UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ODONTOLOGIA DE BAURU

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Influence of psychosocial factors, sleep disturbances and genetic factors on pain sensitivity and temporomandibular disorder

Influência de fatores psicossociais, distúrbios do sono e fatores genéticos na sensibilidade dolorosa e disfunção temporomandibular

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Tese constituída por artigos apresentada a Faculdade de Odontologia de Bauru da Universidade de São Paulo para obtenção do título de Doutor em Ciências no programa de Ciências Odontológicas Aplicadas, área de concentração Reabilitação Oral.

Orientador: Prof. Dr. Paulo César Rodrigues Conti
Co-orientador: Prof. Dr. Gustavo Pompermaier Garlet

(Versão corrigida)

BAURU
2014

Pinto-Fiamengui, Lívia
P658i Influence of psychosocial factors, sleep disturbances and genetic factors on pain sensitivity and temporomandibular disorder/ Lívia Maria Sales Pinto Fiamengui. – Bauru, 2014.
78 p.: il. ; 31cm

Tese (Doutorado) – Faculdade de Odontologia de Bauru. Universidade de São Paulo

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Nota: A versão original desta tese encontra-se disponível no Serviço de Biblioteca e Documentação da Faculdade de Odontologia de Bauru – FOB/USP.

Comitê de Ética da FOB-USP
Protocolo nº: 118/2010
Data: 06 de Julho de 2011

DEDICATÓRIA

Ao meu sobrinho e afilhado, Felipe. Você deu um novo sentido as nossas vidas. Hoje quero ser um exemplo para você, mas sei que você que sempre nos encherá de orgulho. Meu pequeno grande herói, a Tetz te ama!

Aos meus pais, Sérgio e Ligia. Vocês são meu maior exemplo de vida, amor, simplicidade, honestidade e generosidade. Sinceramente, me faltam palavras para agradecer tudo o que vocês fazem e fizeram por mim e por nossa família. Eu simplismente não sou nada sem vocês. Obrigada por tornar a base da nossa família tão sólida. Essa conquista é para vocês! Amo vocês!!

Ao meu marido, grande amor e eterno companheiro, Jorge. Obrigada por me dar forças para correr atrás de todos os meus sonhos e, principalmente, por correr atrás deles comigo. Obrigada por fazer meus dias mais alegres e coloridos. Essa conquista também é para você! Te amo!!!

Aos meus irmãos, Luiz e Leandro, que sempre foram meus maiores orgulhos. Obrigada pelo maior presente que me deram juntos com a Ciça e Lenina: ser tia! Luca, a titia ainda nem te conhece, mas já te ama muito!!

A minha Sogra, Zezé, e ao meu Sogro, Jorjão, pois sei que vocês torcem por nós e vibram com todas as nossas conquistas. Muito obrigada por tudo!!!!

AGRADECIMENTOS ESPECIAIS

Ao meu querido orientador, Prof. Dr. Paulo César Rodrigues Conti. Hoje, mais do que nunca, só tenho a agradecer o professor, orientador e amigo que o senhor é e sempre será! Obrigada por não medir esforços para me ajudar sempre que eu precisei. Obrigada por todos os ensinamentos, incentivos e conselhos. Há 7 anos atrás cheguei a Bauru, e o senhor me recebeu de braços abertos. Me ensinou o caminho e me deu asas para conquistar tudo o que sou hoje. Ao senhor, e de coração, meu eterno agradecimento!!

AGRADECIMENTOS

A Deus, por sempre me abençoar, me dar oportunidades maravilhosas e, acima de tudo, por sempre me dar fé e força para superar todos os desafios.

Ao querido Prof. Dr. Leonardo Rigoldi Bonjardim. Não tenho palavras para agradecer todo o apoio, incentivo, ensinamentos, amizade e atenção dedicados a mim. Muito obrigada por tudo e por me ensinar que, com garra, tudo é possível.

A minha amiga, irmã e eterna companheira de vida e de estudos, Carolina Ortigosa Cunha. Obrigada por estar sempre ao meu lado, e ser extremamente presente, mesmo estando longe! Te amo muito e espero sempre tê-la em minha vida!

A Tia Sylvanira, ao Tio Clóvis, e a toda Família Bramante. Vocês me acolheram e me trataram como filha todos esses anos! Serei eternamente grata!

Aos meus amigos de doutorado, Luana, Lívia, Vitor, Luiz, Max Dória, Max Laurent, Hugo, Laís e Ilana (já viraram da nossa turma). Crescemos juntos e juntos continuaremos. Vocês me ensinaram que amigos também viram família e amigos dizem, sim, "Eu te amo". Amo vocês!

Ao Bauru Orofacial Pain Group, Prof. Conti, Léo, Carol, Juliana, Fernanda, André, Yuri, Nayla, Pri, Aline, Bruno, e a todos que por aqui já passaram. Participar desse grupo me enche de orgulho. Fico feliz em dividir com vocês o amor pela DTM.

Ao co-orientador deste trabalho, Prof. Dr. Gustavo Garlet, e aos demais colaboradores, principalmente Bruno Furquim e Carlos Eduardo Repeke. Sem vocês nada disso seria possível! Obrigada pela paciência e dedicação de cada um ao desenvolvimento dessa pesquisa.

A todos os Professores do Departamento de Prótese, em especial ao Prof. Dr. Luiz Fernando Pegoraro, Prof. Dr. Accácio Lins do Vale, Prof. Dr. Carlos Araújo e Prof. Lucimar Falavinha. Obrigada por transmitir o amor que vocês têm pela Odontologia, em especial pela Prótese Dentária. Muito obrigada por todos os ensinamentos e pelos anos de boa convivência!

Ao Prof. Dr. Lauris por realizar a análise estatística da minha pesquisa e ser sempre tão prestativo.

Aos funcionários da Faculdade de Odontologia de Bauru, em especial aos funcionários do Departamento de Prótese, da pós-graduação e da biblioteca. Obrigada pela disposição em ajudar e dedicação aos alunos!

À Capes, pela concessão da bolsa, imprescindível para a realização desta pesquisa.

À Faculdade de Odontologia de Bauru-USP, por abrir as portas para o início da minha carreira acadêmica. Aqui conheci pessoas incríveis e tive experiências que levarei para o resto da minha vida.

Aos meus alunos da FCRS, pela valorização e incentivo. Vocês me estimulam a querer ser cada vez melhor.

A Cidade de Bauru, pois aqui fui imensamente feliz, conheci grandes amigos, e o grande amor da minha vida!

“Enfrente seus obstáculos e faça alguma coisa em relação a eles. Você descobrirá que eles não têm metade da força que você pensava que eles tinham.”

Norman Vincent Peale

ABSTRACT

Influence of psychosocial factors, sleep disturbances and genetic factors on pain sensitivity and temporomandibular disorder

The present study aimed to evaluate the influence of psychosocial factors – depression and anxiety, sleep disturbances – poor sleep and bruxism, and single nucleotide polymorphisms of COMT Val^{158}Met (rs4680), IL-1β3954 (rs:1143634), IL6-174 (rs:1800795), IL10-592 (rs:1800872), MMP1-1607 (rs:1799750) and TNFα-308 (rs:1800629) as contributors to pain sensitivity and Temporomandibular Disorders. The sample comprised 291 subjects of both genders, with ages ranging from 18 to 65. Psychosocial factors were assessed using Beck Depression Inventory and Beck Anxiety Inventory. Pittsburg Sleep Questionnaire Index Sleep was used to determine sleep quality. Sleep bruxism was diagnosed in accordance with validated clinical diagnostic criteria proposed by American Academy of Sleep Medicine. The saliva samples for the DNA analysis were collected with the Oragene DNA self-collection kit. The single nucleotide polymorphisms analysis was performed using PCR. An algometer was used to record the Pressure Pain Threshold (PPT) value for the TMJ, masseter muscle and anterior temporalis. Linear multiple regression was performed to evaluate the influence of the variables on the PPT. The level of significance was set at $p<0.05$. In order to evaluate the influence of the above mentioned variables as contributors to TMD, all subjects were examined according to the American Academy of Orofacial Pain Guidelines for assessment, diagnosis and management of TMD and divided into two groups: group 1 (n=143) – subjects without TMD and group 2 (n=148) – subjects with TMD myofascial pain. Pearson chi-square test followed by a stepwise multivariate logistic regression was used for statistical analysis. The level of significance was set at $p<0.05$. According to the first analysis, the PPT of TMJ was negatively influenced by SNPs of COMT Val^{158}Met (p=0.013) and IL6-174 (p=0.006). No genetic influence was found for PPT of masticatory muscles, which were significantly influenced by poor sleep (p=0.003) and sleep bruxism (p=0.000). After the second analysis, sleep bruxism (p=0.000), poor sleep (p=0.000) and anxiety (p=0.003) were found to be associated with TMD. No association between TMD and the genetic profiles evaluated was found. The results provide evidence that pain sensitivity of TMJ is related to decreased COMT activity, and increased IL-6 activity, while pain sensitivity of masticatory muscles is influenced by sleep disturbances. On the other hand, sleep disturbances and anxiety were pointed as contributing factors for TMD.

Key words: Pain Threshold. Facial Pain. Polymorphism, Single Nucleotide.

