Escola Superior de Tecnologia da Saúde do Porto

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Oxidative Stress in Diabetic Neuropathy

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Dissertação submetida à Escola Superior de Tecnologia da Saúde do Porto para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica em Saúde – Ramo Biotecnologia, realizada sobre a orientação Mónica Vieira, Professora Adjunta da área técnico-científica das Ciências Químicas e das Biomoléculas, e coorientação de Cristina Prudêncio, Professora Coordenadora com Agregação da área técnico-científica das Ciências Químicas e das Biomoléculas.

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O sucesso nasce do querer, da determinação e persistência em se chegar a um objetivo. Mesmo não atingindo o alvo, quem busca e vence obstáculos, no mínimo fará coisas admiráveis.

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Resumo

Para o tratamento da dor crónica, uma condição multifatorial, é necessário um diagnóstico adequado que permita a diferenciação da dor nas suas diversas componentes. A neuropatia diabética está presente em todo o mundo e tem um grande impacto na sociedade atual. A diabetes poderá desencadear a produção de espécies reativas de oxigénio, associadas ao *stress* oxidativo, que poderão ser um fator-chave no desenvolvimento da neuropatia.

O objetivo principal deste trabalho é averiguar uma eventual associação entre dor crónica, neuropatia diabética e *stress* oxidativo.

Neste sentido, foi desenvolvida uma meta-análise, que permitiu a avaliação da relação causal entre o *stress* oxidativo e a neuropatia diabética, e, também, a aplicação de questionários validados para a avaliação da dor, o *Brief Pain Inventory* (BPI) e o *Douleur Neuropathique* 4 (DN4) a uma amostra de conveniência.

Na meta-análise avaliaram-se resultados para indicadores de *stress* oxidativo, como a peroxidação lipídica, as atividades da superóxido dismutase e da catalase, e, ainda, a glutationa reduzida. Selecionaram-se, de entre 257 artigos, 9 estudos realizados em ratinhos diabéticos neuropáticos e em controlos saudáveis. Os níveis de peroxidação lipídica estão elevados em todos os estudos, enquanto os níveis dos restantes biomarcadores se encontram diminuídos, comparando o grupo diabético com o grupo controlo.

Na avaliação da amostra de conveniência, foram recolhidos 84 inquéritos ao longo das quatro estações: verão, outono, inverno e primavera. As queixas de dor foram descritas em termos do local e intensidade, do alívio da dor com toma de medicação e os efeitos da dor nas atividades diárias, através do BPI. Os *scores* obtidos no BPI indicam a presença de dor moderada a severa, aumentada no outono e na primavera, com implicações nas tarefas diárias, e transversais aos grupos estudados. Para determinar as principais caraterísticas associadas à dor neuropática, utilizou-se o DN4. Este indicou a presença de cerca de 50% de doentes com dor neuropática. Os resultados sugerem, ainda, que ser mulher, o aumento da idade e estar em situação de inatividade profissional são fatores que podem influenciar a dor crónica e, consequentemente, a dor neuropática.

Os resultados obtidos neste estudo apontam para uma possível existência de uma associação entre *stress* oxidativo e dor neuropática, e, ainda, uma possível ligação entre as condições climáticas e queixas/prevalência de dor.

Palavras-chave

Dor crónica, dor neuropática, diabetes, neuropatia diabética.

Abstract

For treating chronic pain, a multifactorial condition, is needed a suitable diagnosis which allows the differentiation in its many components. Diabetic neuropathy is a worldwide disease with great impact in the modern society. Diabetes may leads to the production of reactive oxygen species that are associated to oxidative stress, which may be a key factor in the development of diabetic neuropathy.

The main goal is to inquire a potential association between chronic pain, diabetic neuropathy and oxidative stress.

Thus, was performed a meta-analysis that permitted the causal evaluation between oxidative stress and diabetic neuropathy, and, a pain evaluation was accomplished in a convenience sample using validated surveys – Brief Pain Inventory (BPI) and *Douleur Neuropathique* 4 (DN4).

Through the meta-analysis it was possible evaluate oxidative stress biomarkers, such lipid peroxidation, superoxide dismutase and catalase activities, and reduced glutathione. 9 studies were selected and all were performed in mouse models. The levels of lipid peroxidation were increased in all the studies, however the levels of the other biomarkers were diminished in diabetic models comparatively to healthy controls.

In the evaluation of convenience sample, 84 surveys were collected along the four seasons: summer, autumn, winter and spring. The pain complaints were described in terms of local, intensity, impact, relief by medication and its effect on daily activities using BPI questionnaire. The scores obtained in BPI indicate the presence of moderate to severe pain, with increased complaints in autumn and spring, and implications in daily activities, transversal to all groups. To determine the main features associated with neuropathic pain it was used DN4 questionnaire. The DN4 indicated the presence of nearly 50% of patients with neuropathic pain. The results suggest that being female, the increased age and being retired can influence chronic pain and neuropathic pain in patients.

As main conclusions, it was possible to verify an association between oxidative stress, and neuropathic pain, and, also, that weather conditions may influence the pain complaints and its prevalence.

Key words

Chronic pain, neuropathic pain, diabetes, diabetic neuropathy, oxidative stress.

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Index of abbreviations

Abbreviation	Full name
ADP	Adenosine diphosphate
AGEs	Advanced glycation end product formation
ALA	α-lipoic acid
ATP	Adenosine triphosphate
BPI	Brief Pain Inventory
CAT	Catalase
CMMP	*Clínica Médica Povo Portuense*
CNS	Central Nervous System
CP	Chronic Pain
DHT	Dihydrotestosterone
DN	Diabetic neuropathy
DN4	*Douleur neuropathique* 4
DNNP	Diabetic Non-Neuropathic Pain
DNP	Diabetic Neuropathic Pain
GSH	Reduced glutathione
IASP	International Association for the Study of Pain
Jac/STAT	Janus kinase/signal transducer and activator of transcription
LDL	Low density lipoprotein
LPO	Lipid peroxidation
MAPK	Mitogen-activated protein kinase
MDA	Malondialdehyde
MetS	Metabolic syndrome
NADPH	Nicotinamide adenine dinucleotide phosphate
NDNP	Non-Diabetic Neuropathic Pain
NF-κb	Nuclear transcription factor κb
NNP	Non-Neuropathic Pain
NOS	Nitric oxide synthase
NP	Neuropathic pain
NSAIDs	Nonsteroidal anti-inflammatory drugs
OS	Oxidative stress

PARP	Poly ADP-ribose polymerase
PKC	Protein kinase C
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
QoL	Quality of Life
ROS	Reactive oxygen species
SD	Standard Deviation
SOD	Superoxide dismutase
T1D	Type 1 diabetes
T2D	Type 2 diabetes

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Chapter I – State of the art

Chronic Pain

Usually chronic illness patients have pain and other associated symptoms that are often ineffectively managed and, therefore, some pain variations occur over the time (Effiong & Effiong, 2012).

Pain is a useful sensation that has a protective effect in our body, working as a warning signal. It is characterized as an individual experience which is often hard to determine (Calcutt, 2013; Robison-Papp, George, Dorfman, & Simpson, 2015).

A painful stimuli normally begins in the periphery and reaches the central nervous system (CNS) through pain pathways. An injury in the neuronal network may lead to an amplified reaction to noxious stimuli (hyperalgesia), and nociceptive response to harmless stimuli (allodynia) (Hagenston & Simonetti, 2014).

Chronic pain (CP), as Diabetes, is considered a major public health problem worldwide with a huge impact in social and economic areas (De Moraes Vieira, Garcia, Da Silva, Mualem Araújo, & Jansen, 2012; Poliakov & Toth, 2011).

Pain, particularly CP, has a great influence concerning daily activities, because induces alterations in mood, cognition and emotional functioning, which potentiates limitations in basic activities, as normal work or walking, among others (Poliakov & Toth, 2011; Reid et al., 2011).

The International Association for the Study of Pain (IASP) defines CP as “persisting continuously or intermittently for longer than 3 months” (L. F. Azevedo, Costa-Pereira, Mendonça, Dias, & Castro-Lopes, 2012; Inoue et al., 2015). In terms of intensity, pain may be classified as mild-to-severe (L. F. Azevedo et al., 2012; Reid et al., 2011).

CP may be nociceptive – related with tissue injury; neuropathic – when it corresponds to a failure in nerves or in a part of the nervous system; or both, also called mixed pain (Baker, Collett, Fischer, & Herrmann, 2010).

In the past 10 years, several studies worldwide presented an estimative of people affected by CP (Table I). This estimative varies from 4.2 to 48% mainly due to (i) different definitions of CP, (ii) target population, and (iii) applied methodology for the investigation (De Moraes Vieira et al., 2012; Reid et al., 2011). In Europe, 19% of the population suffers from CP (Baker et al., 2010).

Table I: Prevalence of CP worldwide.

Study	Year	Population	% of CP
(Torrance, Smith, Bennett, & Lee, 2006)	2006	United Kingdom	48%
(Breivik, Collett, Ventafridda, Cohen, & Gallacher, 2006)	2006	Europe and Israel	19%
(Bouhassira, Lantéri-Minet, Attal, Laurent, & Touboul, 2008)	2008	France	31.7%
(Toth, Lander, & Wiebe, 2009)	2009	Canada	35.0%
(Reid et al., 2011)	2011	Europe	19%
(De Moraes Vieira et al., 2012)	2012	Brazil	42%
(Kurita, Sjogren, Juel, Hojsted, & Ekholm, 2012)	2012	Denmark	26.8%
(L. F. Azevedo et al., 2012)	2012	Portugal	36.7%
(Mohamed Zaki & Hairi, 2014)	2014	Malaysia	15.2%
(Inoue et al., 2015)	2015	Japan	39.3%
(Bernfort, Rahmqvist, Husberg, & Ake, 2015)	2015	Sweden	4.2-18.9%

The most common sites of pain are the upper and lower back, joints, head and neck. Also, spinal complications, pain after trauma and surgery are pointed as common causes of CP (Baker et al., 2010; Breivik, Eisenberg, & O'Brien, 2013; Reid et al., 2011). A European report about CP indicates NP (11%) and diabetes (4%) as common causes of CP (Figure I) (Baker et al., 2010)

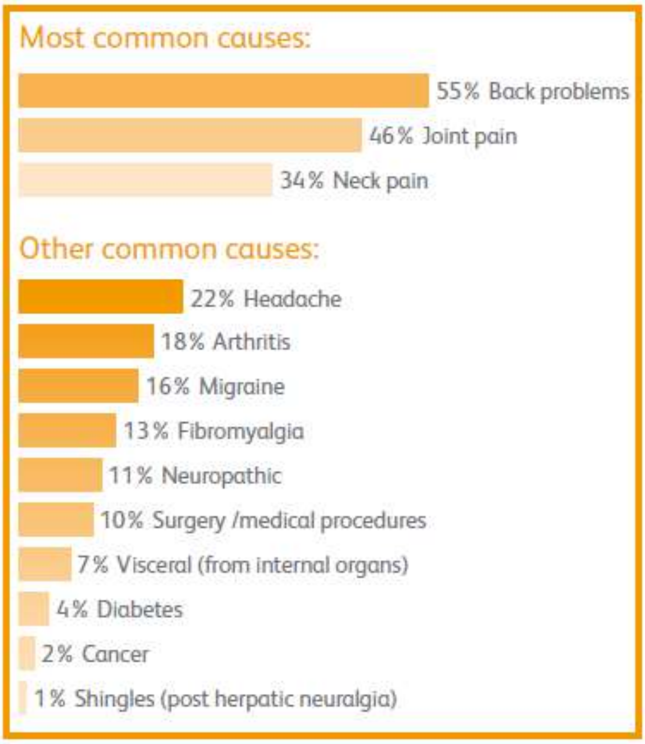


Figure I: Chronic pain origins – most common causes (Baker et al., 2010).

A CP condition may implicate alterations in different organs and systems, as well as psychiatric disorders (L. Azevedo, Pereira, Dias, & Agualusa, 2007).

For the care of CP, it is necessary to implement a multidisciplinary intervention, establishing a cognitive behavioral methodology with therapeutic dialogues and training addressing the psychosocial aspects of CP and physical activity. Often the time until diagnosis is larger than expected, situated between 3 months and 5 years, which is a long period to manage pain without the proper treatment (Figure II). In Portugal, it takes about 2.5 years (Baker et al., 2010; Jouini et al., 2014; Lapane, Quilliam, Benson, Chow, & Kim, 2014) to diagnose CP.

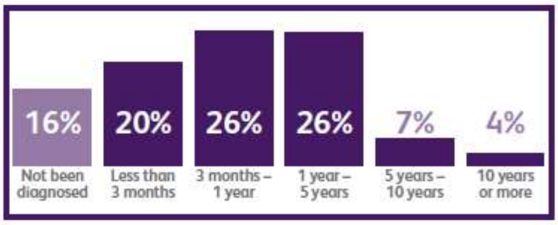


Figure II: Time until diagnose (Baker et al., 2010).

In 2030-2050 it is expected that the range of the population over 60 years will be 1.3-2 billion. With the population aging, the incidence and prevalence of pain is expected to rise. However, pain could be underestimated due to the association of elderly and pain as a normal process in this age group (Bernfort et al., 2015; Kaye, Baluch, & Scott, 2010; Mohamed Zaki & Hairi, 2014).

Several times, CP is associated with other comorbidities. Diabetes is one of the most affected conditions, and CP is a diminishing factor for patients' quality of life (QoL) (Chang, Ma, Lee, & Hsieh, 2015; Liberman, Peleg, & Shvartzman, 2014; Nenke et al., 2015)

Neuropathic pain

The IASP defines neuropathic pain (NP) as "pain arising from a direct consequence of a lesion or disease affecting the somatosensory system", and may result from a variety of different illnesses (Barbosa, Bennett, & Carvalho, 2014; Paul C Langley, Van Litsenburg, Cappelleri, & Carroll, 2013; Schaefer et al., 2014)

The estimated prevalence of occurrence of NP in general population varies among authors:

- 3 - 17% (Mathieson, Maher, Terwee, Folly de Campos, & Lin, 2015);
- 3 - 8% (Barbosa et al., 2014);
- 7 - 8% (De Andrés, De La Calle, Pérez, & López, 2014; De Moraes Vieira et al., 2012);
- 6.9–8.2% (Hamdan, Luna, Del Pozo, & Gálvez, 2014).

NP is associated with nerve damage and it is characterized by spontaneous and induced pain, along with paresthesias, dysesthesias, and normal sensation impairment. Many patients describe numbness, burning, tingling in the extremities and slow nerve conduction as the main symptoms (Jain, Bansal, Dalvi, Upganlawar, & Somani, 2014; Mathieson et al., 2015; Papanas & Ziegler, 2014; Schaefer et al., 2014). It is also related with some comorbidities such as depression, anxiety and sleep disorders (Cortez, Reis, Cardoso, Onofre, & Piovezan, 2014; García-Campayo, Caballero, Perez, & López, 2012).

The appearance of NP is associated to degenerative or age-related factors, being the elderly the most affected population. This condition arises in a wide and heterogeneous number of clinical conditions (De Andrés et al., 2014; Lekpa et al., 2013).

Distinguish neuropathic from nociceptive pain is not often easy, therefore, questionnaires for NP have been developed and applied when there is suspicion of NP, because NP diagnosis and treatment are a challenge, and, consequently, this condition is not treated optimally. (Barbosa et al., 2014; Erdemoglu & Koc, 2013; Harifi et al., 2013; Mathieson et al., 2015; Radat, Margot-Duclot, & Attal, 2013).

Antidepressants, anticonvulsants and opioid analgesics are among the selected drugs with known efficacy in the control of NP. Nevertheless, some unsatisfactory side effects are known and new approaches are being designed such as dihydrotestosterone (DHT) and 3α-diol (Calabrese et al., 2014; Hamdan et al., 2014; Moulin et al., 2015; Oosterling, Boveldt, & Verhagen, 2015).

