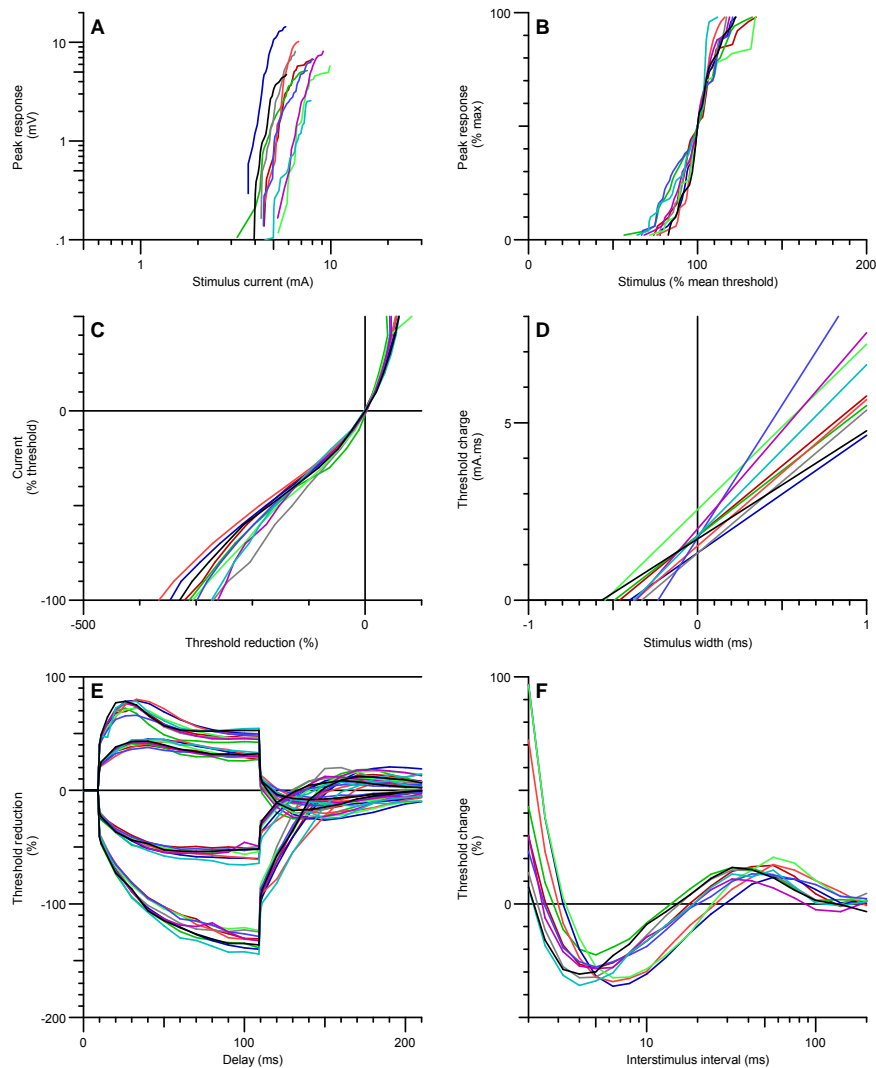
Figure 8: Excitability data of the 10 TTR-FAP patients at entry – before treatment with Tafamidis.

Tafamidis was well tolerated and there was no drop-out. Six patients reported improvement of the sensory symptoms, in particular neuralgic pain, one noticed reduced cramps and mild increased BMI was observed in three. But diarrhea and weight loss was more severe in one patient.

We repeated the same experimental protocol three months and six months after study entry, all patients were taking the drug and no missing value was found.