RESUMO

Influência de fatores psicossociais, distúrbios do sono e fatores genéticos na sensibilidade dolorosa e disfunção temporomandibular

O presente estudo teve como objetivo avaliar a influência de fatores psicossociais – depressão e ansiedade; distúrbios do sono – má qualidade do sono e bruxismo; e os polimorfismos de nucleotídeo único (SNP) da COMT Val^{158}Met (rs4680), IL-1β3954 (rs:1143634), IL6-174 (rs:1800795), IL10-592 (rs:1800872), MMP1-1607 (rs:1799750) e TNFα-308 (rs:1800629) na sensibilidade dolorosa da Articulação Temporomandibular (ATM) e dos músculos mastigatórios; e como fator contribuinte para Disfunção Temporomandibular (DTM). A amostra foi composta por 291 indivíduos, ambos sexos, com idade entre 18 e 65 anos. Os fatores psicossociais foram avaliados através dos Inventários de Depressão e Ansiedade de Beck. O Índice de Qualidade de Sono de Pittsburg foi utilizado para determinar a qualidade do sono, e o bruxismo do sono foi diagnosticado de acordo com critério de diagnóstico validado proposto pela Academia Americana de Medicina do Sono. Para análise do DNA, utilizou-se amostras de saliva coletadas utilizando-se o kit de auto-coleta Oragene® DNA. A análise dos SNPs foi realizada através de PCR. As medições do Limiar de Dor à Pressão (LDP) da ATM, masséter e temporal anterior foram realizadas utilizando-se um algômetro. Regressão múltipla linear foi realizada para avaliar a influência das variáveis no LDP. Com o objetivo de avaliar a influência das variáveis acima mencionadas como contribuintes para DTM, os indivíduos foram examinados de acordo com o guia de avaliação, diagnóstico e tratamento das DTMs da Academia Americana de Dor Orofacial e divididos em dois grupos: grupo 1 (n=143) – indivíduos sem DTM e grupo 2 (n=148) – indivíduos com DTM. O teste de correlação de Pearson seguido de regressão logística multivariada foi utilizado para análise estatística. O nível de significância foi de 5%. De acordo com a primeira análise, o LDP da ATM foi negativamente influenciado pelos SNPs da COMT Val^{158}Met (p=0,013) e IL6-174 (p=0,006). O LDP da musculatura mastigatória foi negativamente influenciado pela má qualidade de sono (p=0,003) e bruxismo do sono (p=0,000), mas não sofreu influência de nenhum SNP avaliado. Após a segunda análise, bruxismo do sono (p=0,000), má qualidade de sono (p=0,000) e ansiedade (p=0,003) foram associados com a presença de DTM. Nenhuma associação entre DTM e os genótipos avaliados foi encontrada. Os resultados sugerem que a sensibilidade dolorosa da ATM está relacionada com a atividade diminuída da COMT, e com a atividade aumentada da IL-6, enquanto a sensibilidade dos músculos mastigatórios está relacionada com distúrbios do sono. Por outro lado, distúrbios do sono e ansiedade parecem ser fatores contribuintes para DTM, independentemente de fatores genéticos.

Palavras- chave: Limiar da dor. Dor facial. Polimorfismo de nucleotídeo único.

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1 Introduction

1 INTRODUCTION

According to the International Association for the Study of Pain (IASP), pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage (BONICA, 1979).

In 2008, about 100 million adults in the United States were affected by chronic pain, limiting their functional status and adversely impacting their quality of life. Besides, it was estimated that the total financial cost of pain to society, which combines the health care cost and the productivity estimates, ranged from $560 to $636 billion (INSTITUTE OF MEDICINE (US), 2011).

Temporomandibular Disorder (TMD) comprises a collective term embracing a number of clinical problems that involve the masticatory muscles and/or Temporomandibular Joints (TMJ) and associated structures (DE LEEUW, 2013). It is a common disorder, occurring in about 10% of the general population (LERESCHE, 1997) and is recognized as the most common non-odontogenic-related chronic orofacial pain condition confronted by dentists and other healthcare providers. Pain during palpation and oral functions are frequent symptoms (MCNEILL, 1997).

Over the years, several theories to explain TMD etiology have been described in the literature. Those theories range from biomedical models related to temporomandibular joints, muscles of mastication and occlusal factors, psychological models and biopsychosocial models (SUVINEN et al., 2005). Bell (1990) stated that a single etiologic agent could never be isolated, and multiple factors, in terms of predisposing conditions, activating factors and perpetuating influences should be recognized, which included structural and psychological factors.

Multifactorial etiological concepts consider several initiating, predisposing, and aggravating biomechanical, neuromuscular, biopsychosocial and neurobiological factors in TMD etiology (SVENSSON, GRAVEN-NIELSEN, 2001), including genetic variations. Studies have indicated that patients with TMD demonstrate increased somatization, stress, anxiety, and depression when compared to healthy individuals (ROLLMAN, GILLESPIE, 2000; SUVINEN et al., 2005). Current studies also suggest that specific genetic alterations may play an important role in the pathogenesis of the TMD (DIATCHENKO et al., 2006) and also with experimental pain perception (ZUBIETA et al., 2003; DIATCHENKO et al.,

2005,2006; KIM et al., 2004; FILLINGIM et al., 2005). It has been hypothesized that these changes associated with certain environmental exposures can influence the course and outcome of the disorder (DIATCHENKO et al., 2006).

Candidate genes studies have found polymorphic genetic variants to be associated with chronic diseases, such as fibromyalgia (FERNÁNDES-DE-LAS-PENAS et al., 2012; LEE, KIM, SONG, 2014), arthritis (AREND, DAYER, 1990; LI et al., 2014; SONG et al., 2014; ZHANG et al., 2014), periodontitis (TREVILATTO et al., 2010; BRAOSI et al., 2012; CLAUDINO et al., 2012) and also TMD (DIATCHENKO et al., 2005; MELOTO et al., 2011; SMITH et al., 2011,2014; MICHELOTTI et al., 2014). Most studies are focusing on genes that are able to influence the activity of peripheral afferent pain fibers, central nervous system pain processing and activity of peripheral cells that release proinflammatory mediators and the production of proinflammatory mediators from cells within the central nervous system (DIATCHENKO et al.,2006).

Smith and cols. (2011) evaluated 358 genes involved in pain processes and compared allelic frequencies between 166 cases with chronic TMD and 1.442 controls. To enhance statistical power, 182 TMD cases and 170 controls from a similar study were included in the analysis. Their findings provided evidence supporting previously reported associations between TMD and genes COMT and HTR2A. Catechol-O-Methyltransferase (COMT) is an enzyme that metabolizes catecholamines, including the neurotransmitters norepinephrine and dopamine. A diminished activity of COMT is associated with sustained elevation in catecholamines levels, which, in turn, contribute to heightened pain sensitivity and persistent pain states (NACKELEY et al., 2007). Functional polymorphisms in the COMT gene have been associated with fibromyalgia (GURSOY et al., 2003), TMJD onset (DIATCHENKO et al., 2005), experimental pain sensitivity (ZUBIETA et al. 2003; DIATCHENKO et al., 2005), and morphine efficacy in cancer pain treatment (RAKVAG et al. 2005). A functional polymorphism of the COMT gene that codes the substitution of valine (val) by methionine (met) at codon 158 ($val^{158}met$) causes difference in COMT enzyme thermostability, leading to a reduction in its activity (LOTTA et al. 1995).

Previous studies have shown that cytokines contribute to complex chronic pain conditions, such as irritable bowel syndrome (PARK, CAMILLERI 2005), low back pain (SOLOVIEVA et al., 2004) and also TMD (SLADE et al., 2011). Cytokines are small intracellular regulatory proteins secreted by immune cells in the periphery and neurons and

glia in the central nervous system. Elevated levels of proinflammatory cytokine have been found in TMJ fluid of patients with TMD (TAKAHASHI et al., 1998; KANEYAMA et al., 2002; KANEYAMA et al., 2005; MATSUMOTO et al., 2006; SLADE et al., 2011). These cytokines stimulate the production, release, and/or activation of matrix-degrading enzymes, leading to production of inflammatory mediators such as prostaglandin and leukotriene (AREND, DAYER, 1990) and are probably involved in the pathogenesis of synovitis and degenerative changes of the cartilaginous tissue and bone of the TMJ (TAKAHASHI et al., 1998). IL-1 and TNF-a can cause cartilage degradation through up-regulation of Matrix Metalloproteinases (MMPs) gene expression, metal dependent endopeptidases that are capable of cleaving most constituents of the extracellular matrix including collagen, fibronectin and proteoglycans. Another problem is added when genetic polymorphisms that influence the synthesis and release of cytokines occur (WARZOCHA et al., 1997; WILSON et al., 1997). The study of cytokine gene polymorphisms is highly significant since it is important to enhance the understanding of the etiology and pathology of human disease; to identify potential markers of susceptibility, severity, and clinical outcome; to identify potential markers for responders versus non-responders in therapeutic trials and to identify targets for therapeutic intervention (BIDWELL, 1999**).**

The studies presented here are intended to evaluate the simultaneous influence of psychological phenotypes – depression and anxiety, sleep quality –poor sleep and bruxism, and single nucleotide polymorphisms of COMT Val^{158}Met (rs4680), IL-1β3954 (rs:1143634), IL6-174 (rs:1800795), IL10-592 (rs:1800872), MMP1-1607 (rs:1799750) and TNFα-308 (rs:1800629) as risk factors for TMD in a multiple logistic regression analysis. The influence of those variables on the mechanical sensitivity of TMJ and masticatory muscles using a pressure algometer were also evaluated.