Diabetes is pointed as a major cause of NP. This condition affects up to 25 % of diabetic patients and has a great impact on QoL (Barbosa et al., 2014; Freeman, 2013; Lapane et al., 2014; Wang et al., 2014). However, there are other causes such as infection-associated neuropathy (i.e. postherpetic neuralgia and HIV), cancer, stroke, lumbar or cervical radiculopathies, complex regional pain syndrome, spinal cord injury pain, trigeminal neuralgia, drug-induced polyneuropathies, multiple sclerosis, traumatic or post-surgical nerve injuries and central pain syndromes secondary to vascular lesions (De Andrés et al., 2014; Lekpa et al., 2013; Snedecor et al., 2013).

Diabetes

Diabetes is an epidemic disease, considered a public health issue of great relevance, that has a huge impact in nowadays society, being in the top five main causes of death in developed countries (Bandeira et al., 2013; Chopra et al., 2012; Matough & Budin, 2012). Several authors estimated that about 366 million people worldwide suffer from this condition and that it is predictable, until 2030, an increase of 1.5 times (Chopra et al., 2012; J. Han et al., 2014; Kandhare, Raygude, Ghosh, Ghule, & Bodhankar, 2012).

Diabetes is associated with complications at macro and microvascular levels such as retinopathy, neuropathy and nephropathy (Bandeira et al., 2013; Chilelli, Burlina, & Lapolla, 2013; Singh, Kishore, & Kaur, 2014). Chronic hyperglycemia, a rise of blood sugar, is the major contributor for this condition (Hinder, Vivekanandan-Giri, McLean, Pennathur, & Feldman, 2013; Jain et al., 2014; Singh et al., 2014). Alterations in cellular homeostasis have also a great importance, causing diffuse vascular injury and multi-organ dysfunction (Chilelli et al., 2013).

Diabetes has two distinct types, type 1 diabetes (T1D) and type 2 diabetes (T2D) that are both characterized by impaired insulin signaling and hyperglycemia. T2D is the most prevalent, representing 90% to 95% of known cases (Bandeira et al., 2013; Hinder et al., 2013; Y.-B. Wu et al., 2013). T1D patients have autoimmune lesions, which lead to pancreatic b-cells destruction, and loss of insulin production, while T2D patients have a remarkable increase in insulin production and insulin resistance in several tissues (muscle, fat and liver) (Hinder et al., 2013; Premkumar & Pabbidi, 2013).

Diabetes is responsible for structural/functional damages in several organs (i.e. heart, blood vessels, eyes, kidneys, and nerves) (J. Han et al., 2014)

Diabetic Neuropathy

Vinik and colleagues (2003) have defined diabetic neuropathy (DN) as ‘‘a demonstrable disorder, either clinically evident or subclinical, that occurs in setting of diabetes mellitus without other causes for peripheral neuropathy”.

DN is a long-term disorder and the most common complication of diabetes worldwide (Al-Enazi, 2013; Kasznicki et al., 2012; Singh et al., 2014). It appears as one of the predominant causes of diabetes-related hospital admissions, foot ulcers and non-traumatic amputations in Western countries (Figueroa-Romero, Sadidi, & Feldman, 2008; Hinder et al., 2013). 30-40% T2D patients are affected by DN, but those with T1D have a more severe outcome. This condition causes incapacities, poor QoL secondary to pain and a high mortality rate (Al-Enazi, 2013; Figueroa-Romero et al., 2008; Kasznicki et al., 2012; Singh et al., 2014).

Some disorders are closely related to DN, i.e. nerve fiber loss, demyelination and axonal degeneration of myelinated fibers (Al-Enazi, 2013; Chopra et al., 2012). Specific structural changes (nodal and paranodal swelling) in myelinated fibers are noted primary, and leads to disturbed axonal transport and progressive axonal atrophy (Singh et al., 2014). Relying on the cause, the injury may appear in the axons or in the myelin sheaths (Hosseini & Abdollahi, 2013).

DN is associated with some painful symptoms. Paraesthesia (spontaneous pins and needles sensation), hyperalgesia (increased pain responses to normally noxious stimuli) and allodynia (abnormal hypersensitivity to normally innocuous stimuli) are present in around 20% of all diabetic patients (Areti, Yerra, Naidu, & Kumar, 2014; Gong et al., 2015; Singh et al., 2014).

One of the key factors in the progress of DN is the generation of free radicals by amplified glycolytic processes. The physiological intermediaries and metabolic initiators, resulting of oxidative stress (OS) and ROS, have a preponderant weight in progressive nerve fiber damage, dysfunction and loss in DN (Hosseini & Abdollahi, 2013).

In diabetes the nerve tissues are in a proinflammatory state and this is a leading cause for the development of DN (Hosseini & Abdollahi, 2013; Kumar, Negi, & Sharma, 2012).

Among other methods of diagnosis, the clinical assessment, quantitative sensory testing, and electrophysiological study (latency, amplitudes and nerve conduction velocity of sensory and/or motor nerve) are the key points for determination of DN (Rajanandh, Kosey, & Prathiksha, 2014).

To monitor the progression of DN, in terms of oxidative stress measurement, and the response to therapeutics it is important to have some reliable biomarkers. Thus, malondialdehyde (MDA), reduced glutathione (GSH), superoxide dismutase (SOD) and activities of mitochondrial enzymes (citrate and ATP synthase) are considered valuable biomarkers (Areti et al., 2014).

The most common therapeutics is the use of agents that modulate pathogenetic mechanisms (glycemic control), foot care and relieve the symptoms of DN (Figueroa-Romero et al., 2008; Hinder et al., 2013; Singh et al., 2014). It is desirable that treatment initiates when patients notice that painful neuropathy is impairing activities of daily living and their QoL (Hosseini & Abdollahi, 2013; Singh et al., 2014).

In early stages, the progress of DN should be prevented and accompanied with a suitable treatment (Catanzaro et al., 2013). However, DN is known as a multifactorial disease which undermines the choice of an effective treatment. Thus, it is very important the discovering of novel therapeutic agents (Cameron, 2013; Kumar et al., 2012; Papanas & Ziegler, 2014).

According to Snedecor and colleagues (2013), the European Federation of Neurological Societies subdivided the treatment in two categories:

- First line: tricyclic antidepressants, gabapentin, pregabalin, and serotonin-norepinephrine reuptake inhibitors;
- Second- or third-line: tramadol or opioids.

The current treatment of DN includes antioxidants, antidepressants, polyphenols, selective serotonin reuptake inhibitors (SSRIs), antiarrhythmics, opioids, anticonvulsants and analgesics, but its effectiveness has limited success in clinical trials (Dieleman, Kerklaan, Huygen, Bouma, & Sturkenboom, 2008; Kandhare et al., 2012; Snedecor et al., 2013).

Pathak and colleagues (2014) has suggested that a therapeutic with atorvastatin may reestablish the levels of enzymatic and non-enzymatic antioxidants, helping in the relief of NP symptoms.

The use of α-lipoic acid (ALA) is also suitable in the treatment DN (T. Han, Bai, Liu, & Hu, 2012; Papanas & Ziegler, 2014)

Oxidative Stress

The overproduction of free radicals and/or reduced antioxidant protection potentially leads to an increase of oxidative stress (OS) (Liu et al., 2014).

An excess of blood glucose leads to an overload of electron transport chain and, therefore, to the production of superoxides species. Subsequently mitochondrial and cytosolic OS is generated (Bandeira et al., 2013; Choi, Chandrasekaran, Inoue, Muragundla, & Russell, 2014; Schnell et al., 2013).

Chopra and colleagues (2012) defined OS as an "imbalance between oxidants and antioxidants in favor of the oxidants, potentially leading to damage".

Currently, OS is a main concern among biomedical research and clinical practice areas. OS is pointed as an important phenomenon associated with several disease states, as diabetes, obesity, cancer, ageing, inflammation, neurodegenerative disorders, hypertension, apoptosis, cardiovascular diseases, and heart failure (Pitocco et al., 2010).

ROS are produced in cells continuously during aerobic metabolism (Li, O, Li, Jiang, & Ghanbari, 2013). In mammalian cells, several enzyme systems generate ROS, comprising (i) nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, (ii) uncoupled nitric oxide (NO) synthase and (iii) the mitochondrial electron transport chain (Chopra et al., 2012).

The beginning of diabetes is deeply connected with OS. ROS are present and increased in both T1D and T2D (Singh et al., 2014). In fact, the CNS is significantly affected by OS because of its needs of oxygen, diminished antioxidant capacity and neurons characteristics, making OS one of the main causes of neurodegenerative diseases (Li et al., 2013).

In several illnesses, and specially in diabetes, the mitochondrial metabolism and the cascade of oxidative phosphorylation are pointed as significant contributors of ROS generation (Chopra et al., 2012; Figueroa-Romero et al., 2008; Li et al., 2013). This increased generation of ROS in cells and/or an inefficient elimination results in vascular dysfunction, damage in several cellular components (proteins, membrane lipids and nucleic acids), which potentiates the appearance of cancer, diabetes and other OS-mediated dysfunctions (Bandeira et al., 2013; Matough & Budin, 2012). Several studies declared that OS was already identified as a main cause of neuronal injury in several neuropathies (i.e. DN, acrylamide induced neuropathy and Charcot–Marie neuropathy) (Areti et al., 2014; Tang et al., 2012).

Despite its negative impact on health, ROS have important cellular activities, namely, as mediators of intracellular signaling cascades and as defense mechanism (i.e. phagocytosis, neutrophil function, macrophages and other cells of immune system) (Li et al., 2013; Matough & Budin, 2012).

The protective cellular machinery, through antioxidant enzymes and non-enzymatic antioxidants, allows the regularization of ROS levels in all cells (Li et al., 2013; Matough & Budin, 2012). Nevertheless, an imbalance between the production of ROS and the decrease of endogenous antioxidant power spread the effects of OS, causing endothelial dysfunction, insulin resistance and alterations in pancreatic β-cells (Bandeira et al., 2013). Li and colleagues (2013) reported that the free radicals of ROS might easily pass through the plasma membrane, leading to cell membrane injuries via lipid peroxidation, modification of the signal and alterations of the structural proteins conducting to misfolding and aggregation, and oxidation of RNA/DNA interrupting transcription and resulting in gene mutation.

In diabetic state, the increased production of ROS is related with hyperglycemia, and is indicated as a major complication of diabetes (Matough & Budin, 2012).

In OS conditions, mitochondria has a major role, once it normalizes this metabolic dysregulation state. An increase in ROS (Table II) results in ions in the intramitochondrial space, which leads to damage in cellular functions (Bandeira et al., 2013).

Table II: Overview of different types of reactive oxygen species (adapted from (Bandeira et al., 2013)).

ROS	Reactivity	Source	Activity
Superoxide anion radical ($^\bullet O_2^-$)	Low	Phagocytic cells, autoxidation reactions, enzymatic reactions, smoking	Vascular regulation, response to OS and maintenance of redox homeostasis
Hydrogen peroxide (H_2O_2)	Low	Mitochondrial matrix, $^\bullet O_2^-$ dismutation by SOD, xanthine dehydrogenase, smoking	May generate $^\bullet OH$ radical in the presence of transition metals, inactivate enzymes through oxidation of essential –SH groups.
Hydroxyl radical ($^\bullet OH$)	High	Water radiolysis, reaction of $^\bullet O_2^-$ with H_2O_2 and in the presence of transition metals, water ozonation	May damage DNA, proteins, lipids and carbohydrates
Singlet oxygen (O_2^1)	High	Phagocytes, light induction, radiation, reactions catalyzed by peroxidases, smoking and others	Direct DNA and protein damage, initiates lipids peroxidation and produces alcoxyl and peroxyl radicals

The pathways dysregulation indicated above, and caused by DN, are the major source of cellular damage, producing an imbalance in body's redox homeostasis, resulting in OS (Areti et al., 2014; Negi, Kumar, Joshi, & Sharma, 2011; Singh et al., 2014), which damage the exposed neurons, conducts to apoptosis and prevents neurite outgrowth, consequently resulting in an impair of normal neuronal process (Chopra et al., 2012).

OS may increase neurodegeneration through different paths, such as (i) bioenergetic failure, (ii) depletion of antioxidant defenses, (iii) biomolecular damage, (iv) microtubular disruption, (v) ion channel activation, (vi) demyelination, (vii) neuroinflammation, (viii) mitophagy impairment and (ix) neuronal death through apoptosis (Areti et al., 2014).

Along with OS, several crucial factors in regulation of homeostasis (metabolic and vascular pathways) are altered in DN (Figure III), such as:

- advanced glycation end product formation (AGEs) (Al-Enazi, 2013; Bandeira et al., 2013; Chilelli et al., 2013; Chopra et al., 2012; T. Han et al., 2012; Hosseini & Abdollahi, 2013; Jain et al., 2014; Kandhare et al., 2012; Liu et al., 2014; Negi, Kumar, & Sharma, 2011; T. Zhang, Gong, & Zhou, 2013);
- increased protein kinase C (PKC) activity (Bandeira et al., 2013; Chopra et al., 2012; T. Han et al., 2012; Hosseini & Abdollahi, 2013; Jain et al., 2014; Kandhare et al., 2012; Liu et al., 2014; Negi, Kumar, Joshi, et al., 2011; Singh et al., 2014; T. Zhang et al., 2013);
- activation of poly ADP-ribose polymerase (PARP) (Al-Enazi, 2013; T. Han et al., 2012; Hosseini & Abdollahi, 2013; Jain et al., 2014; Kandhare et al., 2012; Negi, Kumar, Joshi, et al., 2011);
- hexosamine pathway (Bandeira et al., 2013; Chopra et al., 2012; T. Han et al., 2012; Hosseini & Abdollahi, 2013; Jain et al., 2014; Kandhare et al., 2012; T. Zhang et al., 2013);
- polyol pathway (Al-Enazi, 2013; Bandeira et al., 2013; Chopra et al., 2012; Hosseini & Abdollahi, 2013; Kandhare et al., 2012; Liu et al., 2014; Singh et al., 2014; T. Zhang et al., 2013);
- inflammation (Hosseini & Abdollahi, 2013; Kumar et al., 2012; Negi, Kumar, Joshi, et al., 2011).

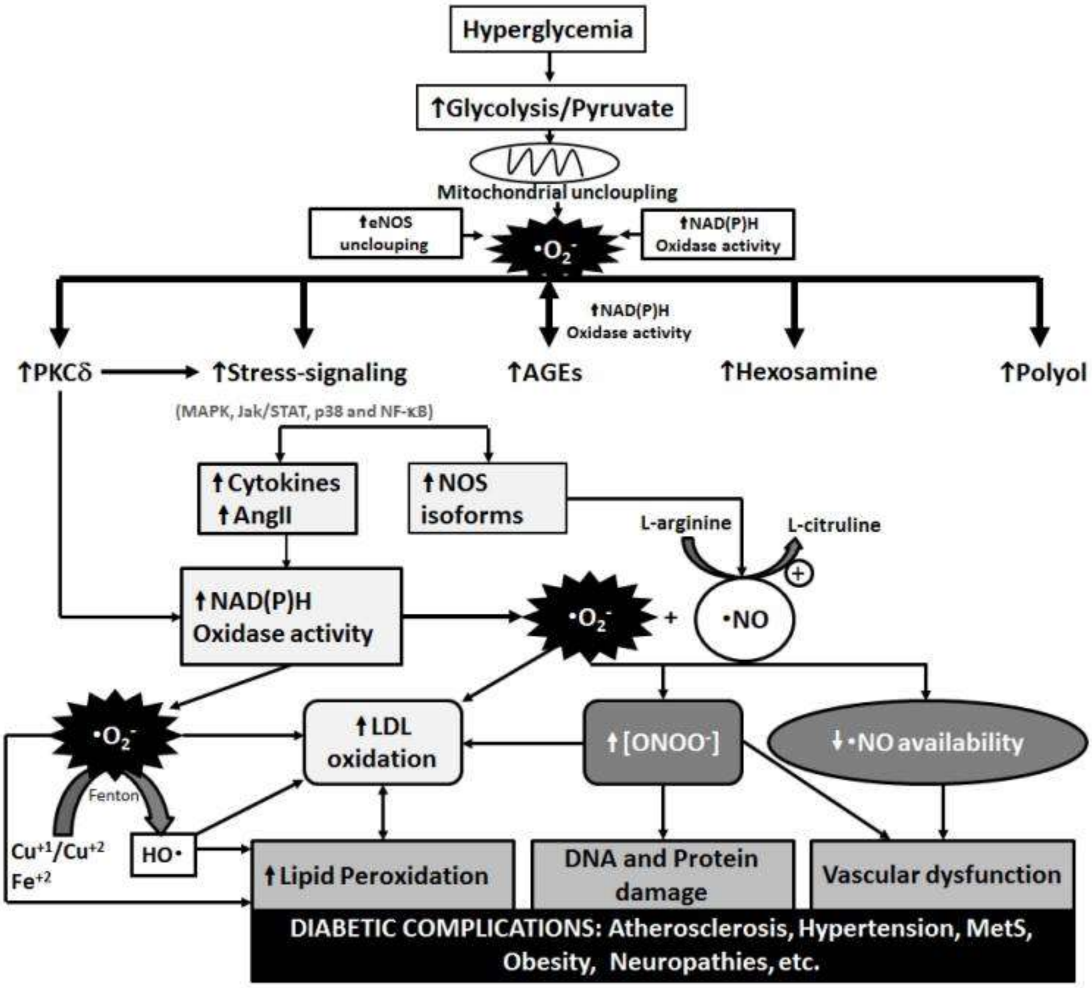


Figure III: In diabetes, several OS pathways are impaired due to hyperglycemia.