4.2.2 Before vs after treatment

There was a trend for higher stimulus intensity for 50% of the maximal motor response and rheobase between baseline (T0) and after 3 months (T1), and for higher rheobase between 3 months (T1) and 6 months on treatment (T2). However, between T0 and T2 both stimulus intensity for 50% of the maximal motor response and rheobase increased significantly ($p<0.001$). Indeed, rheobase augmented by 190% and the stimulus intensity for 50% of the maximal motor response by 170% (figure 10). SDTC decreased significantly from T1 to T2 ($p=0.009$) and showed a trend to decrease between T0 and T2 ($p=0.03$). The results are summarized in Table 4.
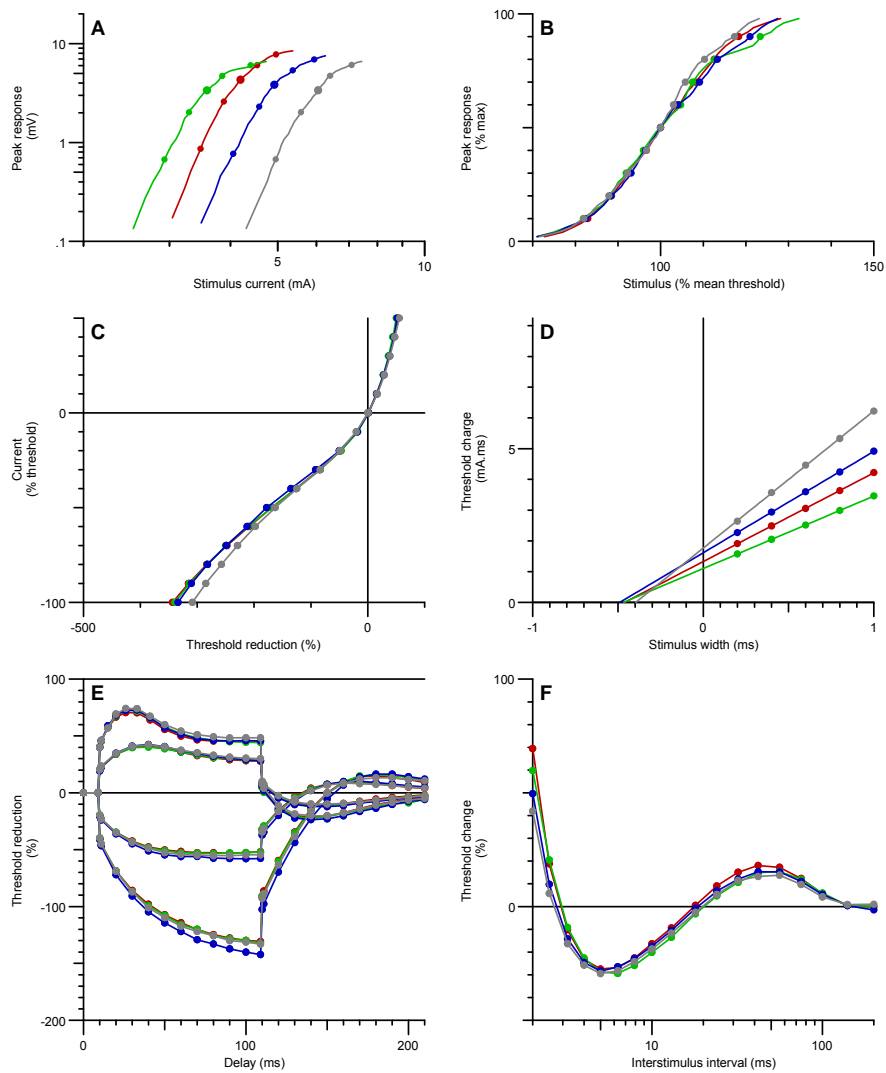
Figure 9 – Red represents healthy controls, green TTR-FAP patients at study entry (T0), blue 3-months after treatment onset (T1) and grey at 6 months after entry (T2). It is represented the absolute stimulus-response relationship (top left), the normalized stimulus-response relationship (top right), the current-threshold relationship (middle-left), threshold change-stimulus width ratio (middle right), threshold-tracking (bottom left) and recovery-cycle (bottom right).
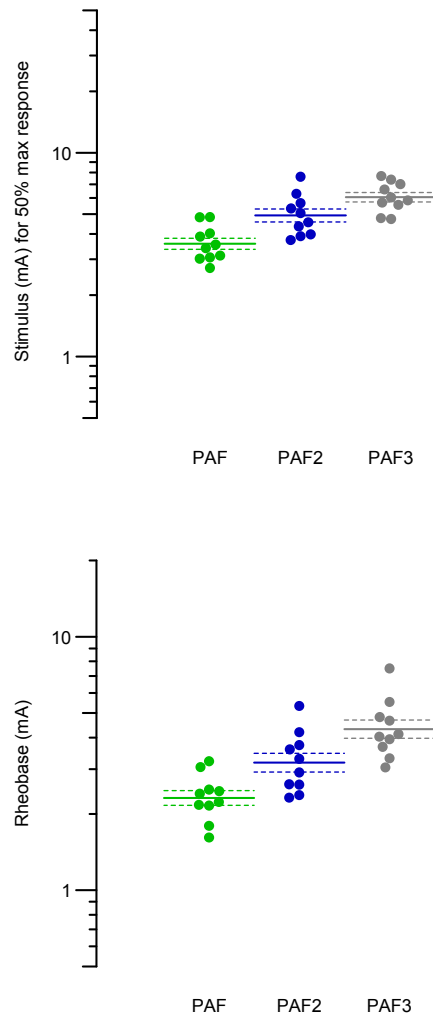
Figure 10 – Excitability studies of TTR-FAP patients on tafamidis. Mean and standard deviation of stimulus intensity for 50% maximal compound muscle action potential (top) and rheobase (bottom) of the TTR-FAP patients at T0 (PAF), T1 (PAF2) and T2 (PAF3), showing progressive increasing values.