2 Articles

2 ARTICLES

The articles presented in this Thesis were written according to *The Clinical Journal of Pain* instructions and guidelines for articles submission.

2.1. ARTICLE 1

Influence of psychosocial phenotypes and genetic profiles on Pressure Pain Threshold (PPT) of masticatory muscles and Temporomandibular Joint (TMJ).

Authors:

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- Gustavo Pompermaier Garlet- Bauru School of Dentistry, University of São Paulo, Brazil.
- Paulo César Rodrigues Conti - Bauru School of Dentistry, University of São Paulo, Brazil .

ABSTRACT

Objective: to examine the simultaneous influence of psychosocial phenotypes - depression, anxiety, sleep disturbances – poor sleep and sleep bruxism, and single nucleotide polymorphisms of COMT Val^{158}Met (rs4680), IL-1β3954 (rs:1143634), IL6-174 (rs:1800795), IL10-592 (rs:1800872), MMP1-1607 (rs:1799750) and TNFα-308 (rs:1800629), on mechanical sensitivity of TMJ and masticatory muscles using a pressure algometer.

Methods: The sample comprised 291 subjects, both genders, with ages ranging from 18 to 65. Psychological phenotypes were assessed using Beck Depression Inventory and Beck Anxiety

Inventory. Pittsburg Sleep Questionnaire Index Sleep was used to determine sleep quality, and sleep bruxism was diagnosed in accordance with validated clinical diagnostic criteria proposed by American Academy of Sleep Medicine. The saliva samples for the DNA analysis were collected with the Oragene DNA self-collection kit. The single nucleotide polymorphisms analysis was performed using PCR. An algometer was used to record the Pressure Pain Threshold (PPT) value for the TMJ, masseter muscle and anterior temporalis. Linear multiple regression was performed to evaluate the influence of the variables in the PPT. The level of significance was set at $p<0.05$.

Results: The PPT of TMJ was negatively influenced by SNPs COMT Val^{158}Met ($p=0.013$) and IL6-174 ($p=0.006$). No genetic influence was found for PPT of masticatory muscles, which were significantly influenced by poor sleep ($p=0.003$) and sleep bruxism ($p=0.000$).

Discussion: The results provide evidence that pain sensitivity of masticatory muscles and TMJ are differently associated with psychological phenotypes and genotypes, highlighting the need for particular managements.

Key words: Pain Threshold. Facial Pain. Polymorphism, Single Nucleotide.

Introduction

There is a wide variation in the appreciation of pain between individuals in human populations[1]. Several genetic polymorphisms and environmental factors have been identified to contribute to differences in pain[1-6].

Temporomandibular disorders (TMDs) collectively embrace alterations or dysfunctions on the masticatory muscles, the Temporomandibular Joint (TMJ), and on its associated structures[7]. Pain during palpation and oral functions are common symptoms of TMD. The pathophysiologic mechanism responsible for lowered pain thresholds in deep craniofacial tissues could be a sensitization of peripheral nociceptors[8], central hyperexcitability[9] and/or problems in the descending pain control[10]. The influence of environmental and genetic profiles on that screening, however, should also be recognized. Because of the frequent overlap between joint and muscle-related disorders, several epidemiological investigations do not distinguish those conditions, resulting in study bias.

Recent studies have identified genes that are associated with the predictive risk of developing TMD, and also with experimental pain perception[11-18]. Possibly, multiple genes,

each with a small individual effect, interact among themselves and with a variety of environmental factors - such as anxiety, depression and sleep disorders - to influence pain sensitivity and the expression of chronic pain conditions[4].

Among those "pain genes", recent reports have shown the involvement of single nucleotide polymorphism of Catechol-O-Methyltransferase (COMT), an enzyme that metabolizes catecholamines, in the regulation of pain perception[11,12,15,17-19]. The participation of cytokines polymorphisms has also gained notability in this scenario. Previous studies have shown its association with chronic widespread pain[20], low back pain[21] and juvenile rheumatoid arthritis[22]. The literature also suggests heightened levels of cytokines in the TMJ in individuals with TMD[23-27] and also the involvement of cytokines polymorphisms with this disorder[27].

The present study aimed to examine the simultaneous influence of psychosocial phenotypes - depression, anxiety, sleep disturbances – poor sleep and sleep bruxism, and single nucleotide polymorphisms of COMT Val^{158}Met (rs4680), IL-1β3954 (rs:1143634), IL6-174 (rs:1800795), IL10-592 (rs:1800872), MMP1-1607 (rs:1799750) and TNFα-308 (rs:1800629) on mechanical sensitivity of TMJ and masticatory muscles using a pressure algometer. It was hypothesized that the interaction between environmental and genetic profiles could influence masticatory muscles and TMJ pain sensitivity differently.

Material and Methods

The study described below was approved by the local Human Research Committee (protocol nº 118/2010) and was developed along with another one in the same university (Furquim BD. Potential influence of genetic variant in temporomandibular disorders. Bauru. Thesis [Applied Dental Sciences] – Bauru School of Dentistry; 2013). All patients signed an informed consent before entering the study.

Subjects and study settings

Subjects from both sexes with ages ranging from 18 to 65 years old searching for dental treatment were selected from the Bauru School of Dentistry and recruited from Bauru, São Paulo area through media advertisements. Subjects presenting with odontalgia, neuropathic pain, rhinosinusitis, history of drug or alcohol abuse, cognitive and neurologic issues were excluded from the study. Two hundred and ninety one subjects who were

included and the overall sample was 90% female. After the initial screening, all subjects were equally evaluated by a trained dentist according to the following questionnaires and – exam methods.

Pittsburg Sleep Questionnaire Index (PSQI)

The PSQI consists of 19 self-rated questions and five questions rated by the bed partner or roommate. The 19 self-rated questions assess a wide variety of factors relating to sleep quality, including estimates of sleep duration and latency and frequency and severity of specific sleep-related problems. These 19 items are grouped into seven component scores, each weighted equally on a 0-3 scale. The seven component scores are then summed to yield a global PSQI score, which has a range of 0-21; higher scores indicate worse sleep quality.

The seven components of the PSQI are standardized versions of areas routinely assessed in clinical interviews of patients with sleep/ wake complaints. These components are subjective sleep quality, sleep latency, sleep duration, habitual sleep efficiency, sleep disturbances, use of sleeping medications, and daytime dysfunction. A PSQI global score > 5 provides a sensitive and specific measure of poor sleep quality and indicates that a subject is having severe difficulties in at least two areas, or moderate difficulties in more than three areas[28].

Beck Anxiety Inventory (BAI)

The BAI consists of 21 items, which subjects should rate themselves according to how much they are bothered by the particular symptom. Each item is on a four-point scale ranging from 0 (not at all) to 3 (severely, I could barely stand it). Thirteen items describe physical PR physiological symptoms, five represent clearly cognitive aspects of anxiety and three have physical as well as cognitive connotation. The subjects' level of anxiety is classified according to the sum of individual items scores in: minimal level of anxiety (0-7), mild anxiety (8-15), moderate anxiety (16-25) and severe anxiety (26-63)[29].

Beck Depression Inventory (BDI)

The BDI is a self-administered instrument comprising 21 items based in symptoms and attitudes to assess the intensity of depression. Each item can be rated from 0 to 3 in terms of intensity. The 21 symptoms and attitudes are mood, pessimism, sense of failure, lack of satisfaction, guilt feeling, sense of punishment, self-dislike, self-accusation, suicidal wishes, crying, irritability, social withdrawal, indecisiveness, distortion of body image, work inhibition, sleep disturbance, fatigability, loss of appetite, weight loss, somatic preoccupation and loss of libido. The BDI cut-off scores are: none or minimal depression (0-10), mild to moderate depression (10-18), moderate to severe depression (19-29), and severe depression (30-63) [30].

Sleep Bruxism assessment

Sleep Bruxism (SB) was diagnosed in accordance with validated clinical diagnostic criteria proposed by American Academy of Sleep Medicine (AASM)[31]. The criteria are as follows: (i) the patient reports or is aware of the sounds of grinding teeth during sleep, confirmed by a roommate (ii) and presents at least one of the following adjunctive criteria: (a) observation of abnormal tooth wear; (b) reports masticatory muscle fatigue or pain on waking the morning; (c) masseteric hypertrophy upon digital palpation. Added to this, there is no better explanation for the jaw muscle activity given by another current sleep disorder, medical or neurological disorder, medication use or substances use disorder.