AngII: angiotensin II; eNOS: endothelial nitric oxide synthase; Jac/STAT: janus kinase (Jac)-signal transducer and activator of transcription (STAT); LDL: low density lipoprotein cholesterol; MAPK: mitogen-activated protein kinase; MetS: metabolic syndrome; NF-κb: nuclear transcription factor κb; •NO: nitric oxide; NOS: nitric oxide synthase; •O2−: superoxide anion radical; •OH: hydroxyl radical; ONOO−: peroxynitrite; PCKδ: protein kinase C δ. (Bandeira et al., 2013).

AGEs and polyol pathways cause the modification of redox ability by disturbing glutathione recycling or through increasing ROS production. Other pathways like hexosamine, PKC and PARP have an important role in injury due to increased expression of inflammatory proteins (Brownlee, 2005; Hosseini & Abdollahi, 2013).

Objectives

1. To investigate the eventual relation between chronic pain, diabetes and oxidative stress.
2. To describe the average biomarkers effect and summarize its statistical significance.
3. To predict likely clinical benefit for future groups of patients.
4. To determine the socio-demographic characteristics of the convenience sample in each season (summer, autumn, winter and spring).
5. To determine the duration and etiology of pain, the associated pathologies and usual medication in each season (summer, autumn, winter and spring).
6. To characterize the pain: local, intensity, impact, relief by medication and its effect on daily activities, using Brain Pain Inventory (BPI) questionnaire.
7. To determine the main features associated with neuropathic pain, using *Douleur Neuropathique* 4 (DN4) questionnaire.

In figure IV are represented the three key points of this thesis. We intend to study an eventual relation between this three major and current subjects: chronic pain, diabetes and oxidative stress

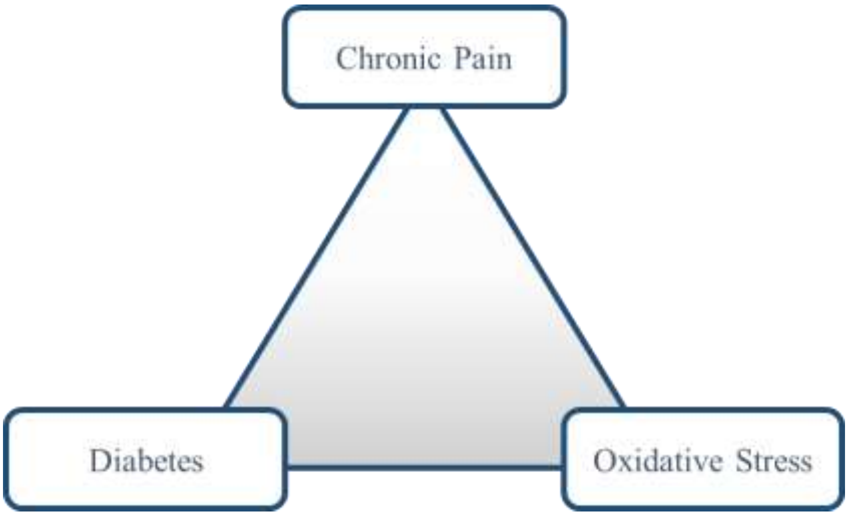


Figure IV: Key points of thesis.

Chapter II – Oxidative Stress in Diabetic Neuropathy – a meta-analysis

Abstract

Several studies reported a connection between oxidative stress (OS) dysregulation and diabetic neuropathy (DN). This meta-analysis intend to analyze the clinical data quantitatively by comparing the OS biomarkers between diabetic neuropathic models and healthy controls.

A PubMed search was conducted to collect the studies that related OS and DN from 1995 to 2015. The effect sizes for the standardized mean differences were expressed through Hedges's G and a Z-score; a p-value of <0.05 for Z was considered statistically significant.

The search originated 275 studies, and 9 studies met the inclusion criteria. The biochemical parameters assessed were: superoxide dismutase (SOD, n=6); lipid peroxidation (LPO, n=4); reduced glutathione (GSH, n=2) and catalase (CAT, n=2).

Compared to health control, DN models showed significantly higher LPO ($\rho < 0.05$), and significantly lower CAT, SOD and GSH ($\rho < 0.05$). It was verified high heterogeneity between the studies ($I^2 > 60\%$), except for CAT analysis ($I^2 = 0\%$).

Thus, data indicated that the evaluated OS biomarkers may have clinical significance and may be useful in DN diagnostic, representing a possible relation between OS and DN.

Introduction

Currently, diabetes corresponds to an epidemic disease characterized by chronic hyperglycemia. This may conduct to the development of DN with macro and microvascular damages (Bandeira et al., 2013; Jain et al., 2014; Premkumar & Pabbidi, 2013).

Neuropathy is an emergent condition and yet it’s still not established any theory as the main cause for this condition. Nevertheless neurons are very sensitive to reactive oxygen species (ROS) and its elevation intracellularly may lead to cell injury (Hassler, Johnson, & Hulsebosch, 2014; Nikolic et al., 2014; Visnagri et al., 2014). Neuropathy has an elevated prevalence which is enhanced with the duration of the disease (Premkumar & Pabbidi, 2013).

DN arises as a chronic common and significant complication of diabetes. 50–60 % of diabetic patients may have associated neuropathies (Kumar et al., 2012). Hence, its evolution has great effect in the diabetes prognosis and it’s a burden for health economy (Jain et al., 2014; Kumar et al., 2012; Liu et al., 2014). Age and duration of diabetes are key factors in the development of neuropathy (Tang et al., 2012)

DN is a condition described by a sensitivity loss (i.e. chemical, thermal, and mechanical sensations) (Mathieson et al., 2015; Premkumar & Pabbidi, 2013). Spontaneous pain, allodynia and hyperalgesia are among the complaints of DN patients (Kandhare et al., 2012; Stavniichuk, Shevalye, Hirooka, Nadler, & Obrosova, 2012; Zhao et al., 2014).

Several studies pointed hyperglycemia as the main source of OS, which may be a leading cause in the development of sciatic nerve dysfunction in diabetic models (Y. B. Wu et al., 2012). OS is pointed in several studies as having an important role on the pathogenic mechanism of DN. OS may arise from a hyperglycemic state, and is described as one of the responsible mechanisms for the neuronal injury and loss of function in diabetes (Areti et al., 2014; T. Han et al., 2012; Premkumar & Pabbidi, 2013).

Diabetes has a strong association with increased OS levels, as a result of either amplified production of free radicals or reduced antioxidant defenses (T. Han et al., 2012; Pitocco et al., 2010).

The maintenance of ROS levels in all cells is accomplished by the production of antioxidant enzymes and non-enzymatic antioxidants, leading to the regulation of homeostasis (Li et al., 2013; Matough & Budin, 2012). Some antioxidant enzymes are described as part of the OS regulation, such as SOD and CAT, with protection functions (Bandeira et al., 2013; Kasznicki et al., 2012).

To monitor the progression of DN and its response to therapeutics it is important to evaluate some reliable biomarkers. Several OS biomarkers could be assessed to evaluate the cellular oxidative status, such as CAT, GSH, LPO and SOD, among others (Areti et al., 2014).

CAT is a key enzyme which converts hydrogen peroxide into water and oxygen, transforming a ROS in a non-toxic molecule (Bandeira et al., 2013; Kasznicki et al., 2012; Nikolic, Stanimirovic, Bjelogrlic, & Isenovic, 2014).

GSH is a key enzyme in cellular protection against sulfahydryl group of cysteine in proteins, being an extremely efficient scavenger for ROS. It stands out as non-enzymatic antioxidant, being present in all tissues. When its concentration is higher in the cell it becomes more resistant to OS, and, consequently, GSH becomes an excellent OS biomarker (Guedes et al., 2009; Kulkarni, Acharya, Ghaskadbi, & Goel, 2014; Pathak et al., 2014; Visnagri, Kandhare, Chakravarty, Ghosh, & Bodhankar, 2014). Moreover, hydroxyl radicals are only scavenged by GSH (Pathak et al., 2014).

SOD is a protecting enzyme for aerobic organisms from the toxicity of superoxide radical, and also responsible for redox balance in neuron (Hybertson, Gao, Bose, & McCord, 2011; Visnagri et al., 2014). Superoxide radicals are transformed into oxygen and hydrogen peroxide by SOD, through a dismutation reaction (Bandeira et al., 2013; Kasznicki et al., 2012; Pathak et al., 2014).

Free radicals attack several biomolecules (amino acids, lipids, proteins and DNA), being the lipids the most vulnerable (Bandeira et al., 2013; Ilechukwu, Ebenebe, & Ubajaka, 2014; Tiwari, Pandey, Abidi, & Rizvi, 2013). Malondialdehyde (MDA) acts in cell membranes promoting nerve damage. Thus, LPO, measured in terms of MDA production, is a reliable biomarker being frequently used in the assessment of cellular OS status (Bandeira et al., 2013; Tiwari et al., 2013; Visnagri et al., 2014). Lipoperoxides and reactive aldehydes, as MDA, are produced by breakdown of polyunsaturated fatty acids present in cell membrane by ROS (Bandeira et al., 2013; Catanzaro et al., 2013; Jakuš & Sándorová, 2014).

To our knowledge, a meta-analysis of studies about oxidative stress biomarkers in DN is missing. This study intends to analyze the clinical data quantitatively by comparing oxidative stress biomarkers between healthy and diabetic neuropathic models, through a systematic review and meta-analysis of OS biomarkers, as predictive of poor prognostic for diabetic neuropathy.

Materials and Methods

We performed a systematic review and meta-analysis of published works about oxidative stress markers, as risks factors for diabetic neuropathy, according to Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (Hutton et al., 2015).

i. Search procedures

The study was conducted according to PRISMA directives. In order to identify the studies, an electronic search in September 2015, with no linguistic restriction, was conducted, researching one database – Medline (PubMed). The Boolean search was conducted according to the following keywords: Diabetic Neuropathies [AND] Oxidative Stress. Two investigators evaluated, independently, the titles and abstracts of the papers identified as of potential relevance in the inclusion criteria. Where a title or abstract could not be included for certainty, a third corrector helped decide whether to reject it or not.

ii. Inclusion criteria

The papers which satisfied the following inclusion criteria were selected: (i) presence of following keywords in the abstract: diabetic neuropathy and oxidative stress or reactive oxygen species; (ii) performed in human or in mouse models; (iii) studies that presented clinical data for OS biomarkers, such as CAT, GSH, LPO, SOD and 8-hydroxy-2'-deoxyguanosine (8-OHdG); (iv) studies that provided subject numbers, means and standard deviations for controls and diabetic neuropathic models; (v) studies performed in sciatic nerve samples; and (vi) studies presented the same or convertible units for each OS biomarker.

iii. Exclusion criteria

Studies were excluded from our analysis if they met the following criteria: (i) reviews; (ii) aren't written in English; and (iii) studies with subjects' numbers defined as an interval (i.e. n=5-7).

iv. Statistics

A separated meta-analysis was performed for each OS biomarker, which >2 studies provided data, comparing levels in diabetic neuropathic models with those in healthy controls. A fixed-effects model was used and standardized mean differences weighted for sample size were calculated, using RevMan version 3.3.070. The effect sizes for the standardized mean differences were expressed through Hedges's G and a Z-score; a p-value of <0.05 for Z was considered statistically significant. The between-study heterogeneity was determined using the Cochrane's Q statistic and expressed using I^2.

Results

Figure V shows the results of the literature search, screening and review for studies eligible for inclusion in the meta-analysis. The search generated 257 papers between 1994 and 2015: of these, 29 were excluded because were reviews and 31 because weren't written in English; 197 publications were reviewed for eligibility: 120 articles presented the terms DN and OS or ROS in the abstract, 74 articles were included for the meta-analysis, 13 studies were excluded because presented subjects number defined as an interval, and 52 full-text articles were excluded based on the inclusion/exclusion criteria.

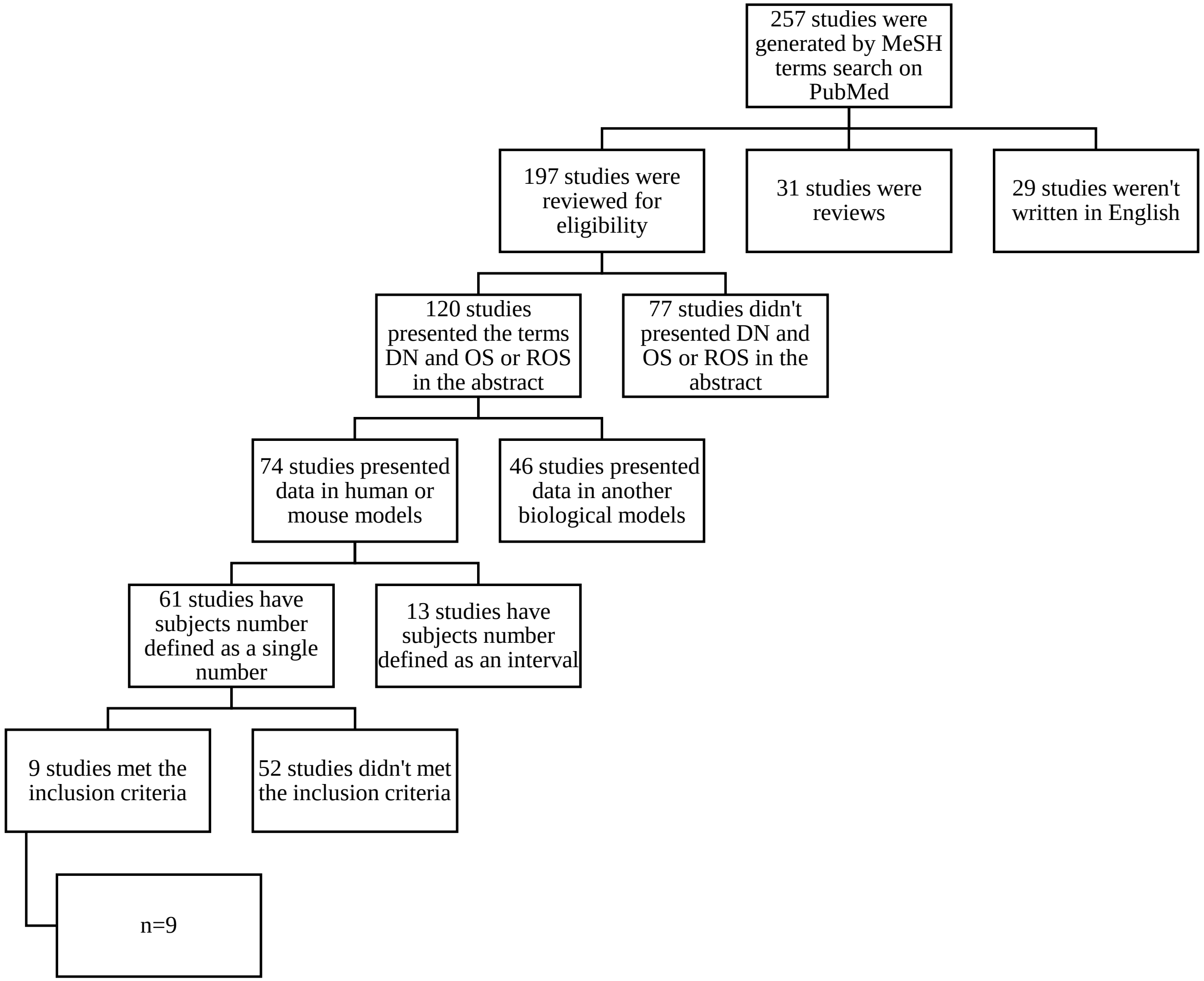


Figure V: Literature search results

MeSH – medical subject headings; DN – diabetic neuropathy; OS – oxidative stress; ROS – reactive oxygen species

Our research identified 9 studies which measured antioxidant enzymatic activities (SOD and CAT), antioxidant non-enzymatic activity (GSH) and LPO in sciatic nerve of mouse model, streptozotocin-induced (STZ), according to the inclusion criteria. These studies included a total of 66 diabetic neuropathic models and 71 healthy controls.