Table 4 – Measures of axonal excitability in healthy controls and familial amyloid polyneuropathy (FAP) patients

Excitability parameters	Controls (21)	Controls vs FAP	FAP T 0	FAP T0 vs FAP T1	FAP T1	FAP T1 vs FAP T2	FAP T2	FAP T0 vs FAP T2
Mean values (Standard Error)								
1. Peak CMAP (mV)	8.641(1.06)	NS	6.725(1.12)	NS	7.077(1.14)	NS	6.748(1.16)	NS
2. Stimulus for 50% CMAP (mA)	4.199(1.06)	NS	3.578(1.06)	p=0.0038	4.922(1.08)	NS	6.052(1.05)	p<0.001
3. Stimulus-response slope	5.121(1.04)	NS	5.235(1.1)	NS	4.985(1.06)	NS	5.856(1.09)	NS
4. Rheobase (mA)	2.759(1.07)	NS	2.311(1.07)	p=0.0083	3.187(1.09)	p=0.0333	4.319(1.09)	p<0.001
5. Strength duration time constant	0.4817(0.0251)	NS	0.4704(0.0334)	NS	0.5320(0.0231)	p=0.009	0.4146(0.0324)	p=0.03
I/V parameters								
6. Resting I/V slope	0.5929(0.0161)	NS	0.628(0.0499)	NS	0.5880(0.024)	NS	0.5520(0.031)	NS
7. Minimum I/V slope	0.2332(0.0083)	NS	0.2311(0.0099)	NS	0.2293(0.0095)	NS	0.2492(0.0104)	NS
8. Hyperpolarizing I/V slope	0.3513(0.0194)	NS	0.3599(0.0175)	NS	0.3920(0.0153)	NS	0.4150(0.0363)	NS
Threshold Electrotonus								
9. Accommodation half time	39.17(0.983)	NS	41.09(1.34)	NS	39.33(1.54)	NS	41.69(0.801)	NS
10. S2 accommodation	23.34(0.989)	NS	26.46(1.32)	NS	25.37(1.85)	NS	24.52(1.23)	NS
11. TEd peak	68.96(0.848)	NS	70.77(1.47)	NS	71.19(1.4)	NS	72.63(1.31)	NS
12. TEd20 (peak)	40.38(0.886)	NS	39.99(0.846)	NS	41.78(0.876)	NS	41.92 (10.90)	NS
13. TEd40 (Accom)	24.46(0.647)	NS	26.23(1.3)	NS	25.83(1.8)	NS	24.52(1.23)	NS
14. TEd20 (10-20 ms)	36.93(0.71)	NS	36.86(0.661)	NS	38.01(0.891)	NS	37.88(0.719)	NS
15. TEh20 (10-20 ms)	-38.61(0.759)	NS	-38.85(0.929)	NS	-40.51(0.656)	NS	-39.22(0.715)	NS
16. TEd (10-20 ms)	69.26(0.936)	NS	71.05(1.33)	NS	71.25(1.62)	NS	72.6 (1.26)	NS
17. TEd (90-100 ms)	45.62(0.85)	NS	44.31(1.41)	NS	45.81(1.51)	NS	48.41(1.12)	NS
18. TEd (undershoot)	-20.9(0.701)	NS	-21.96(1.42)	NS	-23.29(1.59)	NS	-20.5 (1.28)	NS
19. TEh (10-20 ms)	-77.23(1.45)	NS	-77.76(1.3)	NS	-81.38(1.5)	NS	-77.98(1.26)	NS
20. TEh (90-100 ms)	-130.2(4.91)	NS	-131(3.97)	NS	-141.2(4.93)	NS	-132. 2(2.11)	NS
21. TEh (overshoot)	15.21(0.872)	NS	16.59(1.47)	NS	16.99(2.27)	NS	14.48(1.36)	NS
Recovery cycle parameters								
22. RRP (ms)	2.878(1.04)	NS	2.897(1.04)	NS	2.721(1.04)	NS	2.595(1.05)	NS
23. Refractoriness at 2 ms	69.44(9.11)	NS	59.79(7.77)	NS	49.6(8.33)	NS	41.92(10.9)	NS
24. Refractoriness at 2.5 ms	18.77(4.07)	NS	20.51(7.28)	NS	9.806(5.69)	NS	5.745(6.64)	NS
25. Superexcitability at 5 ms	-27.5(1.11)	NS	-28.74(1.3)	NS	-28.39(1.4)	NS	-29.5(1.04)	NS
26. Superexcitability at 7 ms	-24.75(0.956)	NS	-27.67(1.52)	NS	-24.71(1.21)	NS	-26.26(1.86)	NS
27. Superexcitability (%)	-26.66(1.01)	NS	-28.9(1.3)	NS	-27.42(1.11)	NS	-29.49(1.33)	NS
28. Subexcitability (%)	17.04(0.936)	NS	14.78(1.09)	NS	14.5(1.18)	NS	13.69(0.788)	NS