PPT Recording

PPT determination was carried out with the aid of a digital algometer (KRATOS, Cotia, Brazil) containing a rod with a 1 cm^2 flat circular tip at one end, which was used to apply the pressure over the muscle. The pressure application rate was set at approximately 0.5kgf/cm^2/s. PPT was assessed bilaterally over anterior temporalis, masseter muscle and lateral pole of the TMJ. The use of the algometer was demonstrated and the procedure explained to all individuals. It was emphasized that the purpose of the study was to measure PPT and not pain tolerance[32]. PPT was recorded when the subject felt the pressure just beginning to cause pain. Throughout the examination, the individual's head was firmly supported by the operator's hand and each area was tested twice. The participants pressed a

button when the PPT level was reached, and at that moment the pressure was stopped and the value displayed. The device used in the present study has a button, controlled by the patient, who was asked to press it at the very beginning of pain sensation. The values from both sides were averaged to obtain one PPT per anatomical site.

DNA collection and single nucleotide polymorphism analysis

Saliva samples were collected according to the manufacturer's instructions using the Oragene DNA self-collection kit. DNA was extracted from epithelial buccal cells with sequential phenol/chloroform solution and precipitated with sal/ethanol solution[33]. DNA integrity was checked as previously described[34,35]. The allelic discrimination of variants SNPs COMT Val^{158}Met (rs4680), IL-1β3954 (rs:1143634), IL6-174 (rs:1800795), IL10-592 (rs:1800872), MMP1-1607 (rs:1799750) and TNFα-308 (rs:1800629) was performed in 3 mL reactions using Taqman (Applied Biosystems, Warrington, UK) chemistry, as previously described[35]. Genotyping was performed blinded to group status. For reaction quality control, a sample of known genotype was included in the plate and a DNA template sample was included as negative control. Only genotypes with an automatic cell rate >95% were considered. Samples that failed to provide a genotype were repeated in additional reactions.

The polymerase chain reaction (PCR) was performed using 10ng of sample DNA, 1X TaqMan™ SNP genotyping assays, 1X TaqMan™ Universal MasterMix, H20 q.s.p. 5uL. The PCR cycle conditions 60ºC for 30s, 95ºC for 10s, followed by 40 cycles at 92ºC for 15s, 60ºC for 60s, and 60ºC for 30s.

Statistical Analysis

Linear multiple regression was performed to evaluate the influence of the above-mentioned variables in the PPT of TMJ, anterior temporalis and masseter muscles, separately. For this analysis, the dependent variable was the PPT of each site. The level of significance was set at $p<0.05$.

Results

Table 1 shows values and standard deviation of PPT of TMJ, anterior temporalis and masseter.

Table 1. Minimum, maximum, mean values and Standard Deviation of PPT values.

	Minimum	*Maximum*	*Mean*	*Std. Deviation*
TMJ	.110	4.86	1.89	.714
Anterior Temporalis	.520	6.5	2.32	.862
Masseter	.39	4.1	1.65	.629

As shown on table 2, the SNPs COMT Val^{158}Met (p=0.013) and IL6-174 (p=0.006) have negatively impacted PPT of TMJ. No genetic influence was found for masticatory muscles PPT, which were significantly influenced by poor sleep and bruxism (Tables 3 and 4).

Table 2. Linear multiple regression with the PPT of TMJ as dependent variable.

	Unstandardized Coefficients		Standardized Coefficients	P	95% Confidence Interval for B	
	B	Std. Error	Beta		Lower	Upper
(Constant)	2.26	0.11		0.00000	2.04	2.48
SNP COMT Val^{158}Met	-0.28	0.11	-0.14	0.013*	-0.5	-0.05
SNP IL6-174	-0.23	0.08	-0.16	0.006*	-0.4	-0.06

*Statistically significant

Table 3. Linear multiple regression with the PPT of Anterior Temporalis as dependent variable.

	Unstandardized Coefficients		Standardized Coefficients	p	95% Confidence Interval for B	
	B	Std. Error	Beta		Lower	Upper
(Constant)	2.74	0.08		0.00000	2.57	2.91
Poor sleep	-0.31	0.1	-0.17	0.003*	-0.52	-0.1
Bruxism	-0.40	0.1	-0.23	0.000*	-0.6	-0.2

*Statistically significant

Table 4. Linear multiple regression with the PPT of Masseter as dependent variable.

	Unstandardized Coefficients		Standardized Coefficients	p	95% Confidence Interval for B	
	B	Std. Error	Beta		Lower	Upper
(Constant)	1.88	0.06		0.0000	1.75	2.01
Poor sleep	-0.18	0.07	-0.14	0.02*	-0.33	-0.02
Bruxism	-0.21	0.07	-0.16	0.006*	-0.36	-0.05

*Statistically significant

Discussion

The present study evaluated the simultaneous influence of psychological phenotypes - depression, anxiety, sleep disturbances – poor sleep and sleep bruxism, and single nucleotide polymorphisms of COMT Val^{158}Met (rs4680), IL-1β3954 (rs:1143634), IL6-174 (rs:1800795), IL10-592 (rs:1800872), MMP1-1607 (rs:1799750) and TNFα-308 (rs:1800629) on mechanical sensitivity of TMJ and masticatory muscles, using a pressure algometer. The mechanical sensitivity was obtained by measuring the PPT with a pressure algometer. This method is reliable[36] and widely used for analyzing pain sensitivity and pain threshold.

It is well know that multiple factors may influence pain sensitivity in humans, however, to our knowledge, the present study is the first to perform a multifactorial analysis regarding the association of the variables presented here and the PPT of the masticatory muscles and TMJ. Once muscles and joints are presented with different structures and physiology, it is reasonable and expected that its pain sensitivity would be influenced by different factors. As main results, the present study found that SNPs COMT Val^{158}Met and IL6-174 influenced PPT of TMJ, but it was not influenced by psychological phenotypes or sleep disturbances. On the other hand, the PPT of masseter and anterior temporalis muscles were influenced by the presence of poor sleep and bruxism, but were not influenced by any SNP evaluated.

SNP COMT Val^{158}Met and pain sensitivity

Several SNPs within COMT locus and its relationship with pain sensitivity are presented in the literature. In the present study, a functional polymorphism of the COMT gene that codes the substitution of valine (*val*) by methionine (*met*) at codon 158 (*val^{158}met*) was analyzed. This replacement causes difference in COMT enzyme thermostability, leading to a

three to four fold reduction in its activity[37]. The alleles are codominant so that individuals with the *val/val* genotype have the highest activity of COMT, those with the *met/met* genotype have the lowest activity of COMT, and heterozygous individuals are intermediate[38].

The present study is the first to suggest the association between COMT Val^{158}Met gene polymorphism and lowered PPT values of the TMJ, however, the exact mechanism to explain this phenomenon is not known. Previous studies have shown controversial findings regarding COMT polymorphisms and pain sensitivity. First, we did not find any previous study regarding the influence of COMT polymorphisms on pain sensitivity of the TMJ alone. Most studies[2,12], evaluate its influence on a global pain index, including bilateral TMJ, masseter, anterior temporalis and also extra-cranial sites. Diatchenko et al., 2006[12], found association between COMT Val^{158}Met and temporal summation of heat pain, suggesting some issues on descending pain modulation, however, no influence on global pain outcome was found. It has been suggested that the reduction in COMT activity leads to a reduction in the content of encephalin in certain regions of the Central Nervous System (CNS) associated with pain and mood[11] and in elevated levels of catecholamines, such as epinephrine, which promote the production of persistent pain states via the stimulation of b2-adrenergic receptors in the peripheral and central nervous system[39].

In 2007, a prospective cohort study evaluated healthy females volunteers regarding the influence of psychological characteristics and COMT haploblocks on tolerance to thermal pain, ischemic pain and PPT of temporalis muscles, masseter muscles, TMJs and ventral surfaces of the wrists. The measured pain sensitivity phenotype was obtained by summarizing responses to 13 standardized noxious stimuli, yielding a single index of pain sensitivity. Depression, perceived stress and mood were associated with pain sensitivity and were predictive of 2 to 3-fold increases in risk of TMD. The results remained unchanged after adjustment for the COMT haplotype[40]. Their findings should be compared to the present study with caution since different methods of pain sensitivity assessment were used and a global pain index was obtained, which did not allow a separate evaluation of the variables' influence on masticatory muscles and TMJ. Furthermore, haploblocks within the COMT gene were analyzed, while in the present study, only polymorphism in COMT Val^{158}Met gene was evaluated.

SNP IL6-174 and pain sensitivity

The concentrations of proinflammatory cytokines, such as tumor necrosis factor TNF-α, interleukin IL-6, and IL-1b, are elevated in the synovial fluid of patients with TMD, suggesting that those cytokines may be involved in the pathogenesis of these disorders[25]. Rheumatoid arthritis (RA) is a chronic synovial inflammation resulting in progressive joint damage and may share some pathophysiological mechanisms with chronic TMJ disorders. Studies have shown the role of cytokines such as TNF-α and interleukins IL-1, 6 and 15 in the pathogenesis of RA and its potential therapeutic targets[41].