Table III presents the included studies and their OS biochemical indicators. Five of the included studies analyzed just one OS biomarker and the others analyzed two or more.

The biochemical parameters assessed were: (i) SOD – 6 studies; (ii) LPO – 4 studies; (iii) GSH – 2 studies, and (iv) CAT – 2 studies.

Table III: Included studies in the meta-analysis for diabetic neuropathy and oxidative stress in mouse model, and their biochemical indicators for oxidative stress.

Study	Biological model	N		LPO (nmol MDA/mg protein)		SOD (units/mg)		CAT (µmoles of H_2O_2 decomposed/min/mg)		GSH (nmol/mg protein)	
		Control	DN	Control	DN	Control	DN	Control	DN	Control	DN
(Visnagri et al., 2014)	Sprague-Dawley rat	6	6	ND	ND	24,12±1,53	6,14±0,52	ND	ND	ND	ND
(Rao et al., 2014)	Wistar rat	6	6	ND	ND	32,00±2,00	10,00±0,00	ND	ND	ND	ND
(Y. B. Wu et al., 2012)	Sprague-Dawley rat	15	13	1,97±0,18	4,55±0,52	82,95±7,17	59,93±7,23	ND	ND	ND	ND
(Kandhare et al., 2012)	Wistar rat	6	6	ND	ND	29,46±0,83	5,78±0,89	ND	ND	ND	ND
(Baluchnejadmojarad, Roghani, & Khastehkhodaie, 2010)	Wistar rat	8	8	4,00±1,00	10,50±1,5	ND	ND	ND	ND	ND	ND
(Kamboj, Vasishta, & Sandhir, 2010)	Wistar rat	6	6	1,63±0,14	2,31±0,22	2,17±0,30	1,11±0,12	1,05±0,08	0,5±0,17	5,15±0,24	3,21±0,24
(Kuhad & Chopra, 2009)	Wistar rat	7	7	1,94±0,04	3,58±0,11	12,44±0,09	4,32±0,51	ND	ND	ND	ND
(Stevens, Obrosova, Cao, Van Huysen, & Greene, 2000)	Wistar rat	9	8	ND	ND	ND	ND	0,11±0,01	0,08±0,01	ND	ND
(Nagamatsu et al., 1995)	Mouse (without model definition)	8	6	ND	ND	ND	ND	ND	ND	8,00±0,80	5,00±0,60

ND – not defined; DN – diabetic neuropathic LPO – lipid peroxidation; SOD – superoxide dismutase; CAT – catalase; GSH – reduced glutathione; MDA – malondialdehyde.

Figures VI to IX represented the forest plots for LPO, CAT, SOD and GSH, respectively.

Results displayed a significant increase in LPO levels in DN (Figure VI), ρ-value < 0.05, with a large effect size.

Concerning CAT activity, the results showed a significant decrease in this biomarker in DN (Figure VII), ρ-value < 0.05, with a large effect size.

Results for SOD activity presented a significant decrease in this biomarker in DN (Figure VIII), ρ-value < 0.05, with a large effect size.

Regarding GSH, the results showed a significant decrease in this biomarker in DN (Figure IX), ρ-value < 0.05, with a large effect size.

Lipid peroxidation

Study name	Hedges's g	Standard error	Variance	Lower limit	Upper limit	Z-Value	p-Value
Kuhad 2009	18,55	4,17	17,39	10,38	26,72	4,45	0,00
Kamboj 2010	3,40	1,03	1,05	1,39	5,41	3,32	0,00
Baluchnejadmojarad 2010	4,82	1,10	1,	[illegible]	6,98	4,38	0,00
Wu 2012	6,64	1,03	1,	[illegible]	8,66	6,45	0,00
	5,24	0,60	0,36	4,06	6,42	8,73	0,00

Statistics for each study

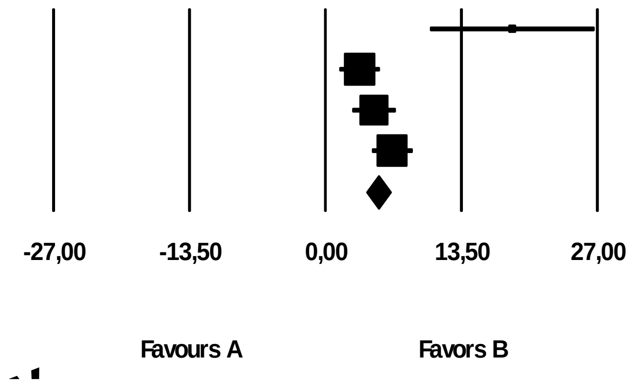


Figure VI: Lipid peroxidation in diabetic neuropathy by study.

Effect size (Hedges's g) estimates and 95% confidence intervals (CIs) for LPO parameter.

Catalase

Study name	Hedges's g	Standard error	Variance	Lower limit	Upper limit	Z-Value	p-Value
	Statistics for each study						
Stevens 2000	-2,85	0,67	0,45	-4,16	-1,53	-4,24	0,00
Baluchnejadmojarad 2010	-3,91	0,84	0,70	-5,56	-2,27	-4,67	0,00
	-3,26	0,52	0,27	-4,29	-2,24	-6,23	0,00

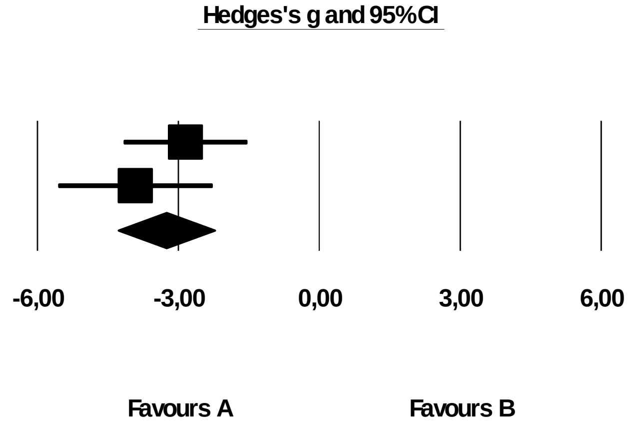


Figure VII: Catalase activity in diabetic neuropathy by study.

Effect size (Hedges's g) estimates and 95% confidence intervals (CIs) for CAT activity.

Superoxide dismutase

Study name	Hedges's g	Standard error	Variance	Lower limit	Upper limit	Z-Value	p-Value
Kuhad 2009	-20,76	4,66	21,70	-29,89	-11,63	-4,46	0,00
Kamboj 2009	-4,28	1,21	1,47	-6,66	-1,91	-3,53	0,00
Kandhare 2012	-25,40	6,35	40,36	-37,85	-12,95	-4,00	0,00
Wu 2012	-3,11	0,59			-1,96	-5,29	0,00
Rao 2014	-14,36	3,62	13,12	-21,46	-7,26	-3,96	0,00
Visnagri 2014	-14,52	3,66	13,42	-21,71	-7,34	-3,96	0,00
	-4,12	0,51	0,26	-5,13	-3,12	-8,05	0,00

Hedges's g and 95%CI

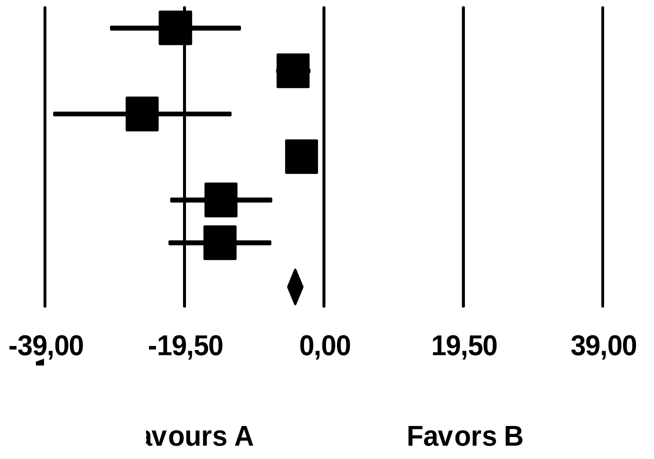


Figure VIII: Superoxide dismutase activity in diabetic neuropathy by study.

Effect size (Hedges's g) estimates and 95% confidence intervals (CIs) for SOD activity.

Reduced glutathione

Study name	Statistics for each study						
	Hedges's g	Standard error	Variance	Lower limit	Upper limit	Z-Value	p-Value
Nagamatsu 1995	-3,88	1,02	1,04	-5,88	-1,88	-3,81	0,00
Kamboj 2010	-7,46	1,95	3,79	-11,28	-3,65	-3,83	0,00
	-4,65	0,90	0,82	-6,42	-2,88	-5,15	0,00

Hedges's g and 95%CI

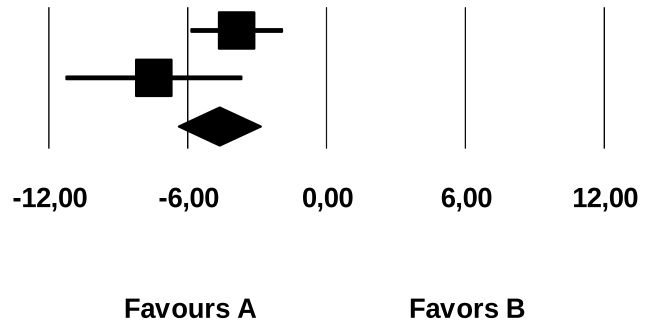


Figure IX: Reduced glutathione in diabetic neuropathy by study.

Effect size (Hedges's g) estimates and 95% confidence intervals (CIs) for GSH parameter.

Overall, the heterogeneity of studies was high, except for CAT activity (I2 = 0%), (Table IV), which led us to investigate whether the sources of sampling or the assays were the potential contributors for such heterogeneity.

Table IV: Heterogeneity of standardized mean group differences for oxidative stress markers in diabetic neuropathic models compared with healthy controls.

Biomarker	Heterogeneity
	I^2 (%)
LPO	81
SOD	88
CAT	0
GSH	62

LPO was increased in all the studies; while SOD, GSH and CAT biomarkers were diminished in all the studies; when comparing the diabetic group with the healthy controls. Table V shows the sensitivity and specificity of each biomarker and it was possible verify that all the biomarkers are specific and sensitive.

Table V: Values of sensitivity and specificity of each analyzed biomarker.

Biomarker	Sensitivity	Specificity
LPO	34/34 = 1	36/36 = 1
SOD	44/44 = 1	46/46 = 1
CAT	16/16 = 1	17/17 = 1
GSH	12/12 = 1	14/14 = 1

Discussion

The main outcome of this meta-analysis is the presence of amplified OS in DN; our results exhibited increased LPO and decreased CAT, GSH and SOD concentrations in DN models compared to healthy controls.

Based on our meta-analysis results, four biomarkers more often used (LPO, GSH, SOD and CAT) were selected.

In accordance with our results, most of studies that analyzed CAT found that its concentration was decreased in diabetic models both experimental (Kumar, Kaundal, Iyer, & Sharma, 2007; Ranjithkumar, Prathab Balaji, Balaji, Ramesh, & Ramanathan, 2013; Saini, Kumar, & Sharma, 2007; Sayyed, Kumar, & Sharma, 2006; Sharma & Sayyed, 2006; Valsecchi et al., 2011) and clinical (Arya, Pokharia, & Tripathi, 2011; Kasznicki et al., 2012), but Kelloog and Pop-Busui (2005) reported an elevation in this enzyme in their study about peripheral nerve dysfunction in diabetes.

Several studies, in mouse models, reported a decrease in the activity of antioxidant enzyme – SOD (Catanzaro et al., 2013; Jain et al., 2014; Jin, Lee, Wu, Baek, & Park, 2014; Kelloog & Pop-Busui, 2005; Mirshekar, Roghani, Khalili, Baluchnejadmojarad, & Moazzen, 2010; Saini et al., 2007; Sayyed et al., 2006; Sharma & Sayyed, 2006; Zhao et al., 2014), in agreement with our data. Also, in peripheral blood of T2D patients with distal symmetric polyneuropathy a reduction in SOD concentration was found (Kasznicki et al., 2012). Nevertheless, other studies indicated a raise in SOD activity both in diabetic patients (lymphocytes lysate) (Arya et al., 2011) and diabetic mouse models (Ilnytska et al., 2006; Valsecchi et al., 2011).

In our study, the GSH levels were diminished in diabetic rat models, which is in accordance with several studies (Al-Enazi, 2013; Comelli et al., 2010; Low, Nickander, & Tritschler, 1997; I G Obrosova, Fathallah, Lang, & Greene, 1999; Irina G Obrosova, Fathallah, & Stevens, 2001; Song et al., 2003; Yu et al., 2014). The GSH levels are, also, decreased in diabetic patients (El Boghdady & Badr, 2012). Camera and Picardo (2002) identified some limitations related to the family of GSH biomarkers: the available results in the literature fluctuate over 10-fold, which may happen in consequence of GSH autoxidation and the fast proteolysis of GSH and GSSG in biological samples. Guedes *et al.* (2009) worked with the sciatic nerve of Wistar rats and verified that GSH variations were independent of neuropathic pain. Nevertheless, an increase in GSH/GSSG ratio was confirmed only in a sciatic nerve rat model, which indicate a positive association with NP.

ROS are generated in an OS environment, which leads to cellular LPO (Rao et al., 2014). Thus, lipids are molecules with several unsaturated bonds and consequently larger. Due to these characteristics they are suitable to oxidative damage, causing end products of lipoperoxidation, which are toxic, leading to tissue damaging by free radicals (Brown, Andreazza, & Young, 2014; Rao et al., 2014). Several of the reviewed studies reported a higher level of LPO markers in diabetic rodents, which is in accordance with our data (Hosseini et al., 2010; Pabreja, Dua, Sharma, Padi, & Kulkarni, 2011; Y. P. Zhang et al., 2013), as well as in diabetic patients (Arya et al., 2011; El Boghdady & Badr, 2012; Hoeldtke, Bryner, & Vandyke, 2011; Sayın et al., 2014).

The sciatic nerve becomes more affected by OS in presence of low levels of SOD and GSH, and high levels of LPO. This evidence increases with the duration of diabetes (Kamboj et al., 2010; Visnagri et al., 2014).

In agreement with the abovementioned for each OS biomarker, the pooled effect sizes showed that alterations in antioxidant enzymes (SOD and CAT), LPO and GSH were significant, when comparing the diabetic neuropathic group with the healthy controls.