Table 4 - Measures of axonal excitability in healthy controls and familial amyloid polyneuropathy (FAP) patients. NS – non-significant comparison; p< 0.01 = trend for significant; p <0.001 = significant difference. FAP (patients with familial amyloid polyneuropathy (TTR V30M), T0 = at study entry, one day before the onset of tafamidis treatment, T1 = 3 months after treatment onset; T2 = 6 months after treatment onset.

To test if local edema overlying the nerve on the first session could originate the observed findings by its gradual reduction over treatment, the subcutaneous tissue of two investigators were infiltrated with physiologic saline. The excitability studies were performed before and after infiltration (control MdeC with 0.5 and 1 ml; control MB with 0.5 ml), it was noticed that 0.5 ml was enough to create a bulging area over the nerve at wrist. No significant changes could be observed (figure 10). Stimulus for 50% of the maximal motor response increased in MB from 4.023 to 4.931mA, and varied from 5.853 to 5.210 (0.5ml) to 4.445mA (1 ml) in MdeC. Rheobase changed from 2.690 to 3.409mA in MB and from 3.853 to 3.497 (0.5ml) to 4.475mA (1 ml) in MdeC. For both measurements the trends were not consistent.
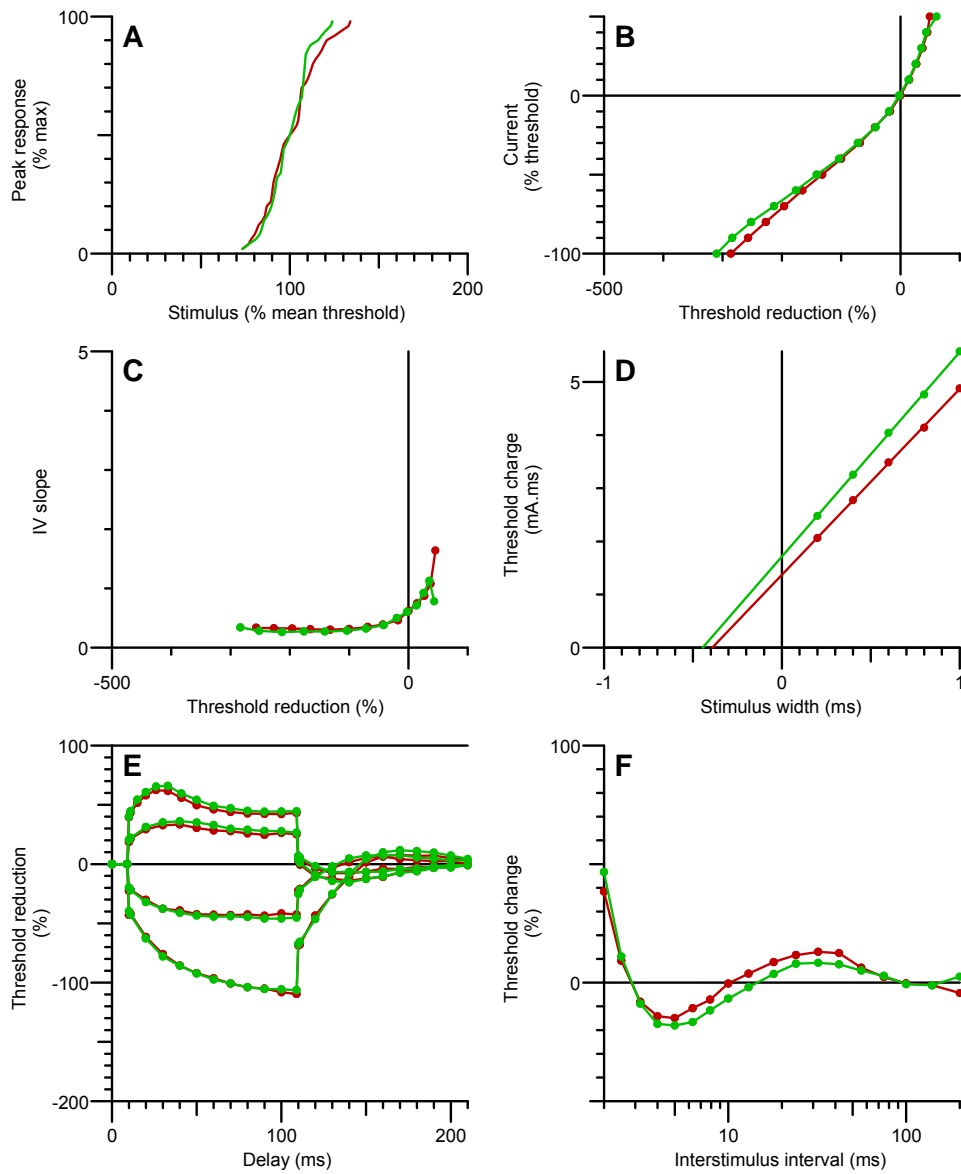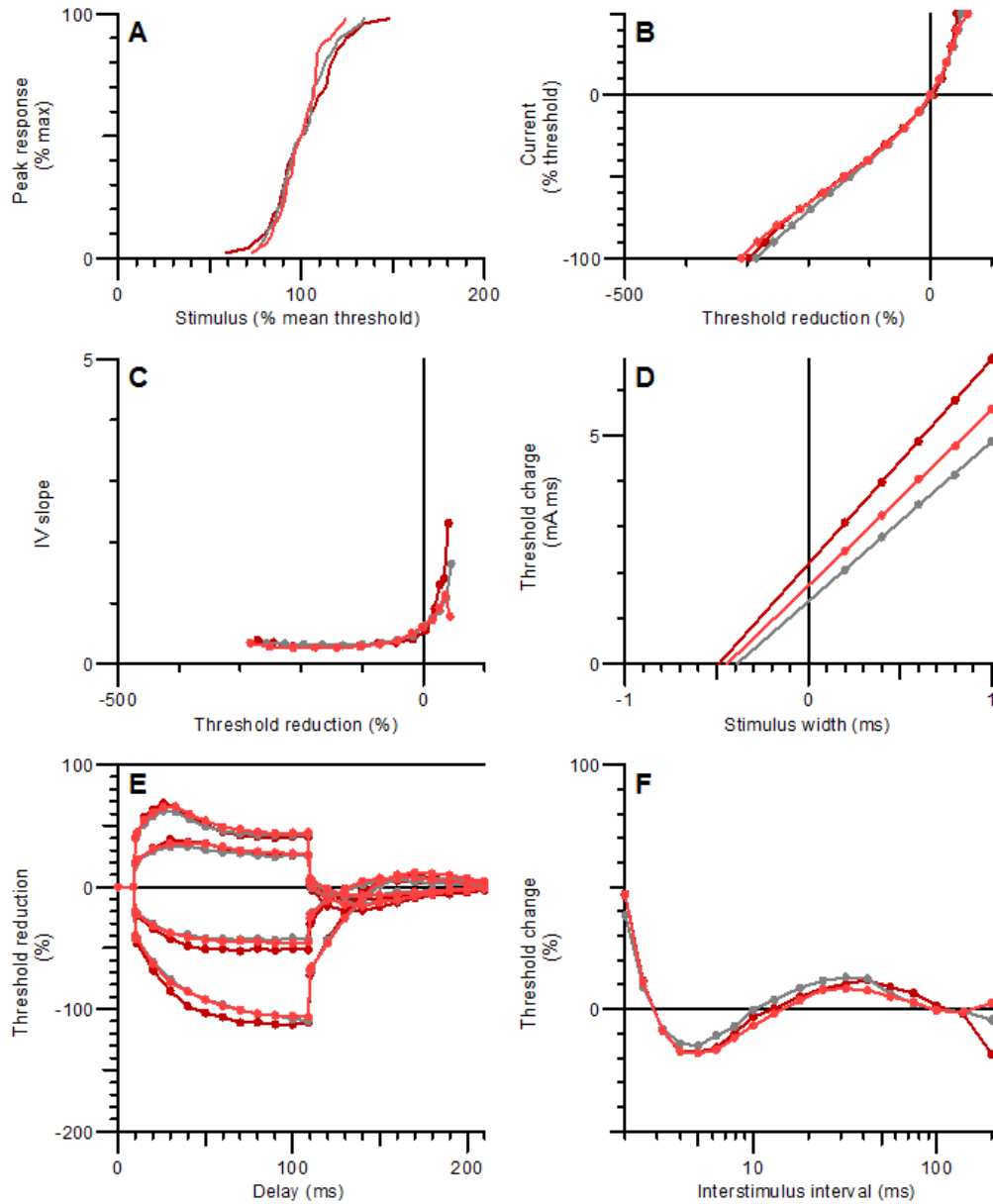
Figure 11 – Infiltration of physiologic saline in the subcutaneous tissue of healthy controls (A1 – 0.5 ml, green before and red after; B1 – 0.5 and 1 ml, before red, after 0.5 ml grey and after 1 ml dark red).Graphic order is described in figure 9.