Previous studies have shown that the *IL6* -174 G/C polymorphism is associated with functional differences in IL-6 levels[42,43] and with inflammatory diseases[44]. In the present study, *IL6*-174 G/C polymorphism influenced only the PPT of TMJ, which could be explained by its own physiology, making it more susceptible to inflammatory processes. Increased concentrations of inflammatory mediators have been identified in the synovial fluid of affected patients with TMD, suggesting an underlying degenerative or inflammatory process[26]. IL-6 seems to be produced by the macrophages and T lymphocytes, which infiltrate the synovium, as well as by chondrocytes and fibroblasts[45]. The presence of such peripheral sensitizing substances could decrease the primary afferent neurons threshold and be responsible for the decreased mechanical sensitivity, when judged by pressure algometry. In other words, there is a possible predisposition for mechanical hypersensivity in patients with the above-mentioned polymorphisms. In the present study, the influence of polymorphisms in TNFα-308, IL-1β3954, IL-10-592 and MMP1 on the PPT of masticatory muscles and TMJ was also evaluated, but no association was found. A recent study[46] found that TNFα is consistently detected in the TMJ synovial fluid of healthy individuals, while cytokines IFN-y and IL-2 are sporadically detected and IL-10, IL-1b and IL-6 are not frequently detectable, suggesting those cytokines are more specific for inflammatory environments.

Sleep disturbances, psychosocial phenotypes and pain sensitivity

Previous study has shown that poor sleep and depression have been associated with reduced pain threshold[47]. In the present study, association between poor sleep and lowered PPT of masticatory muscles was found, however, no influence on PPT of TMJ was detected. Vazquez-Delgado et al., 2004[48], investigated whether individuals with chronic daily headache, myofascial pain TMD and intracapsular pain TMD present with different

psychological and sleep quality characteristics. Sleep quality and psychological distress were significantly worse in individuals with myofascial pain and chronic daily headache than in those with intracapsular pain TMD. According to the same author, the more diffuse nature of pain and its higher capacity to generate central excitatory effects, may account for the sleep quality differences between groups, what could explain the results of the present study, where the PPT of TMJ suffered no influence from sleep quality. Sleep disorders and painful conditions are among the most common complaints in society. Painful disorders interfere with sleep, but disturbances in sleep also contribute to the experience of pain[49]. Since Growth hormone (GH) is known to play a crucial role in skeletal muscle synthesis and repair, deep sleep deprivation, which alters GH synthesis, may compromise muscle healing[48]. Furthermore, evidence suggest that a dysfunction of the central serotonergic system, which has been implicated in pain control and sleep regulation, may be strongly related to hormonal hypothalamic- pituitary-adrenal axis (HPA) alterations[50].

Pain and disturbed sleep might be secondary phenomena due to a common neurobiological dysfunction[51]. It is suggested that normal duration of REM sleep is of importance for the anti-nociceptive activity of endogenous and exogenous opiates[52]. Also, sleep deprivation may cause an inhibition of opioid protein synthesis[53] and a reduced affinity of m- and d-opioid receptors[54].

In 2009, Smith and colleagues[55] characterized the spectrum of sleep disorders in a well-described sample of myofascial TMD patients using polysomnography and conducted algometric measures on the masseter muscle and forearm to evaluate possible association between observed sleep disorder indexes and laboratory measures of pain threshold. Primary insomnia was associated with reduced pain thresholds at all sites, while no relationship between the sleep bruxism and pain sensitivity was found.

In the present study, we found association between self-report of sleep bruxism and lowered PPT of masticatory muscles. Self-report studies have found positive associations between sleep bruxism and orofacial pain severity[56-58], however, recent laboratory-based polysomnographic study failed to support an association between sleep bruxism and myofascial TMD[59,60]. Furthermore, Rompre et al., in 2007[61], reported that a subgroup of sleep bruxers who demonstrate reduced bruxism events are at increased risk for reporting pain. The data presented here need to be interpreted with caution, since sleep bruxism was diagnosed by a self-reporting questionnaire, with no polysomnography recording. Previous studies have shown that individuals with facial pain believe they have bruxism more often then asymptomatic individuals[59], leading to potential bias.

The present study has some limitations. The aim of the study was to analyze the influence of the variables on the PPT of TMJ and masticatory muscles, however, TMD complains were not assessed, resulting in a heterogeneous group. Also, the data presented are cross-sectional and therefore preclude causal interpretations. Future case-control studies, with a more representative population, are needed to confirm the result. For the moment, the results provide evidence that pain sensitivity of masticatory muscles and TMJ are differently associated with psychological phenotypes and genotypes, highlighting the need for particular managements. Maybe, the control of sleep disturbances, including bruxism, and associated comorbidities may be an effective strategies to prevent or treat muscle TMD, while interventions to compensate decreased COMT activity and increased IL-6 activity may be more beneficial for individuals presenting with TMJ pain complains.

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2.2. ARTICLE 2

Evaluation of psychosocial phenotypes and multiple Single Nucleotide Polymorphisms (SNPs) as risk factors for Temporomandibular Disorders (TMD)

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ABSTRACT

Objective: to evaluate the influence of certain psychosocial phenotypes - depression, anxiety; sleep disturbances - poor sleep and sleep bruxism; and single nucleotide polymorphisms of COMT Val^{158}Met (rs4680), IL-1β3954 (rs:1143634), IL6-174 (rs:1800795), IL10-592 (rs:1800872), MMP1-1607 (rs:1799750) and TNFα-308 (rs:1800629) as contributing factors for Temporomandibular Disorders (TMD).

Methods: A sample of 291 subjects of both genders, with age ranging from 18 to 65 years old was selected. All subjects were examined according to the American Academy of Orofacial Pain Guidelines for assessment, diagnosis and management of TMD and divided into two groups: group 1 (n=143) – subjects without TMD, and group 2 (n=148) – subjects with TMD myofascial pain. TMD diagnosis was based on anamnesis and physical examination. Psychsocial phenotypes were assessed using Beck Depression Inventory and Beck Anxiety Inventory. Pittsburg Sleep Questionnaire Index Sleep was used to determine sleep quality, and sleep bruxism was diagnosed in accordance with validated diagnostic criteria proposed by American Academy of Sleep Medicine. The saliva samples for the DNA analysis were

collected with the Oragene DNA self-collection kit. The single nucleotide polymorphisms analysis was performed using PCR. Pearson chi-square test followed by a stepwise multivariate logistic regression was used for statistical analysis. The level of significance was set at $p<0.05$

Results: Sleep bruxism ($p=0.000$), poor sleep ($p=0.000$) and anxiety ($p=0.003$) were found to be associated with TMD. No association between TMD and the genetic profiles evaluated was found.

Discussion: Sleep disturbances and anxiety, independent of the individuals' genotype, were pointed as contributing factors for TMD. TMD treatment programs should focus on cognitive-behavioral therapy and good sleep strategies.

Key words: Facial Pain. Polymorphism, Single Nucleotide. Bruxism.

Introduction

Temporomandibular Disorder (TMD) is a collective term embracing a number of clinical problems that involve the masticatory muscles and/or Temporomandibular Joints (TMJ) and associated structures. Pain is the most frequent symptom, which is usually aggravated by chewing and other jaw functions[1].

Multifactorial models consider several initiating, predisposing, and aggravating biomechanical, neuromuscular, biopsychosocial and neurobiological factors in TMD etiology[2]. However, it is still unclear and difficult to analyze which of those factors are more associated with TMD etiology since most individuals with TMD often present with more than one.

Recent studies have tried to identify psychological and physiological risk determinants and genetic polymorphisms that mediate the onset, maintenance and pain amplification in TMD[3-12]. Abundant evidence demonstrates that psychosocial factors contribute significantly to the experience of pain. Patients with chronic pain conditions show elevations on measures of psychosocial distress, environmental stress, catastrophizing, and somatic awareness[8] .

In addition to psychosocial factors, multiple studies are searching for a number of genes that could be associated with TMD and pain sensitivity. Most studies are focusing on genes that are able to influence the activity of peripheral afferent pain fibers, central nervous

system pain processing, activity of peripheral cell that release proinflammatory mediators and the production of proinflammatory mediators from cells within the central nervous system[5].

The aim of this study was to evaluate the influence of certain psychosocial phenotypes - depression, anxiety; sleep disturbances - poor sleep and sleep bruxism; and single nucleotide polymorphisms of COMT Val^{158}Met (rs4680), IL-1β3954 (rs:1143634), IL6-174 (rs:1800795), IL10-592 (rs:1800872), MMP1-1607 (rs:1799750) and TNFα-308 (rs:1800629) as risk factors for TMD in a multiple logistic regression study.

Material and Methods

Sample selection

A sample of 291 subjects of both genders, with age ranging from 18 to 65 years old was selected from the Bauru School of Dentistry and recruited from Bauru - São Paulo area through media advertisements. Subjects presenting with dental pain, neuropathic pain, sinusitis, cognitive and neurologic issues were excluded from the study.

All subjects were examined according to the American Academy of Orofacial Pain Guidelines for assessment, diagnosis and management of Temporomandibular Disorders and divided into two groups: group 1 (n=143) – subjects without TMD and group 2 (n=148) – subjects with TMD myofascial pain. TMD diagnosis was based on anamnesis and physical examination.

The present study was developed along with another one in the same University (Furquim BD. Potential influence of genetic variant in Temporomandibular Disorders. Bauru. PhD Thesis [Applied Dental Sciences Graduate Program] – Bauru School of Dentistry; 2013). The local Human Research Committee approved the project. All patients signed an informed consent before entering the study.

Instruments

Pittsburg Sleep Questionnaire Index (PSQI)

The PSQI consists of 19 self-rated questions and five questions rated by the bed partner or roommate. The 19 self-rated questions assess a wide variety of factors relating to sleep quality, including estimates of sleep duration and latency and the frequency and severity

of specific sleep-related problems. These 19 items are grouped into seven component scores, each weighted equally on a 0-3 scale. The seven component scores are then summed to yield a global PSQI score, which has a range of 0-21; higher scores indicate worse sleep quality.