All the OS biomarkers presented high values for heterogeneity, except CAT that showed no heterogeneity. Nevertheless: (i) in the forest plot for LPO, the error bars for each study include the summary result (except one), which may suggest that statistical heterogeneity is not a problem ; (ii) for SOD, only 1/3 of the studies presented the error bars for each study include the summary result, which suggests that statistical heterogeneity could be a problem in the evaluation of this OS biomarker; (iii) in the forest plots for CAT and GSH, the error bars for each study include the summary result, which may suggest that statistical heterogeneity is not a problem, accordingly to Fletcher (2007). The $I^2 = 0\%$ for CAT may indicate that all variability in effect size estimates is in consequence to sampling error within studies. On the other hand, values above 50% for I^2 may indicate true heterogeneity between studies (Huedo-Medina, Sánchez-Meca, Marín-Martínez, & Botella, 2006).

Rücker and colleagues (2008) pointed some major causes for heterogeneity: (i) clinical baseline heterogeneity ; (ii) statistical heterogeneity, and (iii***)*** heterogeneity from other causes.

The assessment of diagnostic tests quality is a point of interest of clinical and epidemiological research. Thus, concerning the obtained sensitivity and specificity values for each OS biomarker – 1 for each, which is the possible maximum value. So, with the appropriate validation, there is a possibility that the OS biomarkers could be used as a diagnostic biomarkers for DN along the clinic evaluation, as well as prevention strategies and to monitor the response for the treatment. We proposed the use of LPO, SOD, GSH and CAT as OS biomarkers in prognostic/diagnostic of DN.

The STZ diabetes is considered a trustworthy, reproducible and well described animal model (rodent) for assessment of diabetes and DN. The STZ rats show biochemical, oxidative and metabolic changes that mimic the humans. (Catanzaro et al., 2013; Kandhare et al., 2012; Rao et al., 2014; Visnagri et al., 2014).

The main limitations in this meta-analysis are (i) the expression of the results is a recognized constraint manly due to the use of diverse parameters (i.e. fluid or lysate volume, tissue weight, cell number) which difficult the comparison between methods (Camera & Picardo, 2002); (ii) the study of GSH and other OS stress biomarkers in biological samples is problematic because of the lack of standard values (Monostori, Wittmann, Karg, & Túri, 2009); (iii) the low number of included studies (Brown et al., 2014; Flatow, Buckley, & Miller, 2013; Solmi et al., 2015); (iv) between-study heterogeneity (Brown et al., 2014; Flatow et al., 2013; Solmi et al., 2015); and (v) the laboratory methodology (Brown et al., 2014; Flatow et al., 2013).

In conclusion, the achieved results are in agreement with the majority of the reviewed literature, which reported an increase in OS as an important factor in DN development. The reduction of SOD is considered an evidence of amplified damage by ROS and reduced oxidative defense in experimental DN, therefore it was expected a decreased in SOD, CAT and GSH activities due to the impairment of antioxidant defenses caused by the elevation of ROS. SOD concentration decrease results in increased production of superoxide radicals. The transformation of hydrogen peroxide from water and oxygen which results into hydroxyl radical production is impaired in consequence of CAT activity reduction. Thereby, an increase of hydroxyl radicals is a leading cause for increased LPO.

Thus, knowing the cause and effects in daily life of DN patients will improve their treatments and care. These biomarkers should be detected to support a crucial diagnosis and treatment of DN.

Chapter III – Main Characteristics of Pain Assessed in Patients Using BPI and DN4 Surveys

Abstract

Data collections on chronic pain (CP) are scarce in Portugal. This study aims to investigate the prevalence of neuropathic pain (NP) and possible associated factors in patients of a medical clinic in Porto.

The data were collected during four seasons (summer, autumn, winter and spring), between July 2014 and June 2015 in a Porto medical clinic. In addition to sociodemographic and clinical data collection, two internationally validated questionnaires, Brief Pain Inventory (BPI) and *Douleur Neuropathique* 4 (DN4), were applied.

A total of 84 patients reported pain symptoms. The patients mean age was 63±15.5 years old, 82% were women, had pain for more than 3 months, indicated often more than one associated comorbidity and use of medication regularly. The most frequent pain locations were the lumbar, hip, thigh and leg locations, with the highest peaks on autumn. Shoulder, arm and forearm were transversal to 4 seasons. The autumn is the season in which the patients declared to have more pain, referring to the minimal pain in previous week with a score of 4±2 (0-10 pain scale). Nearly 50% of evaluated participants had NP as determined by a DN4 score > 4. Spring was the season more associated with NP complaints.

Our results support the need of an accurate diagnose for CP with neuropathic characteristics, hence the treatment is different from the one used for nociceptive pain. Uncontrolled pain is associated with a huge impact on patients daily routine. Also, results obtained support the belief that weather may influence pain complaints.

Keywords: Chronic pain, neuropathic pain, diabetes, diabetic neuropathy

Introduction

Pain is as a warning signal to the body, acting as a protective response from an injury (Calcutt, 2013; Robison-Papp et al., 2015).

CP, due to its persistent daily interferences, is a condition with a huge impact in QoL (Erdemoglu & Koc, 2013; Poliakov & Toth, 2011).

CP is defined by the International Association for the Study of Pain (IASP) as “persisting continuously or intermittently for longer than 3 months” (L. F. Azevedo et al., 2012;

Inoue et al., 2015) . Concerning intensity, pain may be classified as mild-to-severe (L. F. Azevedo et al., 2012; Reid et al., 2011).

According to De Moraes Vieira and colleagues (2012) the different values found for CP prevalence may vary mainly in consequence to (i) different definitions of CP, (ii) target population, and (iii) applied methodology for the investigation. Some recent studies indicate that prevalence of CP range from 4.2 to 42 % (L. F. Azevedo et al., 2012; Bernfort et al., 2015; De Moraes Vieira et al., 2012; Inoue et al., 2015; Kurita et al., 2012; Mohamed Zaki & Hairi, 2014; Reid et al., 2011) of the population.

CP is often related with other comorbidities, such as diabetes, hypercholesterolemia and hypertension, decreasing the patient's quality of life (QoL) (Chang et al., 2015; Liberman et al., 2014; Nenke et al., 2015).

NP is defined by IASP as "pain arising from a direct consequence of a lesion or disease affecting the somatosensory system", and may result from a variety of different diseases. Its prevalence varies among the authors between 3 and 17 % (Barbosa et al., 2014; De Andrés et al., 2014; De Moraes Vieira et al., 2012; Hamdan et al., 2014; Mathieson et al., 2015).

This study will permit the achievement of more insight in the prevalence, severity, and treatment of neuropathic symptoms in patients with moderate to severe pain and its interference with daily activities. The study was conducted in a Portuguese medical clinic: *Clínica Médica Povo Portuense* (CMPP), Porto – Portugal.

Methods

i. Study design

The population was a convenience sample, which included patients of CMPP that voluntarily and by personal contact agreed to participate in the study. The sample was a group of selected patients from medical consultation at CMPP, between July 2014 and June 2015 at specific periods: (i) summer (July – September); (ii) autumn (October – December); (iii) winter (January – March); and (iv) spring (April – June). The patients were excluded from the study if they were unable to understand the study objectives or complete the questionnaires. This was an observational, cross-sectional epidemiologic study. This study was approved by *Escola Superior de Tecnologia da Saúde do Porto* Ethics Commission and each patient signed an informed consent.

ii. Data Collection Instruments

Two surveys were applied during the consultations: (i) a Portuguese version of BPI (Short Form), with translation, cultural adaptation and validation by *Faculdade de Medicina da Universidade do Porto*, with the permission of the author, Charles Cleeland, PhD. BPI permits the assessment of important pain characteristics (i.e. intensity and location, and the efficacy of pain therapy) and also allows the evaluation of the pain interference in patient's daily routine (Erdemoglu & Koc, 2013). This questionnaire is validated and widely used to evaluate the severity of pain and the effect of pain on daily occupations. It is specified if the pain varies in intensity during the day and defined the highest, lowest, and average intensity of pain in the previous week, on a 0-10 scale (0 = no pain, 10 = worst pain imaginable); (ii) a Portuguese version of Neuropathic Pain Questionnaire from French Neuropathic Pain Group, with translation, cultural adaptation and validation by *Faculdade de Medicina da Universidade do Porto*, with the permission of the author, Didier Bouhassira, PhD. DN4 is a validated screening tool for NP. It consists of a scale with 10 items and is divided into four sections. A score of one is given for each positive item and zero for each negative item, and the total is calculated as the sum of the 10 items. A total score of four or more indicates NP.

Prior the application of the surveys above mentioned a socio-demographic characterization that includes gender, age and professional occupation; duration and etiology of the pain; associated pathologies and usual medication was collected.

iii. Statistical analysis

The analysis was mainly descriptive using the means and standard deviations (SD) for continuous variables, which had all a normal distribution; and categorical variables were expressed as frequency distributions and percentages for the pertinent subject groups. All tests were applied with a 95% confidence interval ($\rho < 0.05$): $\rho<0.05$ is considered significant, $0.001<\rho< 0.01$ very significant, and $\rho<0.001$ extremely significant.

Accordingly Bouhassira *et al.* (2013), pain intensity scores of 1 to 3 were an indicative of mild pain, scores of 4 to 6 were an indicative of moderate pain, and scores of 7 to 10 indicate severe pain.

The data analysis was performed using Statistical Package for the Social Sciences version 22 software and Microsoft Excel.

The Chi-Square test of independence was applied to nominal variables to see whether the proportions of one variable are different for different values of the other variable.

The independent-samples t-test was applied to compare the means between two unrelated groups on the same continuous, dependent variable.

Results

A total of 84 patients reported having pain. The data were collected in four seasons: (i) summer, n=29; (ii) autumn, n=16; (iii) winter, n=18 and (iv) spring, n=21.

Table VI shows the main socio-demographic characteristics, the description of pain duration, associated comorbidities and the use of medication in the studied patients. The patients mean age was 63±15.5 years old, 82% were women, had pain for more than 3 months, indicated often more than one associated comorbidity and use of medication regularly. Despite the majority of the patients were retired, there is a predominance of active individuals (50%) in autumn.

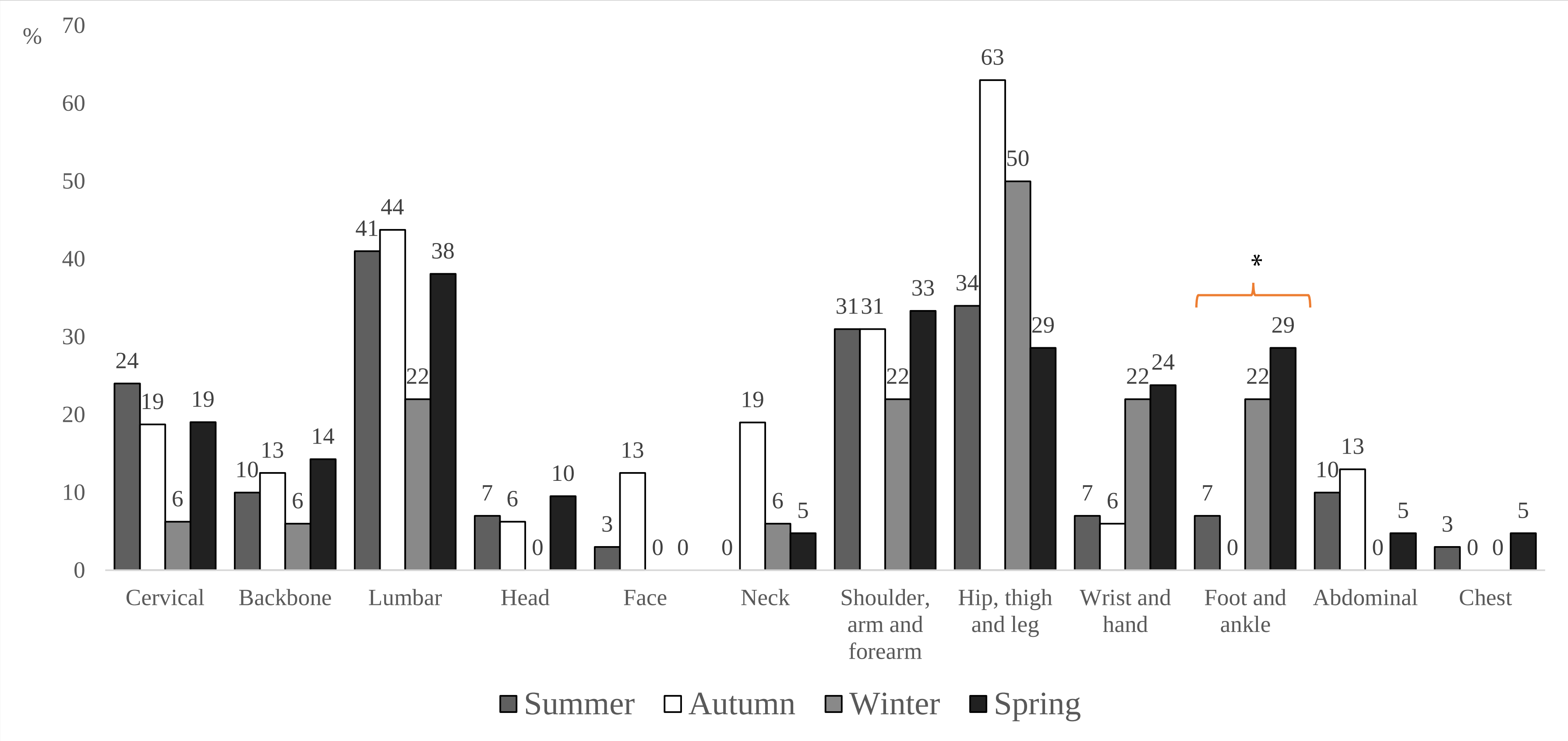
Table VI: Main socio-demographic characteristics of the sample, the description of pain duration and the use of medication.

		Summer	Autumn	Winter	Spring
N		29	16	18	21
Mean age±SD (years old)		64±17	63±17	63±13	62±15
Gender (%)	Female	72	88	83	86
	Male	28	13	17	14
Occupation (%)	Active	28	50	17	33
	Unemployed	3	6	11	5
	Retired	69	44	72	62
Pain duration (%)	Occasional	0	0	0	19
	< 3 months	10	0	17	14
	> 3 months	90	100	83	67
Other comorbidities (%)	Hypertension	24	19	39	33
	Diabetes	21	13	6	24
	Hypercholesterolemia	28	13	44	38
Medication (%)	No medication	10	6	11	10
	Medication	90	94	89	90

SD – Standard Deviation

The prevalence of CP in the population studied, considering the minimum of pain duration of 3 months, is 85 % (n=71); 10 % (n=9) for less than 3 months and 5% (n=4) indicated that the pain was occasional.