Case Study

The figure 12 illustrates an individual case studied at the three time points of the study, where we can observe that Rheobase and the stimulus intensity for 50% of the maximal motor response increased significantly during the treatment period.
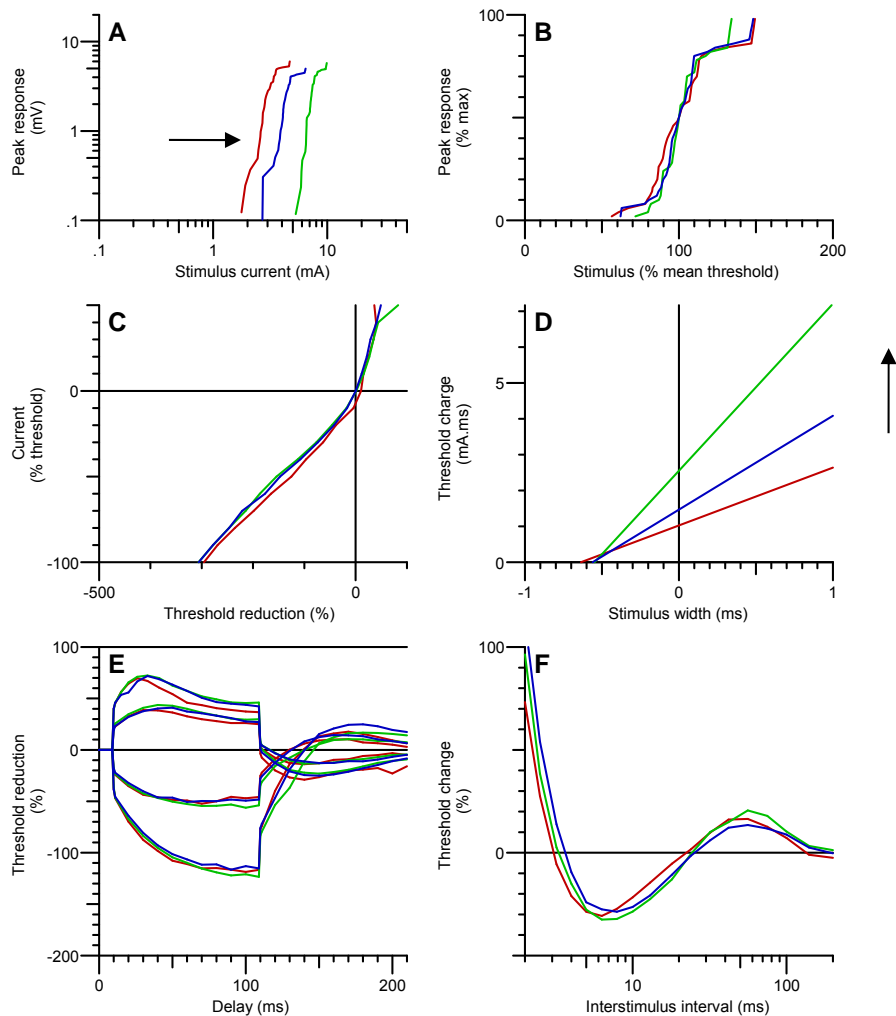