The seven components of the PSQI are standardized versions of areas routinely assessed in clinical interviews of patients with sleep/ wake complaints. These components are subjective sleep quality, sleep latency, sleep duration, habitual sleep efficiency, sleep disturbances, use of sleeping medications, and daytime dysfunction. A PSQI global score > 5 provides a sensitive and specific measure of poor sleep quality and indicates that a subject is having severe difficulties in at least two areas, or moderate difficulties in more than three areas[13].

Beck Anxiety Inventory (BAI)

The BAI consists of 21 items, which subjects should rate according to how much they are bothered by the particular symptom. Each item is on a four-point scale ranging from 0 (not at all) to 3 (severely, I could barely stand it). Thirteen items describe physical PR physiological symptoms, five represent clearly cognitive aspects of anxiety and three have physical as well as cognitive connotation. The subjects' level of anxiety is classified according to the sum of individual items scores in: minimal level of anxiety (0-7), mild anxiety (8-15), moderate anxiety (16-25) and severe anxiety (26-63) [14].

Beck Depression Inventory (BDI)

The BDI is a self-administered instrument comprising 21 items based in symptoms and attitudes to assess the intensity of depression. Each item can be rated from 0 to 3 in terms of intensity. The 21 symptoms and attitudes are mood, pessimism, sense of failure, lack of satisfaction, guilt feeling, sense of punishment, self-dislike, self-accusation, suicidal wishes, crying, irritability, social withdrawal, indecisiveness, distortion of body image, work inhibition, sleep disturbance, fatigability, loss of appetite, weight loss, somatic preoccupation and loss of libido. The BDI cut-off scores are: none or minimal depression (0-10), mild to moderate depression (10-18), moderate to severe depression (19-29), and severe depression (30-63)[15].

Bruxism assessment

SB was diagnosed in accordance with validated clinical diagnostic criteria proposed by AASM[16]. The criteria are as follows: (i) the patient reports or is aware of the sounds of grinding teeth during sleep, confirmed by a roommate and (ii) and presents at least one of the following adjunctive criteria: (a) observation of abnormal tooth wear; (b) reports masticatory muscle fatigue or pain on waking the morning; (c) masseteric hypertrophy upon digital palpation. Added to this, there is no better explanation for the jaw muscle activity given by another current sleep disorder, medical or neurological disorder, medication use or substances use disorder.

Pressure Pain Threshold (PPT) Recording

In order to characterize the sample, it was determined the pain sensitivity of masticatory muscles and TMJ in both groups. PPT values were carried out with the aid of a digital algometer (KRATOS, Cotia, Brazil) containing a rod with a 1 cm2 flat circular tip at one end, which was used to apply the pressure over the muscle. The pressure application rate was set at approximately 0.5kgf/cm^2/s. PPT was assessed bilaterally over anterior temporalis, masseter muscle and lateral pole of the TMJ. The use of the algometer was demonstrated and the procedure explained to all individuals. PPT was recorded when the subject felt the pressure just beginning to cause pain. Throughout the examination, the individual's head was firmly supported by the operator's hand and each area was tested twice. The participants pressed a button when the PPT level was reached, and at that moment the pressure was stopped and the value displayed. The device used in the present study had a button, controlled by the patient, who was asked to press it at the very beginning of pain sensation. The values from both sides were averaged to obtain one PPT per anatomical site.

DNA collection and single nucleotide polymorphism analysis

Saliva samples were collected according to the manufacturer's instructions using the Oragene DNA self-collection kit. DNA was extracted from epithelial buccal cells with sequential phenol/chloroform solution and precipitated with sal/ethanol solution[17]. DNA integrity was checked as previously described[18,19]. The allelic discrimination of variants SNPs COMT Val^{158}Met (rs4680), IL-1β3954 (rs:1143634), IL6-174 (rs:1800795), IL10-592

(rs:1800872), MMP1-1607 (rs:1799750) and TNFα-308 (rs:1800629) was performed in 3 mL reactions using Taqman (Applied Biosystems, Warrington, UK) chemistry as previously described[19]. Genotyping was performed blinded to group status. For reaction quality control, a sample of known genotype was included in the plate and a no DNA template sample was included as negative control. Only genotypes with an automatic call rate >95% were considered. Samples that failed to provide a genotype were repeated in additional reactions.

The polymerase chain reaction (PCR) was performed using 10ng of sample DNA, 1X TaqMan™ SNP genotyping assays, 1X TaqMan™ Universal MasterMix, H20 q.s.p. 5uL. The PCR cycle conditions 60ºC for 30s, 95ºC for 10s, followed by 40 cycles at 92ºC for 15s, 60ºC for 60s, and 60ºC for 30s.

Statistical Analysis

A bivariate analysis was used to compare groups and to evaluate the influence of variables. Pearson chi-square test followed by a stepwise multivariate logistic regression was used to simultaneously assess the association of each one of the contributing factors while controlling the others. The outcome was the experimental group versus the control group. The level of significance was set at $p<0.05$.

Multiple stepwise logistic regression is a method for deciding which of a list of potential predictors are associated with presence or absence of disease. This method identifies which of the potential predictors discriminate between diseased subjects and non-diseased control subjects.

Results

There was no difference in age (37.76 vs 35.84) and gender between groups. In group 2, 42.7% of subjects had muscular TMD while 57.3% had both muscular and articular (TMJ) complaints.

The table below (Table 1) shows the difference on pain sensitivity between groups. When compared to control group, TMD group showed significantly lower PPT values of masticatory muscles ($p<0.0001$) and TMJ ($p<0.01$).

Table 1. PPT of the TMJ, anterior temporalis and masseter muscles for both groups.

	Control Group	TMD group	P value
TMJ	2.08 (0.05)	1.85 (0.06)	0.01*
Anterior Temporalis	2.93 (0.07)	2.04 (0.06)	<0.0001*
Masseter	1.94 (0.05)	1.51 (0.05)	<0.0001*

Unpaired T test; *statistically significant

Phenotypes and genotypes as predictive factors for TMD

The phenotypes and genotypes distributions for all the study variables are expressed on Table 2, Significant differences in the phenotype distribution between groups, and association between TMD and anxiety (p=0.000), depression (p=0.000), sleep disturbance (p=0.000) and bruxism (p=0.000) were found. However, there was no significant difference in the genotype distribution between groups for the polymorphisms COMT $Val^{158}Met$ (p=0.735), MMP1-1607 (p=0.725), TNFa-308 (p=0.064), IL-1β3954 (p=0.864), IL6-174 (p=0.112), IL10-592 (p=0.891). On the multivariate logistic regression, the association between TMD and anxiety (p=0.00327), sleep disturbance (p=0.000) and bruxism (p=0.000) remained. Anxiety, poor sleep and sleep bruxism showed an odds ratio of 2.76, 3.79 and 23.04 respectively for developing TMD.

Table 2. Phenotypes and genotypes distributions for both groups.

	Control Group		TMD group		Pearson Chi-Square	
	No	Yes	No	Yes	Value	P value
Anxiety	64.5%	35.5%	30.3%	69.7%	33.305	.000*
Depression	78.3%	21.7%	53.5%	46.5%	19.519	.000*
Sleep Disturbance	56.8%	43.2%	21.5%	78.5%	37.106	.000*
Bruxism	83.9%	16.1%	17.1%	82.9%	128.919	.000*
SNP COMT Val(158)met	16.1%	83.9%	17.6%	82.4%	0.114	0.735
SNP IL-1□3954	67.8%	32.2%	66.9%	33.1%	0.029	0.864
SNP IL-10 -592	37.1%	62.9%	37.8%	62.2%	0.019	0.891
SNP MMP1-1607	25.9%	74.1%	27.7%	72.3%	0.124	0.725
SNP TNF-□-308	83.2%	16.8%	74.3%	25.7%	3.43	0.064
SNP IL6-174	44.1%	55.9%	53.4%	46.6%	2.53	0.112

* Statistically significant

Table 3. Variables remaining in the final multiple regression equation: control group vs. TMD group.

	B	S.E.	Sig.	OR	95,0% C.I. for OR	
					Lower	Upper
Poor Sleep	1.33	0.37	0.00037	3.79	1.82	7.91
Anxiety	1.05	0.35	0.00327	2.86	1.42	5.79
Bruxism	3.13	0.35	0.00000	23.04	11.5	46.15
Constant	-2.95	0.40	0.00000	0.05		

Variables entered on step 1: Sleep disturbance, anxiety, depression, bruxism and SNPs COMT Val(158)met (rs4680); MMP1-1607 (rs:1799750); TNFa-308 (rs: 1800629); IL-1β3954 (rs:1143634); IL6-174 (rs:1800795); IL10-592 (rs:1800872)

Discussion

For years, the etiology of TMD was based in a biomechanical model that focused on somatic disease and structural dysfunction[1]. Currently, although there is no consensus for an etiological model for TMD, a dual-axis system has been recommended, emphasizing the role of biomechanical and psychosocial factors on the disease initiation, progression and maintenance[20]. Recent studies have shown that association between psychological features and painful disorders, including TMD, could be explained by variations on individual's genetic profile[6,8].