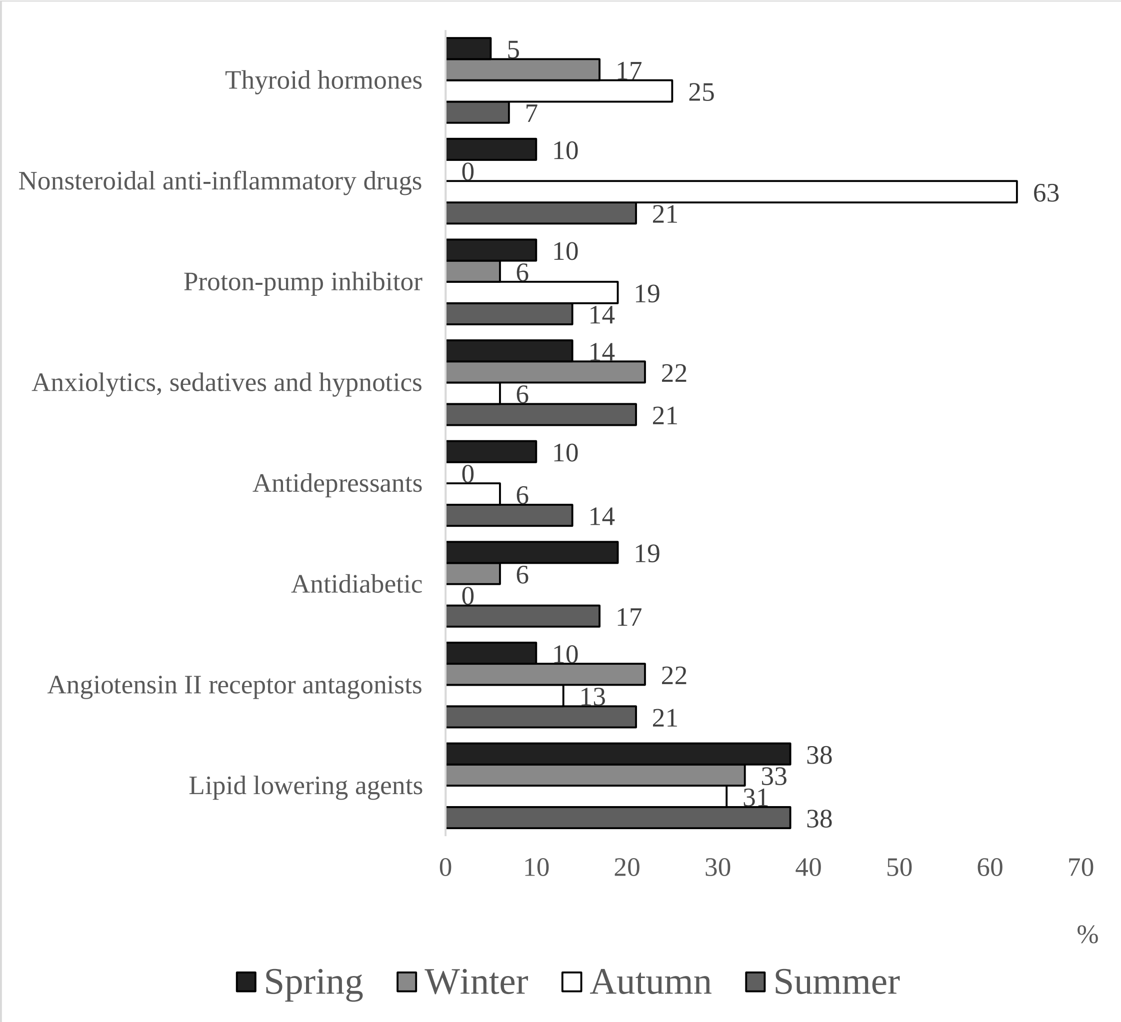
The most frequent pain locations are presented in Graphic I, standing out the lumbar, hip, thigh and leg locations, with the highest peaks on autumn (44% and 63%, respectively), and shoulder, arm and forearm that are transversal to 3 seasons (summer and autumn – 31% each and spring – 33%). It is verified that exist an association between the pain location - foot and ankle and seasons (ρ=0.018).



Graphic I: Most frequent pain locations.

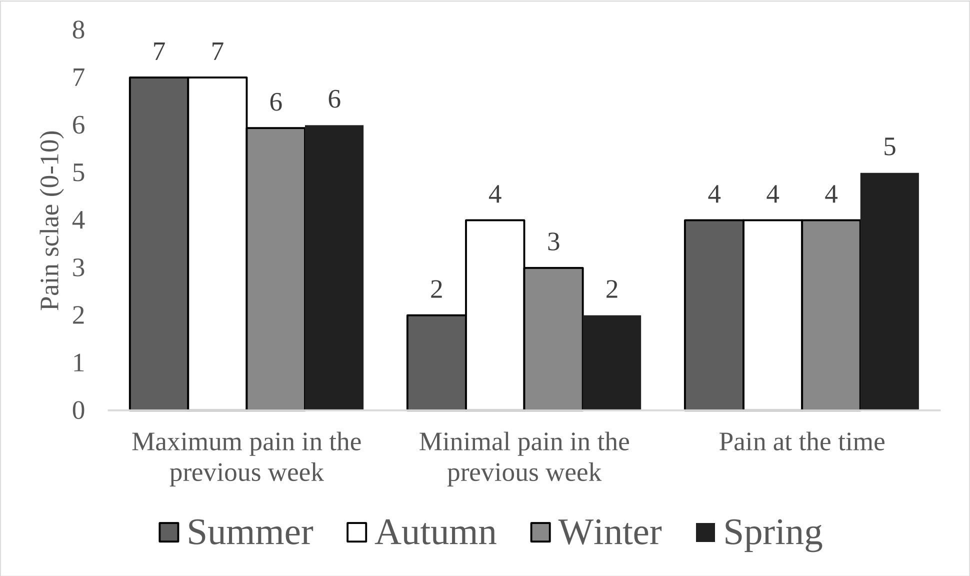
* ρ<0.05 (ρ=0.018)

The usual patient's medication is diverse, nevertheless, it is possible to verify that the nonsteroidal anti-inflammatory drugs (NSAIDs) and the lipid lowering agents consumption are highlighted (Graphic II). NSAIDs have the major percentage of intake on autumn (63%).



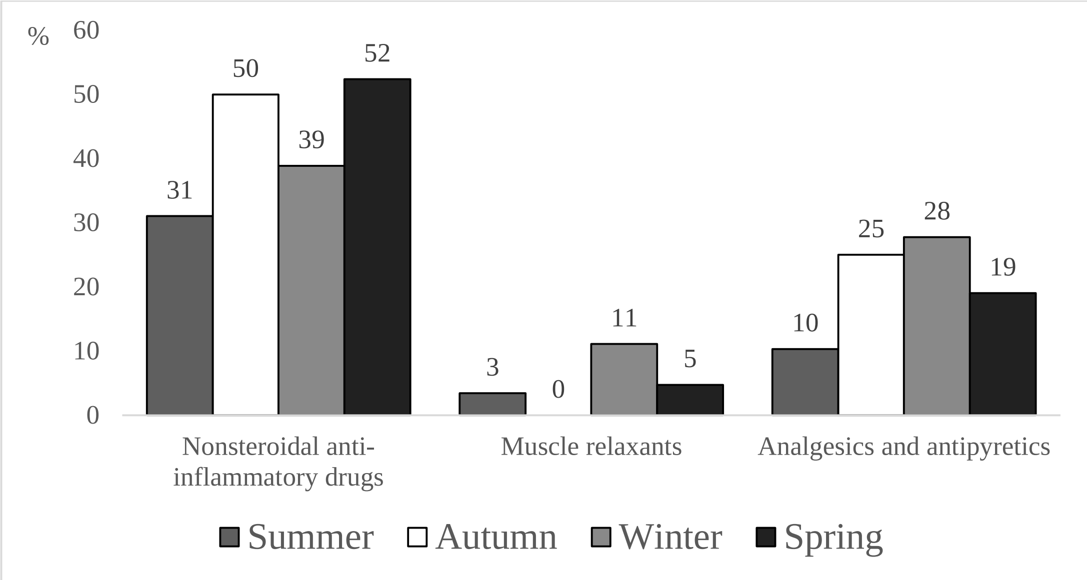
Graphic II: Most used medication distribution.

Concerning BPI results, autumn is the season in which the patients declared to have more pain, referring to the minimal pain in previous week with a score of 4±2 (0-10 pain scale). Concerning the maximum pain felt in previous week, summer and autumn reached the highest scores (7±2), that is considered severe pain. The highest score (5±3) for the pain at the time of the interview was obtained in spring (Graphic III). All diabetic patients (15% of study population) had a maximum score of pain in the previous week greater than 5 (0-10 pain scale), and 54% of those had scores above 8, which indicates presence of moderate to severe pain.



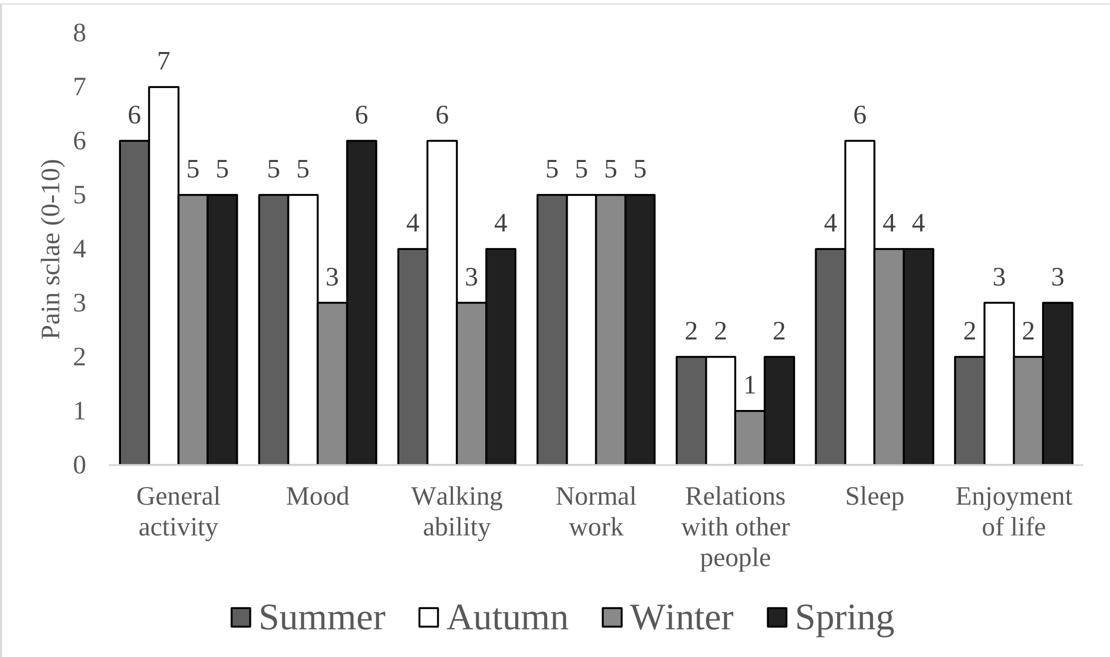
Graphic III: Description of pain in the previous week and at the time (BPI).

Regarding pain relief by medication evaluated with BPI, winter was the season where patients indicated a higher pain relief (65%), followed by spring (61%), autumn (59%) and summer (42%). The pharmacotherapeutic groups commonly used for pain relief are presented on Graphic IV. As above mentioned in the usual medication, the NSAIDs group stands out also as usual medication for pain, with highest percentages of consumption in spring and autumn (52% and 50%, respectively). Other pharmacotherapeutic groups appear as pain drugs, as analgesics and antipyretics (21%, seasons mean) and muscle relaxants (5%, seasons mean).



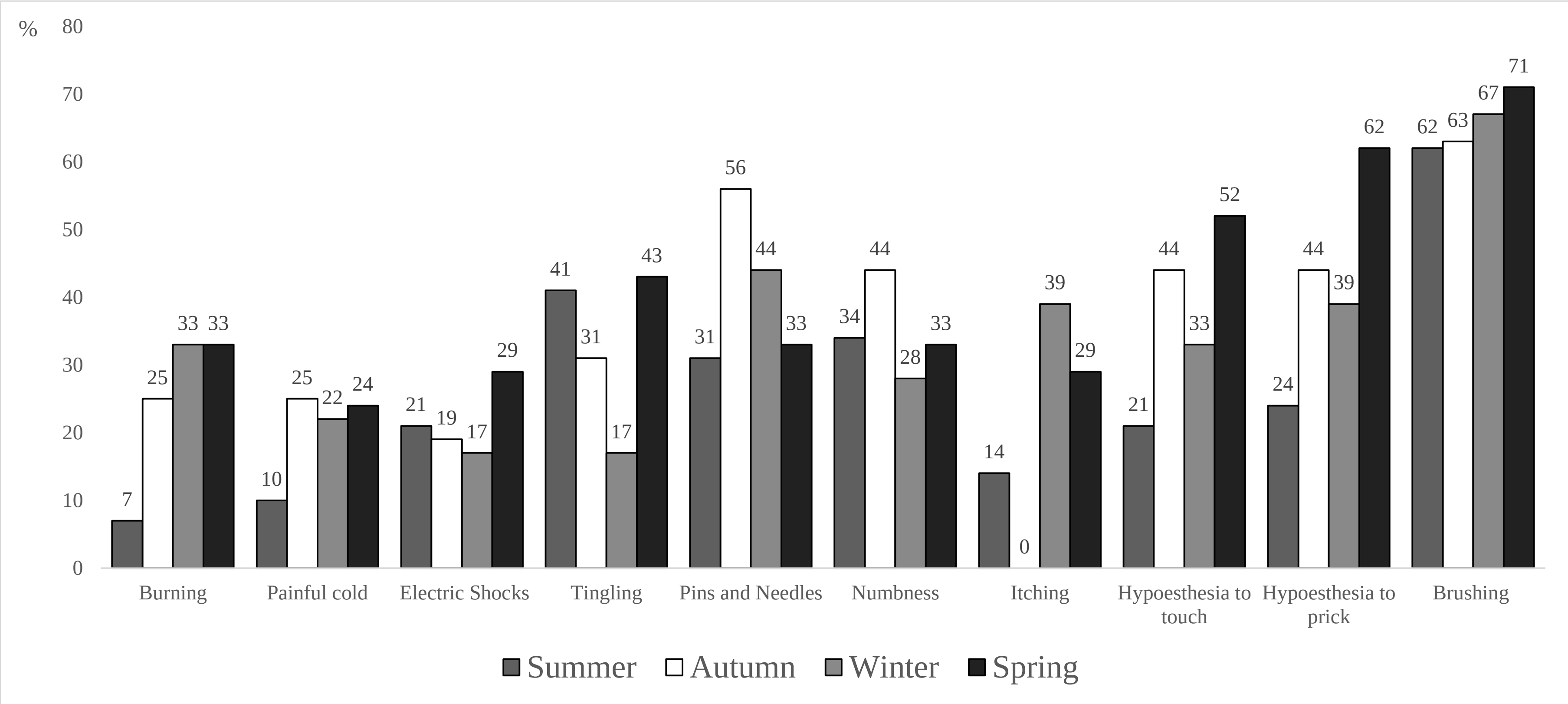
Graphic IV: Specific pain medication (BPI).

Through BPI application is possible to describe the pain interference on patient's daily activities (Graphic V). In almost every daily tasks (sleep, walking ability and general activity), the season with the highest scores is autumn. Concerning the less affected daily task, the patients had affirmed that it is the relation with other people (2.5±3.3). The normal working is the daily task equally affected through the seasons (5.0±3.8).



Graphic V: Pain interference on patients' daily activities (BPI).

Analyzing the results of DN4 questionnaire (Graphic VI), the most common neuropathic complaints are brushing (71% in spring), hypoesthesia to prick (62% in spring), pins and needles (56% in autumn) and tingling (43% in spring). The spring is the season with the highest percentages of neuropathic complaints, contrary to summer that has the lowest values. It was verified an association between itching complaints and seasons ($\rho=0.016$).



Graphic VI: Most common pain complaints (DN4).

* ρ<0.05 (ρ = 0.016)

A total of 39 participants (46%) had NP as determined by a DN4 score > 4. The average score on the DN4 questionnaire for patients with non-neuropathic pain (NNP) was 1.4±1.1 and 5.5±1.3 in the patients with NP. Relating neuropathic patients and diabetes, it was found 6 participants (7%) that may have diabetic neuropathy (DN).

Patients with NNP and NP have similar features (Table VII), concerning season, age, sex, occupation, presence of other comorbidities and pain duration, no statistical significance was found since *p* value was always higher than 0.05. Having hypertension (44% of NP patients), being female (87% of NP patients) and being retired (54% of NP patients) are some factors that may be associated to the presence of NP but with no statistical significance. Concerning the DN4 items, NP patients presented higher scores for DN4 survey than NNP ($\rho<0.05$), showing an association between NP and most usual pain complaints.

Table VII: Socio-demographic characteristics and neuropathic complaints evaluation in patients with neuropathic and non-neuropathic pain.