Figure 12: Excitability data of one TTR-PAF patient. Red – study entry, T0, Blue – after 3 months of treatment, T1, Red – after 6 months of treatment, T2.

5 Discussion

This study investigated the changes in axonal excitability properties related with age, gender, BMI and temperature in healthy controls; and studied excitability properties of motor axons in patients with TTR-FAP neuropathy. In TTR-FAP patients, the excitability measures were assessed before and after Tafamidis treatment.

5.1 Excitability parameters in Healthy Controls

The motor response amplitude reduces with ageing as a consequence of motor unit loss (Kimura, 1984); in addition, lower temperature augments potential amplitude due to lesser temporal dispersion (Bolton et al., 1981; Kiernan et al., 2001; Kimura, 1984). Both these relationships were observed in our study, and are consistent with the findings of McHugh and colleagues (McHugh et al., 2011), who found a significant univariate negative correlation between age and CMAP amplitude (r=-0.52, p<0.001) and a trend towards a significant negative correlation between temperature and CMAP amplitude (r=-0.23, p<0.05).

The influence of BMI on axonal excitability measurements is a less investigated topic. Although McHugh et al. (McHugh et al., 2011) found a significant negative correlation between CMAP amplitude and BMI

(r=-0.30, p<0.01) it did not persist as significant in a multiple regression model. In our population, CMAP amplitude was not influenced by BMI using the same statistical model, but we found a significant negative correlation between stimulus-response slope and BMI (r=-0.441, p=0.002).

In our study, temperature was well controlled but it showed some inter-subject variation (from 31 to 35ºC). This small variation was enough to reveal a significant negative correlation between skin temperature and accommodation half-time (table 3), a relationship that has previously been described by other authors (Kiernan et al., 2001; McHugh et al., 2011). This observation is explained by the sluggishness of slow potassium channel gating at lower temperatures (Kiernan et al., 2001). On the other hand, temperature variation within the limits observed in our subjects did not significantly alter the recovery cycle (Kiernan et al., 2001), nor the refractoriness and the relative refractory period in contrast to other studies (Kiernan 2001; McHugh et al., 2011).

Age was an independent factor decreasing the accommodation half-time to depolarizing currents in our study (r=0.4111, p=0.005). This is in accordance with McHugh and co-workers findings (McHugh et al., 2011). This suggests a trend for lower axonal membrane excitability with ageing (Jankelowitz, et al., 2007). Moreover, ageing was negatively correlated to stimulus-response slope (r=0.437, p=0.002). There was a non-significant

trend for the same negative correlation (univariate analysis, r=-0.19, p<0.05) in McHugh et al. report (McHugh et al., 2011). In contrast to previous studies, we did not find any statistical impact of aging on superexcitability (Bae et al., 2008; McHugh et al., 2011), strength duration time constant (Bae et al., 2008; Jankelowitz, et al., 2007; Yerdelen et al., 2006), IV curve in response to depolarization (Bae et al., 2008; Jankelowitz et al., 2007) or on threshold changes in depolarizing threshold electrotonus (Bae et al., 2008).