The present study evaluated the simultaneous influence of psychological phenotypes – depression and anxiety; sleep disturbances- poor sleep and sleep bruxism; and single nucleotide polymorphisms of COMT Val^{158}Met (rs4680), IL-1β3954 (rs:1143634), IL6-174 (rs:1800795), IL10-592 (rs:1800872), MMP1-1607 (rs:1799750) and TNFα-308 (rs:1800629) as risk factors for TMD. A multiple stepwise logistic regression was used to evaluate which of the potential predictors were associated with presence or absence of the disorder. The aim was to create a model where the association between those environmental and genetic profiles could help to explain the presence of TMD. Sleep bruxism, poor sleep and anxiety were found to be associated with TMD. In individuals with bruxism, the chance of having TMD was found to be 23 times higher. No association between TMD and the genetic profiles evaluated was detected. In the present study, the PPT of masticatory muscles and TMJ was determined in order to improve the samples' characterization, since pain during palpation is one of the most frequent symptoms of individuals with TMD. However, these data did not enter the multiple stepwise logistic regression.

In accordance with the actual findings, previous studies have found positive associations between sleep bruxism and TMD[21-29]. Several techniques, such as questionnaires,

clinical examination, electromyography and polysomnography are available for sleep bruxism diagnosis; however, all of them have advantages and limitations[30]. Self-reports are a common, easy, low- cost method to assess SB, but with elevated risk of study bias[31]. Lobbezoo and collegues (2013)[30] suggested that when sleep bruxism diagnosis is based on self-report, by means of questionnaires and/or the anamnestic part of a clinical examination, it should be classified as "possible" sleep bruxism. 'Probable' sleep or awake bruxism should be based on self-report plus clinical examination and "definite" sleep bruxism should be based on self-report, positive clinical findings, and a polysomnographic (PSG) recording, preferably along with audio/video recordings. In the present study, a validated questionnaire proposed by AASM[16] was used, however, polysomnography was not used due to its high cost and necessity of time investment. For this reason, the bruxism findings presented here should be interpreted with caution.

In general, studies regarding the association between sleep bruxim, TMD and pain sensitivity are controversial. A recent laboratory-based polysomnographic study[32] (Raphael et al., 2012) found similar rates of SB in individuals with TMD (9.7%) and controls (10.9%). In the same study, when self-reported prevalence of SB was analyzed, significant difference between case (55.3%) and control participants (15.2%) was found. It has been observed that patients with TMD believe they have bruxism because specialists told them about their parafunctional habit, even when the evidence is uncertain[33]. In 2008, a laboratory-based polysomnographic study was carried out to verify the association between sleep bruxism and TMD in a sample of 14 TMD patients and 12 healthy control subjects. No association between sleep bruxism, TMD and pain sensitivity was found. Probably, those findings were due to a small and heterogenous TMD group, presenting four patients with articular TMD, three patients with muscular TMD, and seven patients with mixed diagnosis[34]. Intriguingly, Rompre and collegues (2007)[35] demonstrated that a subgroup of sleep bruxers showing reduced bruxism events are indeed at increased risk of reporting pain, consistent with a "pain adaptation" model proposed by Lund et al. (1991) [36].

Polysomnographic[37] and self- report studies[38,39] have also found poor sleep quality in individuals with TMD. If poor sleep quality is cause or effect of pain is still controversial. Experimental studies have shown that reduction in slow wave sleep contributes to pain amplification[40]. Onen and colleagues (2001)[41] reported that healthy males showed hyperalgesia to mechanical stimuli following 40 hours of total sleep deprivation and a robust analgesic effect after selective slow wave recovery sleep. It has been suggested that the same

brain structures associated with sleep are also related to pain modulation. Thalamus, for example, is associated with waking and processing of nociception to the cortex[42].

Several authors have reported that subgroups of TMD are differently associated to psychological issues. Individuals with myogenic TMD have more anxiety traits[43] and sleep disturbances then those with articular TMD[44-46,]. Emotional distress is accompanied by imbalance of neurotransmitters that may be directly or indirectly related with the course of TMD. Disturbance of the noradrenergic system, hypothalamic–pituitary–adrenal axis, mechanism of endogenic opiates and disturbance in the level of serotonin may have an influence on dysfunction by inducing muscular hyperfunction and altered pain perception[47]. In the present study, individuals with myogenic or mixed TMD were included, but it is important to note that muscle related pain was the chief complain in all the cases. Future studies comparing different subgroups of TMD are needed.

Single-nucleotide polymorphisms (SNPs) are common variations among the DNA of individuals. Understanding those human genetic variation will contribute for the understanding of genetic basis of diseases and drug responses[48]. Studies have investigated the association of genetic variants and TMD[10,12,]. The effects of SNPs on experimental treatment outcomes for TMD have also been evaluated[49].

The Catechol-O-Methyltransferase (COMT) gene codes for an enzyme that metabolizes catecholamines and several SNPs within COMT locus and its relationship with pain sensitivity are presented in the literature[9]. In the present study, a functional polymorphism of the COMT gene that codes the substitution of valine (val) by methionine (met) at codon 158 (val^{158}met) was analyzed. This replacement causes difference in COMT enzyme thermostability, leading to a three- to four fold reduction in its activity[50]. The alleles are codominant so that individuals with the val/val genotype have the highest activity of COMT, those with the met/met genotype have the lowest activity of COMT, and heterozygous individuals are intermediate[51]. In the present study, no association between SNPs in COMT Val^{158}Met and the presence of TMD was found. In 2007, Slade and colleagues[52] undertook a prospective cohort study of healthy female volunteers to determine if psychological characteristics associated with pain sensitivity were predictive of TMD risk independently of any effects of COMT haplotype.

At baseline, participants were genotyped for COMT SNPs (rs6269, rs4633, rs4818 and val158met), completed psychological questionnaires and underwent quantitative sensory testing (QST) to determine pain sensitivity. The authors found that psychological factors were linked to pain sensitivity and influenced TMD risk, regardless of COMT haplotypes effects.

Those results are in accordance with the present study, where psychological factors were associated with TMD. On the other hand, the results presented here may also be explained by our study group characteristics. As stated before, myogenic TMD was the chief complaint in all cases. In 2006, a population-based study of 1529 individuals with chronic musculoskeletal complaints and 1488 controls found no difference in genotype and allele frequencies of the Val[158]met between groups[53]. Also, a recent algometric study found influence of SNP COMT Val[158]Met on lowered Pressure Pain Threshold (PPT) of the TMJ, but showed no influence on masticatory muscles sensitivity[54].

The evaluation of cytokine gene polymorphisms is also of high interest in TMD patients. Its importance is based on the understanding of pathologies as well as on the identification of targets for therapeutic intervention[55]. Elevated levels of proinflammatory cytokine have been found in TMJ fluid of patients with TMD[56-60]. Recent studies have shown the involvement of cytokines polymorphisms in joint disorders[61,62,63,], including TMD[60] . These cytokines stimulate the production, release, and/or activation of matrix-degrading enzymes, leading to production of inflammatory mediators such as prostaglandin and leukotriene[64]. According to Takahashi et al. (1998) [56], proinflammatory cytokines are probably involved in the pathogenesis of synovitis and degenerative changes of the cartilaginous tissue and bone of the TMJ. IL-1 and TNF-a can cause cartilage degradation through up-regulation of Matriz Metalloproteinases (MMPs) gene expression. MMPs are metal-dependent endopeptidases that are capable of cleaving most constituents of the extracellular matrix including collagen, fibronectin and proteoglycans. Genetic polymorphisms were reported to influence gene expression levels of related MMPs, and these SNPs were associated with degenerative diseases, such as periodontitis[65] and osteoarthritis[66]. In the present study, no association between SNPs in TNFα-308, IL-1β3954, IL6-174, IL10-592, MMP-1 and TMD was found. When analyzed separately (Pearson chi-square), SNP TNFα-308 showed a strong propensity of association. In 1995, TNF activity in synovial fluid from 27 patients with TMD was evaluated. No detectable TNF levels were found in 5 patients with masticatory muscle disorders, but elevated TNF levels were found in 5 of 11 patients with TMJ disc displacement, and in 9 of 11 patients with degenerative joint disease[67]. In the present study, if a more representative group of individuals with TMJ disorders were included, statistical association would probably be found for this variable. High concentrations of TNF-α have been found in the plasma, synovial fluid and tissue of patients with reumathoid arthritis (RA)[68] and TNF-α polymorphism has also been associated with this

disease[69,70]. Although RA and TMD are distinct pathologies, both present with chronic characteristics and may share some genetic susceptibility. A recent study revealed a significant association between the TNF-α polymorphism and RA in Latin Americans, but not in Europeans, Arabs or Asians[63]. In accordance with the present study, Taskin et al. (2011)[71], investigated the influence of MMP1 promoter polymorphisms in TM joint disorders and could not observe a significant difference in 1G/2G SNP of MMP1 gene between TMJ patients and the control group. Another study, however, revealed an association between the MMP1 2G/2G genotype and TMJ degeneration[72].