	NNP (n=45)	NP (n=39)	ρ value
Season, Su/Au/Wi/Sp (%)	42/20/22/16	26/18/21/36	0.158
Mean age ± SD (years old)	64±16	61±14	0.345
Sex, M/F (%)	24/76	13/87	0.283
Occupation, A/U/R (%)	22/7/71	38/8/54	0.231
Other comorbidities			
Hypertension, Y/N (%)	22/78	44/56	0.063
Diabetes, Y/N (%)	16/84	15/85	0.983
Hypercholesterolemia, Y/N (%)	31/69	33/67	0.828
Pain Duration, O/A/C (%)	4/13/82	5/8/87	0.808
DN4 items, Y/N (%)			
Burning	4/96	43/56	0.000***
Painful cold	7/93	33/67	0.005**
Electric shocks	11/89	33/67	0.027*
Tingling	18/82	54/46	0.001**
Pins and needles	20/80	62/38	0.000***
Numbness	11/89	59/41	0.000***
Itching	9/91	33/67	0.012*
Hypoesthesia to touch	13/87	64/36	0.000***
Hypoesthesia to prick	9/91	74/26	0.000***
Brushing	40/60	97/3	0.000***

NNP – Non Neuropathic Pain; NP – Neuropathic Pain; Su – Summer; Au – Autumn; Wi – Winter; Sp – Spring; M – Male; F – Female; A – Active; U – Unemployed; R – Retired; HT – Hypertension; D – Diabetes; HC – Hypercholesterolemia; Y – Yes; N – No; O – Occasional; A – Acute (< 3 months); C – Chronic (> 3months); * $\rho<0.05$; ** $0.001 < \rho < 0.01$; *** $\rho<0.001$

Diabetic patients with NNP were older ($\rho=0.018$) than with NP (Table VIII). Being retired is more prevalent in diabetic non-neuropathic pain (DNNP) patients than in diabetic neuropathic pain (DNP) patients. Concerning the DN4 items, DNP patients presented more elevated scores for DN4 items, hypoesthesia to prick and brushing than DNNP ($\rho=0.021$ and $\rho=0.005$, respectively), showing an association between DNP and the this two pain complaints (DN4). The other results for DN4 evaluation did not presented any statistical difference and are not shown.

Table VIII: Socio-demographic characteristics and neuropathic complaints evaluation in diabetic patients with neuropathic and non-neuropathic pain.

	DNNP (n=7)	DNP (n=6)	ρ value
Mean age ± SD (years old)	74±6	62±10	0.018*
Sex, M/F (%)	43/57	17/83	0.559
Occupation, A/R (%)	0/100	50/50	0.077
Pain Duration, O/A/C (%)	0/0/100	11/11/78	1.000
DN4 items, Y/N (%)			
Hypoesthesia to prick	0/100	67/33	0.021*
Brushing	14/86	100/0	0.005**

DNNP – Diabetic Non-Neuropathic Pain; DNP – Diabetic Neuropathic Pain; M – Male; F – Female; A – Active; U – Unemployed; R – Retired; HT – Hypertension; D – Diabetes; HC – Hypercholesterolemia; O – Occasional; A – Acute (< 3 months); C – Chronic (> 3months); * $\rho < 0.05$; **$0.001 < \rho < 0.01$

Through the analysis of table IX, neuropathic patients with or without diabetes have identical characteristics. For BPI and DN4 results no statistical significance was found since *p* value was always higher than 0.05. All of the DNP patients referred presence of pain with brushing and presented a maximum score of pain in the previous week higher than 5 (0-10 pain scale) in BPI, that indicates moderate to severe pain.

Table IX: Frequency of positive items in non-diabetic neuropathic and diabetic neuropathic patients.

	NDNP (n=33)	DNP (n=6)	ρ value
Season, Su/A/W/Sp (%)	21/21/21/36	50/0/17/33	0.395
BPI mean > 5 (%)	67	50	0.647
BPI > 5 (%)	88	100	1.000
DN4 items (%)			
Burning	45	33	0.679
Painful cold	33	33	1.000
Electric shocks	36	17	0.643
Tingling	52	67	0.667
Pins and needles	61	67	1.000
Numbness	58	83	0.370
Itching	27	67	0.153
Hypoesthesia to touch	61	67	1.000
Hypoesthesia to prick	79	67	0.636
Brushing	94	100	1.000

NDNP – Non-Diabetic Neuropathic Pain; DNP – Diabetic Neuropathic Pain; Su – Summer; Au – Autumn; Wi – Winter; Sp – Spring; BPI – Brief Pain Inventory; DN4 – *Douleur neuropathique* 4

Discussion/Conclusion

The management of CP, in order to improve pain patient’s healthcare and decrease the associated social burden, is urgent. Thus, the full knowledge of the characteristics of pain becomes crucial.

To our knowledge no study has previously been conducted for chronic and NP in Portugal comprising data from the four seasons.

It is notable that CP, especially NP, has a huge impact in daily activities resulting in an impairment of QoL.

Our data indicate that being female and increasing age are some of the factors associated with CP and NP. These factors are also presented by several studies and are in accordance with the common demographic profile of CP (L. F. Azevedo et al., 2012; Bouhassira et al., 2008; Breivik et al., 2013; Cortez et al., 2014; De Moraes Vieira et al., 2012; Dieleman et al., 2008; Harifi et al., 2013; Kasim, Amar, Sadek, & Gawad, 2010; Lekpa et al., 2013; Reid et al., 2011; Timmerman, Wilder-Smith, van Weel, Wolff, & Vissers, 2014; Torrance et al., 2006).

The subjects presenting NNP are older than those in the NP group, even though the differences in all variables (season, gender, occupation, comorbidities and pain duration) were not statistically significant. According to Langley and colleagues (2013), the NP patients were older than the control group but the other variables are in agreement with our study. Breivik and collaborators (2013) identified that manual workers and the unemployed individuals are more likely to have CP instead of professional workers, but, in a Portuguese study, and in accordance with our data, the retired individuals is the group with more prevalence of CP (L. F. Azevedo et al., 2012). This discrepancy may be explained by the increase age of the participants in the Portuguese study and in our own. Also, in autumn it was noticed a prevalence of active individuals in contrast with other three seasons. This may be explicated since our sample for this season, in particularly, presented more individuals with active occupation than other season.

In this study, the criterion to consider the presence of CP was reporting pain for more than 3 months, thus CP is present in 85 % of the studied population. This results represents an increased percentage compared with previous studies in Portugal which reported only 37% (L. F. Azevedo et al., 2012). This fact may be explained by the low number of the participants (n=84) and by the target population (only participants with pain complaints), which do not fully represents the entire population. Yet, there is noticed an increase in the prevalence of CP globally (Chang et al., 2015).

The most frequent pain locations were the lumbar, hip, thigh and leg, and shoulder, arm and forearm in agreement with results already published (P C Langley, 2011; Reid et al., 2011; Timmerman et al., 2014).

NSAIDs and lipid lowering agents are the medicines with major consumption within usual patients medication which could be justified by the higher percentage of patients with CP and hypercholesterolemia (31%), respectively.

Concerning BPI results, the average maximum pain and the average pain in previous week were 6.5±2 and 5.3±2 (0-10 pain scale), respectively, which may be considered moderate to severe pain which is in agreement with several published studies (L. F. Azevedo et al., 2012; Bouhassira et al., 2013; P C Langley, 2011).

A pain increase has a great impact in the QoL of the patients and often interferes with almost all daily duties, such as family and home tasks, entertaining activities (i.e. exercise) and sleep (Baker et al., 2010; Breivik et al., 2013; Reid et al., 2011; Votrubec & Thong, 2013).

All of diabetic patients presented a maximum score of pain in the previous week regarded as moderate to severe, as well ½ of diabetic patients reached a score above 8 (0-10 pain scale). Over the time, CP has been associated with other comorbidities, and several factors are pointed as significantly associated with an increased risk of neuropathy occurrence: the diabetes duration, poor control of diabetes and associated hypertension, among others. Diabetes up rises as one of the most affected conditions, where the nerve tissues are in a proinflammatory state which is a major cause in the progress of DN, and consequently impacting dramatically the QoL (Barbosa et al., 2014; Chang et al., 2015; Freeman, 2013; Hosseini & Abdollahi, 2013; Kasim et al., 2010; Kumar et al., 2012; Lapane et al., 2014; Liberman et al., 2014; Nenke et al., 2015; Wang et al., 2014).

The pain treatment is a challenging task, even today with the existence of numerous medicines for its treatment. Currently, many patients are unsatisfied with their pharmacological therapy which does not provide reasonable pain relief. This permits the development of new non-pharmacological therapies, less harmful, that could improve significantly the QoL. Our patients indicated 57% in pain relief when they use medicines specific for pain. Once more, and in accordance with several studies, the NSAIDs group highlights as usual medication for pain. Other drugs were emphasized, the analgesics and antipyretics and the muscle relaxants (Baker et al., 2010; De Andrés et al., 2014; Freeman, 2013; Jouini et al., 2014; Lapane et al., 2014; Reid et al., 2011). Reid and colleagues (2011) pointed the use of antidepressants (28.7%) and antiepileptics (51%) as non-standard pain medication but in our population the consumption of these drugs was lower, 7% each. Although many NP patients are regularly medicated with NSAIDs, this group of drugs has more beneficial effects in the treatment of nociceptive pain than NP (Cecil, Goldman, & Schafer, 2012). The highest percentages of NSAIDs consumption were in spring and autumn in accordance with the highest peaks of pain complaints in both groups (NP and NNP).

Lapane and colleagues (2014) concluded that pain intensity is significantly related with diminishing of both physical and mental health components of QoL. Other study refers, in agreement with the previous study and ours, that patients with CP have a reduction in health-related QoL and psychological welfare (Moulin et al., 2015).

Distinguishing neuropathic from nociceptive pain is challenging, thus several instruments were created and standardized with this purpose (Barbosa et al., 2014). DN4, among others, is a useful tool for the evaluation of neuropathic characteristics once NP is typically described as burning, painful, cold or electric shocks and may be associated with tingling, pins and needles, and numbness or itching (Votrubec & Thong, 2013). However, the questionnaires cannot access all the pain specificities and, therefore, the DN4 should not replace a clinical evaluation which provides more specific indications about the pain and its causes. (Mathieson et al., 2015). Almost 50 % of participants in our study had NP as determined by a DN4 score > 4. One of the largest studies realized in Europe indicates that one in five of diabetic patients suffer pain in a daily-based approach, often associated with neuropathic features. (Bouhassira et al., 2013). And other study, indicates that 63 % of the patients evaluated had a NP component (Hamdan et al., 2014). The most common neuropathic complaints are brushing, hypoesthesia to prick, pins and needles and tingling. Comparing NP patients with NNP, it was verified an enormous association between NP and NP complaints. All NP pain complaints were significantly increased in NP patients, which was expected since these complaints are characteristic of this type of pain.

Calcutt, in his study, stated that a percentage of people with diabetes also reports spontaneous tingling, pricking, and pain sensations (Calcutt, 2013). In agreement with Lepka and collaborators (2013), our study presents musculoskeletal pain as the main complaint of patients with DN. Even though DN is often underdiagnosed, less than 1/3 of physicians recognize the cause or discuss this with their patients. This needs to be improved and the physicians must be sensitized for the theme (Vinik, Nevoret, Casellini, & Parson, 2013).

It was verified a significant association between diabetic patients with neuropathic pain and two typical neuropathic pain complaints, hypoesthesia to prick and brushing. It is know that a diabetic state may influence the pain perception, boosting it. This may be explained for the different sensitive pathways. Tactile sensation is divided into (i) crude touch, where the thicker fibers spread impulses such as those arising in touch receptors, and (ii) fine touch, where the thinner fibers, transmit slower transmission, leading to pain impulses (Klaumann, Wouk, & Sillas, 2008; Patestas & Gartner, 2009).

It is known that weather conditions may negatively influence the pain (Smedslund & Hagen, 2011). However there are several potential factors, such as low mood, poor sleep quality and lack of exercise, which may interfere in the relation between weather and pain complaints (Macfarlane, McBeth, Jones, Nicholl, & Macfarlane, 2010). On the other hand, meteorological factors, such as barometric pressure, humidity, and temperature, have been matter of study due to their capability of influence CP. (Cunha Miranda et al., 2007; Funakubo, Sato, Obata, & Mizumura, 2011).

According to several studies, the prevalence of any kind of pain is elevated during the seasons with less sunlight and lower temperatures (autumn and winter, in Northern Hemisphere), and suffers a decrease in summer (Breivik, 2010; Macfarlane et al., 2010; Saps et al., 2013; Erik J Timmermans et al., 2014). But, it is not a consensus subject (McAlindon, Formica, Schmid, & Fletcher, 2007). A study about the influence of humidity on pain suggested that pain was higher in cold meteorological conditions due to the increasing of viscosity of synovial fluid (E. J. Timmermans et al., 2015). Our study reports pain complaints increased during autumn and summer, with a score of 7 (BPI 0-10 pain scale) in the maximum pain on the previous week. And for pain with neuropathic features, the spring is the season with the highest percentages, contrary to summer, that presented the lowest values. This is not completely in agreement with the published studies, but, as we know, the weather influence is partially explained by mood and behavior fluctuations, and all of our subjects reported mild to severe pain, being, therefore susceptible (Macfarlane et al., 2010; Erik J Timmermans et al., 2014). Concerning the itching complaint as being significantly associated to the seasons, it was verified elevated complaints on winter, which could be explained for vasoconstriction phenomena that may lead to an increase of pain perception (Mathias & Bannister, 2013). After all, this discrepancy may be explained due to the diversity of pain components, which are difficult to study in a closed environment (Cunha Miranda et al., 2007).

This study has some limitations: (i) it is based on a convenience sample which does not accurately represent the general population and may under or overestimate population values; (ii) the questionnaires were applied directly in target population, and were based only in the description of pain experienced by the respondents; (iii) it was not assured a clinical confirmation of the presence of pain, pain conditions reported, and the presence of comorbidities.

In order to confirm our results, it is desirable to perform additional studies with larger community-based data which will permit the comparison between the characteristics and the experience of NP patients with other CP conditions.

In conclusion, our results suggest that being a female, the increased age and being retired can influence CP and NP in several patients. The scores obtained in BPI indicate the presence of moderate to severe pain, with implications in almost daily activities. The DN4 indicated the presence of nearly 50% of patients with NP. Thus, CP associated or not with NP is perhaps more predominant in the population than was formerly assumed and normally is characterized as more severe than other CP conditions. Consequently pain evaluation is helpful and with a great importance, as well as the self-management approaches, because of their impact in the improvement of QoL.

Chapter IV

General discussion and conclusion

Through the meta-analysis and the data collection about pain complaints and its characteristics, it was possible to analyze and relate the major actors in the development of diabetic neuropathy (DN): diabetes, chronic pain and oxidative stress (OS).

OS may be a leading cause for progress and aggravation of DN. The meta-analysis and systematic review contributed to the understanding of OS role and importance in patients with DN, as well as to establish several OS biomarkers with potential for the use in diagnose, such as lipid peroxidation, superoxide dismutase, catalase and reduced glutathione. These OS biomarkers allow a more effective accompaniment of the OS status of diabetic neuropathic patients, supporting a better and more accurate treatment.

The application of Brief Pain Inventory and *Douleur Neuropathique* 4 was accomplished in a convenience sample, which included patients of a medical clinic that agreed to participate in the study. The data analysis, resultant from the two surveys application, permitted the achievement of more insight in the prevalence, severity, and treatment of neuropathic symptoms in patients with moderate to severe pain and its interference with daily activities. Our results indicated that a large number of people have daily pain complaints, even under medication, and a high percentage of them presented neuropathic pain characteristics. Concerning the usual pain medication (NSAIDs), it was possible verify that it is unappropriated to treat effectively neuropathic pain, since is more indicated in treatment of nociceptive pain.

The data collection across the four seasons potentiated the current believing that climate alterations and meteorological conditions influence the pain complaints.

Pain arises as a major issue in their daily routines, which has a great impact in QoL. Hence, our results reinforce the importance of pain diagnostic and treatment, among patients, caregivers and medical class.

Future perspectives

As future perspectives we aim to study the influence of infection on processes of the nervous system, including neurodegenerative diseases and pain pathways, particularly in the modulation of the oxidative/inflammatory environment.