Overall, the changes related to demographic features on TRONDE protocol parameters are small and less important than in conventional nerve conduction studies. Nonetheless, our results underscore the relevance of a careful temperature control, and indicate that interpretation of stimulus-response slope and accommodation half-time should take into account age and BMI. In contrast, gender is not of major relevance to appreciate axonal threshold findings in motor nerves.

5.2 Excitability parameters in patients with stage 1 symptomatic polyneuropathy (TTR-PAF) and Tafamidis treatment.

To diagnose early small fiber dysfunction in TTR-FAP is a difficult task (Conceição et al., 2008; Conceição et al., 2013) and threshold tracking technique of the motor fibers of the median nerve does not seem a useful

test regarding this purpose. It is not surprising taking into account that the sensory fibers of the lower limbs are the first somatic fibers to be affected in this condition.

Indeed, recently a study testing threshold tracking of the median motor fibers of patients with primary amyloidosis did not show significant changes as compared with controls (Hafner et al., 2015). The studies of the sensory fibers the only significant changes were related to higher stimulus intensity required for a 50% compound sensory action potential and rheobase (Hafner et al., 2015).

Considering the effectiveness of tafamidis in changing the natural course of the disease and the open questions related to its properties (de Carvalho, 2012) it seemed convenient to test its impact on the axonal membrane function. With this treatment, we noticed a progressive reduction of axon excitability, as demonstrated by the increase of rheobase and stimulus intensity for 50% maximal compound muscle action potential, not related to changes in Na^+ channels conductance or other channels modulation, taking into account the stability of the remaining parameters. It could be derived from decreased resistance of the overlying soft tissue nearby stimulating area, but our experiences infiltrating subcutaneous tissue with physiologic saline seems to exclude this hypothesis. We speculate that physiologic amyloid catabolism (Sousa and

Saraiva, 2003) without formation of new amyloid by the action of the drug could modify nerve structure increasing axon excitability.

6 Conclusions

Nerve excitability techniques provide a novel and powerful noninvasive means of detecting changes in axonal biophysical properties and are a potentially axonal exciting new addition to the armamentarium of the clinical neurophysiologist (Kiernan, 2006).

Our investigation in a large control population enable to establish laboratory normative values, which will permit to compare with diseased population in future studies. In particular, we can explore whether this novel technique has the power to separate normal nerve from affected nerve in an individual patient without the potential overlap of control and patient values.

The study in TTR-PAF patients exemplifies how threshold tracking can contribute to detail the mechanism of action of new drugs and to expand the possibilities of new trials to test it in other diseases.

Nerve excitability testing, with interpretation aided by model fitting, provides an objective, non-invasive means of estimating changes in membrane potential and ion channel function in affected axons.

The global burden imposed by metabolic diseases and associated complications continue to escalate. Neurological complications, most commonly peripheral neuropathy, represent a significant cause of morbidity and disability, like as in patients with familial amyloid polyneuropathy, diabetes and taking neurotoxic drugs. Furthermore, health care costs are substantially increased by the presence of complications making investigation into treatment a matter of high priority.

In conclusion, our study shows that minimally invasive threshold tracking of CMAPs could be useful for study nerve disease models, as the TTR-FAP disease and pharmacological studies.

A word of caution is necessary, because this technique has several limitations. The nerve is tested at the point of stimulation, and so it is not useful for focal neuropathies, unless it is possible to excite the nerve at the lesion site. Tracking threshold test only the axons with thresholds close to the level chosen for tracking, so conditions affecting the excitability of only a minority of axons, whether the least or the more excitable, may go undetected. Finally, the axons that have degenerated, or that are blocked

between stimulation and recording site, will not be evaluated (Bostock et al., 1998).

7. Future Work

This study exemplifies how threshold tracking can contribute to detail the action mechanism of new drugs and to expand the possibilities of new trials to test it in other diseases. Regarding tafamidis, we aim to test sensory nerve fibers in future studies.

Aknowledgments

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