Through the use of bacterial infection/inflammation models, with bacterial strains that are sensitive and resistant to antimicrobial agents, we intend to study their influence on pathological processes of nervous system (i.e. Parkinson's and Alzheimer's diseases). Additionally, to extend these studies to other pathological processes in the nervous system, as the processes underlying the development of chronic pain. We intend to evaluate, through analytical techniques, molecular players, specifically associated to oxidative stress and inflammation, involved in pathologic pathways and to characterize their relation. This evaluation will be carried out using biological models already used at the nervous system level, such as *Caenorhabditis elegans*, among others, and by developing new models and methodologies. The characterization of the potential connection between infection/inflammation and nervous system pathological processes, with high prevalence and impact on Public Health, such as neurodegenerative processes or development of chronic pain, will be extremely relevant. The obtained data may allow early intervention for improving the diagnosis, and also a more timely therapeutic intervention, with great impact on disease pathogenesis, and high gains in health and quality of life.

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Appendix

Appendix I – scientific communications in poster

Title: Oxidative Stress in Diabetic Neuropathy

Poster abstract submitted and accepted at IV *Congresso de Cuidados Paliativos* – IPO, Portugal 2014.

Introduction

Diabetes is an epidemic disease, considered a public health major issue and also in Palliative Care. Diabetic neuropathy (DN) is defined as a demonstrable disorder, either clinically evident or subclinical, that occurs in setting of diabetes without other causes for peripheral neuropathy. Oxidative stress (OS) is related to several physiological and pathological processes and there are several evidence pointing to its involvement in DN.

Objectives

To review the evidences about the relation between OS and DN in order to contribute to the overall understanding of the mechanisms involved.

Material and methods

The keywords used for the search were diabetes, DN, OS, reactive oxygen species and glutathione. Science Direct and PubMed databases were used.

Results

The research made point that the key factor in the progress of DN is the generation of free radicals by amplified glycolytic processes. An excess of blood glucose leads to an overload of electron transport chain and, therefore, to the production of superoxide species. Subsequently mitochondrial and cytosolic OS are generated. Also there are evidence that the beginning of diabetes may be deeply connected with OS.

Discussion/Conclusion

In diabetes, the mitochondrial metabolism and the cascade of oxidative phosphorylation are pointed as significant contributors of ROS generation that are present and increased in both T1D and T2D conditions. This increased production seems to be caused by hyperglycemia. These results contribute to the elucidation of the relation between OS and DN and thus may better allow earlier and better diagnosis and new and optimized treatment.

Title: Study of Duration and Etiology of Pain, Associated Pathologies and Usual Medication in Patients Attending *Clínica Médica Povo Portuense*

Poster abstract submitted and accepted at IV *Congresso de Cuidados Paliativos* - IPO, Portugal 2014.

Introduction

Pain has great impact in quality of life and other associated pathologies and is of great importance in palliative care. Thus the study of its main characteristics contributes to better care.

Objectives

To assess the duration and etiology of the pain, the associated pathologies and usual medication as well as to study its socio-demographic characteristics.

Material and methods

A convenience sample (n=32) of patients of *Clínica Médica Povo Portuense* voluntarily and by personal contact agreed to participate in this study. The parameters assessed were socio-demographic characterization including gender, age and professional occupation, the duration and etiology of the pain, the associated pathologies and usual medication. An informed consent was sign.

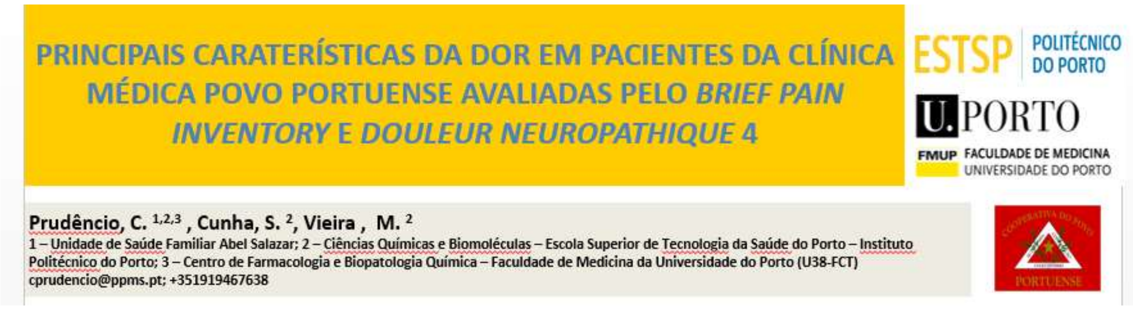
Results

The results were obtained on a sample composed of 24 % males and 76 % females with a mean age of 62 years. Analyzing the pain results, 95% of the respondents have pain for more than 3 months (CP) and 76 % have pain in more than one location. 43% have identified lumbar spine, shoulder, arm and forearm as the more frequent pain location, followed by cervical spine, hip, thigh and leg (29%).Only 19 % declared no other pathologies. 29% indicates more than 2 pathologies, namely diabetes, hypertension and hypercholesterolemia. 10 % refers all the three pathologies diagnosed.

Discussion/Conclusion

Our results indicate an association between age and pain complaints. It was also possible to identify the duration and etiology of the pain and to verify that pain complaints seem to be related with other pathologies.

Title: Main Characteristics of Pain in Patients Attending *Clínica Médica Povo Portuense* Assessed by Brief Pain Inventory and by *Douleur Neuropathique* 4



Poster abstract submitted and accepted at IV *Congresso de Cuidados Paliativos* - IPO, Portugal 2014.

Introduction

Chronic pain is multifactorial and needs correct assessment to distinguish their components with implications in treatment. Thus in the study of pain, neuropathic pain (NP) must be assessed.

Objectives

To characterize pain: local, intensity, impact, relief by medication and its effect on daily activities, using Brief Pain Inventory (BPI).

To determine the main features associated with NP, using *Douleur Neuropathique* 4 (DN4).

Material and methods

A convenience sample (n=32) of patients of *Clínica Médica Povo Portuense* voluntarily and by personal contact agreed to participate in this study. Portuguese validated versions of two surveys were applied: BPI and DN4. An informed consent was signed.

Results

The results (BPI) indicate that 40 % of patients using pain medication have relief symptoms. The mean score for maximum pain in previous week was 7±2 and the minimum was 2±2 in a scale 0-10. General activity, mood and daily work were pointed as the most influenced by the pain. The relationship with others and the pleasure of living were the less influenced by the pain. 1/3 of the sample has NP (DN4). 62 % indicated pain when brushing and also tingling (43 %) and numbness (38 %) are pointed.

Discussion/Conclusion

The maximum pain felt by the patients in the week before survey was very high (7 in 10). Results indicate that pain interferes in several essential tasks. Medication seems not completely effective. An expressive 1/3 of patients seem to have NP. These results further reinforce the need for attention on pain diagnostic and treatment.

Appendix II – oral communication

Oral communication abstract submitted and accepted at II *Congresso Internacional da Saúde Gaia-Porto*, Porto, Portugal 2015 and present in congress proceedings (waiting for ISBN)

Title: Assessment of Pain Characteristics in Patients of *Clínica Médica Povo Portuense*: 3 Seasons Study

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Introduction

Currently, most of the patients having chronic pain (CP) present neuropathic pain (NP). Therefore, the study of the main features of pain contributes to a better and more appropriate treatment

Objectives

To study the duration and etiology of pain, associated pathologies (AP) and usual medication (UM) as well its socio-demographic characteristics (SDC). To assess pain location (PL), intensity, relief by medication and its effect on daily activities (DA). To determine the main features of NP.

Materials and Methods

Data collection was made over 3 seasons: n=63. The convenience sample was CMPP patients that voluntarily and by personal contact agreed to participate in this study, signing an informed consent. The parameters assessed were SCD (gender, age and occupation), pain duration (PD) and pain etiology, AP and UM. Two surveys were applied: Brief Pain Inventory (BPI) and *Douleur Neuropathique* 4(DN4). This study was approved by ESTSP Ethics Commission.

Results and Discussion

Patients mean age was 63years, mostly females (82%), and 63% were retired. The PD is longer than 3months and is transverse to the 3seasons (91%). 49% have identified hip, thigh and leg, and 36% lumbar as the more frequent PL. Concerning AP, patients with hypertension, hypercholesterolemia and diabetes were found. 91% of the patients indicated use of pain medication(PM). The mean score in the 3seasons for maximum pain was 7±2 (scale 0-10). The most influenced DA were general activity, normal work and sleep (8, scale 0-10). The results indicate that 57% of patients using PM have relief symptoms. Brushing was the higher complaint (64%).

Conclusion

This study enables crossing data over 3seasons, where the age seems to be related to pain complaints (PC). Our results indicate that autumn is the season with more PC, having a higher consumption of PM. Presumable NP is present in 44% of population. These results suggest that DA are affected by pain, and medication does not look totally effective.

Appendix III – Applied surveys

i. Informed consent

Declaração de consentimento informado

Conforme a lei 67/98 de 26 de Outubro e a "Declaração de Helsínquia" da Associação Médica Mundial (Helsínquia 1964, Tóquio 1975, Veneza 1983, Hong Kong 1989, Somerset West 1996, Edimburgo 2000, Washington 2002, Tóquio 2004, Seul 2008, Fortaleza 2013) – quando se aplicar

Designação do Estudo:
Caracterização das queixas de dor em utentes adultos que recorreram à Cooperativa do Povo Portuense no 2° semestre de 2014

Eu, abaixo-assinado (NOME COMPLETO DO INDIVÍDUO PARTICIPANTE DO ESTUDO)___________________________________:
Fui informado de que o Estudo de Investigação acima mencionado se destina ao estudo e caracterização das queixas de dor em utentes adultos da Cooperativa do Povo Portuense.
Sei que neste estudo está prevista a realização de questionários tendo-me sido explicado em que consistem.
Foi-me garantido que todos os dados relativos à identificação dos Participantes neste estudo são confidenciais e que será mantido o anonimato.
Sei que posso recusar-me a participar ou interromper a qualquer momento a participação no estudo, sem nenhum tipo de penalização por este facto.
Compreendi a informação que me foi dada, tive oportunidade de fazer perguntas e as minhas dúvidas foram esclarecidas.
Aceito participar de livre vontade no estudo acima mencionado.
Também autorizo a divulgação dos resultados obtidos no meio científico, garantindo o anonimato.

Nome do Investigador e Contacto (Sofia Cunha – ESTSP/IPP)

Data ___/___/_____

Assinatura ___________________________________

Caracterização sócio-demográfica

Sexo: masculino____ feminino____

Idade: ____

Profissão: ____

Duração da dor:

Ocasional____ inferior a 3 meses____ superior a 3 meses____

Etiologia da dor:

Coluna: Cervical___ Dorsal___ Lombar___

Cabeça: ____ Face: ____ Pescoço: ____

Ombro, braço e antebraço: ____ Anca, coxa e perna: ____

Punho e mão: ____ Pé e tornozelo: ____

Abdominal: ____ Genital/Perineal: ___

Peito: ___ Tronco: ____

Outros: ____ Qual(ais) ____

Patologias associadas:

Medicação habitual:

ii. Brief Pain Inventory

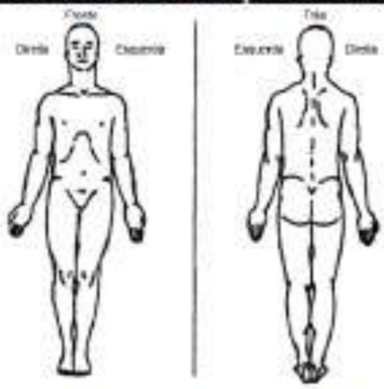
Inventário Resumido da Dor

(Formulário Abreviado)

1 Ao longo da vida, a maior parte de nós teve dor de vez em quando (tais como dores de cabeça de pequena importância, entorses e dores de dentes). Durante a última semana teve alguma dor diferente destas dores comuns?

___ Sim ___ Não

2 Nas figuras marque as áreas onde sente dor. Coloque um X na zona que lhe dói mais.



3 Por favor, classifique a sua dor assinalando com um círculo o número que melhor descreve a sua dor no seu máximo durante a última semana.

0 1 2 3 4 5 6 7 8 9 10

Sem dor — A pior dor que se pode imaginar

4 Por favor, classifique a sua dor assinalando com um círculo o número que melhor descreve a sua dor no seu mínimo durante a última semana.

0 1 2 3 4 5 6 7 8 9 10

Sem dor — A pior dor que se pode imaginar

5 Por favor, classifique a sua dor assinalando com um círculo o número que melhor descreve a sua dor em média.

0 1 2 3 4 5 6 7 8 9 10

Sem dor — A pior dor que se pode imaginar

6 Por favor classifique a sua dor assinalando com um círculo o número que indica a intensidade da sua dor neste preciso momento.

0 1 2 3 4 5 6 7 8 9 10

Sem dor — A pior dor que se pode imaginar

Versão portuguesa do *Brief Pain Inventory (Short Form)*. Tradução, adaptação cultural e validação da responsabilidade da Faculdade de Medicina da Universidade do Porto, com a autorização do autor Charles Cleeland, PhD.

7 Que tratamentos ou medicamentos está a fazer para a sua dor?

____

____

8 Na última semana, até que ponto é que os tratamentos e os medicamentos aliviaram a sua dor? Por favor, assinale com um círculo a percentagem que melhor demonstra o alívio que sentiu.

0%	10%	20%	30%	40%	50%	60%	70%	80%	90%	100%
Nenhum alívio										Alívio completo

9 Assinale com um círculo o número que descreve em que medida é que, durante a última semana, a sua dor interferiu com a sua/seu:

A Actividade geral

0	1	2	3	4	5	6	7	8	9	10
Não interferiu										Interferiu completamente

B Disposição

0	1	2	3	4	5	6	7	8	9	10
Não interferiu										Interferiu completamente

C Capacidade para andar a pé

0	1	2	3	4	5	6	7	8	9	10
Não interferiu										Interferiu completamente

D Trabalho normal (inclui tanto o trabalho doméstico como o trabalho fora de casa)

0	1	2	3	4	5	6	7	8	9	10
Não interferiu										Interferiu completamente

E Relações com outras pessoas

0	1	2	3	4	5	6	7	8	9	10
Não interferiu										Interferiu completamente

F Sono

0	1	2	3	4	5	6	7	8	9	10
Não interferiu										Interferiu completamente

G Prazer de viver

0	1	2	3	4	5	6	7	8	9	10
Não interferiu										Interferiu completamente

Versão portuguesa do *Brief Pain Inventory (Short Form)*. Tradução, adaptação cultural e validação da responsabilidade da Faculdade de Medicina da Universidade do Porto, com a autorização do autor Charles Cleeland, PhD.

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QUESTIONÁRIO ESPECÍFICO PARA RASTREIO DE DOR NEUROPÁTICA – DN4

Por favor, responda às seguintes questões, assinalando uma única resposta para cada alínea.

QUESTIONÁRIO DO DOENTE

Questão 1: A dor apresenta uma, ou mais, das características seguintes?

	sim	não
1 – **Queimadura**		
2 – **Sensação de frio doloroso**		
3 – **Choques eléctricos**		

Questão 2: Na mesma região da dor, sente também um ou mais dos seguintes sintomas?

	sim	não
4 – **Formigueiro**		
5 – **Picadas**		
6 – **Dormência**		
7 – **Comichão**		

EXAME DO DOENTE

Questão 3: A dor está localizada numa zona onde o exame físico evidencia:

	sim	não
8 – **Hipoestesia ao tacto**		
9 – **Hipoestesia à picada**		

Questão 4: A dor é provocada ou aumentada por:

	sim	não
10 – **Fricção leve ("*brushing*")**		

Versão portuguesa do *Neuropathic Pain Questionnaire (DN4)* do *French Neuropathic Pain Group*. Tradução, adaptação cultural e validação da responsabilidade da Faculdade de Medicina da Universidade do Porto, com a autorização do autor Didier Bouhassira, PhD.

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