The study presented here has some limitations. First, it was based on cross-sectional retrospective prevalence data, and despite the sophisticated statistical analysis, cause and effect relationship are difficult to be established. Future prospective studies, with representative samples of different subgroups of TMD patients are needed to confirm the findings presented here. For this moment, data presented emphasizes the importance of TMD treatment programs focusing on cognitive and emotional behavior besides good sleep strategies.

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3 Discussion

3 DISCUSSION

Both papers presented in this thesis aimed to evaluate the simultaneous influence of psychological phenotypes – depression and anxiety; sleep disturbances – poor sleep and bruxism; and single nucleotide polymorphisms of COMT Val^{158}Met (rs4680), IL-1β3954 (rs:1143634), IL6-174 (rs:1800795), IL10-592 (rs:1800872), MMP1-1607 (rs:1799750) and TNFα-308 (rs:1800629) as risk factors for TMD. The influence of those variables on the mechanical sensitivity of TMJ and masticatory muscles, using a pressure algometer, was also evaluated, since pain during palpation is one of the most common symptoms of individuals with TMD. The use of pressure algometer has been considered an objective source to determine the Pressure Pain Threshold (PPT) of masticatory muscles (PINTO et al., 2013; SILVA-SANTOS et al., 2005) and TMJ (CUNHA et al., 2014), and is defined as the minimal amount of pressure when the perception of pressure first changes to discomfort or pain (FISCHER, 1990).

In the present study, in order to evaluate the influence of the same variables on the mechanical sensitivity of TMJ, anterior temporalis and masseter muscles, a linear multiple regression analysis was performed. The PPT of TMJ was influenced by SNPs COMT Val^{158}Met (rs4680) and IL6-174 (rs:1800795), but suffered no influence from psychological phenotypes or sleep disturbances. On the other hand, the PPT of masseter and anterior temporalis muscles were influenced by the presence of poor sleep and bruxism, but had no influence from any single nucleotide polymorphisms evaluated. During this analysis, it is important to notice that participants were not separated into diseased and non-diseased groups, but instead were grouped into one single group.

In order to identify which of the potential predictors discriminate between TMD subjects and controls, a multiple stepwise logistic regression was used. The aim was to create a model where the association between those environmental and genetic profiles could help to explain the presence of TMD signs and symptoms. Sleep bruxism, anxiety and poor sleep were associated with the presence of TMD.

Pain perception is a complex process that is influenced by a variety of environmental and genetic factors (MOGIL, 1999). TMD is a pathological pain condition that may follow a chronic course in several individuals. The literature suggests association with several psychological, behavioral, biomechanical and genetic factors, however, a definitive etiological association is far from being established. Current studies are showing a strong

interest in the discovery of genes that cause individual differences in responses to physical and environmental challenges (ZUBIETA et al., 2003).

SNPs, pain sensitivity and TMD

Studies investigating the potential association of polymorphisms in the COMT gene for different modalities of human pain perception have revealed a complex relationship between COMT genotypes and pain phenotypes (ANDERSEN AND SKORPEN, 2009). The SNP COMT Val^{158}Met (rs4680) evaluated here have been tested in different modalities of nociception. Previous studies have found its influence on sensory and affective pain ratings and diminished activation of mμ-opioid system (ZUBIETA et al., 2003); association with temporal summation of heat pain (DIATCHENKO et al., 2006); and association with global pain score for cutaneous and deep muscle pain based on pressure, thermal and ischemic pain thresholds and tolerance (DIATCHENKO et al., 2005). Others studies found no association between this SNP and muscular complaints (Hagen et al, 2006); neuropathic pain (ARMERO et al., 2005) and acute heat and cold pain (KIM et al., 2004). In the present study, no association between COMT polymorphism, TMD and masticatory muscles sensitivity measured by a pressure algometer was found, however, it was the first study to suggest the association between COMT Val^{158}Met gene polymorphism and lowered PPT values of the TMJ. It has been suggested that the reduction in COMT activity leads to a reduction in the content of encephalin in certain regions of the Central Nervous System (CNS) associated with pain and mood (ZUBIETA et al, 2003) and in elevated levels of catecholamines, such as epinephrine, which promote the production of persistent pain states via the stimulation of b2-adrenergic receptors in the peripheral and central nervous system (KHASAR, 2003). It has also been suggested that COMT plays an important role in balancing extrasynaptic and synaptic dopamine transmission at cortical and subcortical brain regions. Low COMT activity is likely to attenuate synaptic dopamine release and resulting presynaptic D2 receptor-mediated descending pain inhibition, leading to a reduction in pain threshold and an increase in affective ratings of pain (BILDER et al., 2004). Although abundant evidence suggesting an important role of COMT polymorphisms in pain sensitivity, the exact mechanism by which COMT activity influences pain perception is poorly understood. Also, different polymorphisms may occur in the COMT gene, leading to a complex relationship between COMT genotypes and pain (ANDERSEN AND SKORPEN, 2009). In the present study, an association between TMD and COMT polymorphism was expected, but surprisingly it did not

occur. Maybe the findings presented here represent the beginning for a new line of investigation, suggesting COMT activity as a target for TMJ pain specifically.

A previous study have found that cytokines IL-6, IL-10 and IL-1b are not frequently detected in TMJ synovial fluid of healthy individuals, suggesting the possible role of those cytokines in inflammatory environments. Moreover, it has also been found that the concentrations of these cytokines, such as TNF- α, IL-6, and IL-1b, are elevated in the synovial fluid of patients with TMJ disorders (KANEYAMA et al. 2005; NORDAHL, ALSTERGREN, KOPP, 2006). In the present study, IL-6 polymorphism was associated with lowered PPT values of TMJ. The findings of the present study may suggest the influence of individuals' genotype on elevated concentration of proinflammatory cytokines in the TMJ, which increases the risk for TMJ inflammation and pain. Future studies evaluating the local concentration of cytokines in TMJ, plasma and also cytokine polymorphisms in individuals with TMJ arthralgia are suggested.

Psychosocial factors, pain sensitivity and TMD.

Overwhelming evidence demonstrates that patients with chronic pain conditions show elevations on measures of psychosocial distress, environmental stress, catastrophizing and somatic sensitization. (KEEFE et al.. 2004; GATCHEL et al. 2007). In the present study, validated questionnaires for the diagnosis of depression and anxiety were used, and anxiety was pointed as risk factor for TMD, however, no association between those variables and TMJ or masticatory muscle sensitivity was found. Although TMDs are not a life-threatening condition, the patients' quality of life may be reduced due to its chronic pain nature (Liu et al., 2012). Also, whether TMD drives psychological symptoms, or vice versa, is difficult to determine. It has been suggested that individuals with myogenic TMD have more anxiety traits (PALLEGAMA et al., 2005) and higher levels of pain and psychological distress than those suffering from internal derangement or osteoarthritis (JASPERS et al., 1993). Considering the findings presented here, it could be hypothesized that psychological issues are related with the coping of the disease, but not with pain sensitivity itself.

Sleep Distubances, pain sensitivity and TMD.

The present study found association between self-reported sleep bruxism and poor sleep with lowered PPT values of masticatory muscles and also with elevated risk of TMD.

Previous self-report studies have found positive associations between sleep bruxism and TMD (LASKIN, 1969; MOLINA et al., 1997; CIANCAGLINI, GHERLONE, RADAELLI, 2001; MACFARLANE et al., 2001; HUANG et al., 2002; PERGAMALIAN et al., 2003; MANFREDINI et al., 2003; FERNANDES et al., 2012, 2014). In the present study, a validated diagnostic criteria for sleep bruxism proposed by AASM was used, however, only polysomnographic recordings are considered the gold standard for definitive SB diagnosis (AAMS, 2005). As proposed by Lobbezoo and cols (2013), the findings presented here should consider that "possible bruxism" was associated with TMD, due to SB diagnosis by means of questionnaires.

In 2012, a large laboratory polysomnography study of SB (RAPHAEL et al., 2012) found similarly rare SB levels in both myofascial TMD patients and controls, and rejected SB as myofascial TMD maintenance factor. The study also suggested that individuals with moderate frequencies of SB could be at greatest risk of experiencing facial pain. In 2013, another laboratory polysomnography study (RAPHAEL et al., 2013) evaluated sleep masticatory muscle electromyographic activity occurring outside SB events as candidate risk factor for myofascial TMD pain maintenance. The results provided evidence that those eletromyographic activity are significantly elevated in patients with myofascial TMD compared to controls. These data encourage studies that focus on more subtle but prolonged EMG elevations in the masticatory muscles, as a path to further understanding the cause and persistence of myofascial pain. The psychophysiological model of myofascial TMD (LASKIN, 1969) postulates a vicious cycle in which stress leads to muscle hyperactivity leading to bruxism which leads to pain, which feeds back to a cycle of increased stress, bruxism and pain. In contrast, the pain adaptation model (LUND et al., 1991) leads to a decrease in muscle activity in the painful area. More recent data suggest that cumulative effect of long time periods of mild elevations in EMG activity may eventually cause persistent pain (RAPHAEL, 2013). Future studies evaluating the influence of nocturnal and diurnal parafunctional habits on EMG activity, TMD and pain sensitivity are needed.

In the present study, poor sleep quality was associated with pain sensitivity of masticatory muscles and also with elevated risk of TMD. Copperman and cols. (1934), first evaluated the effects of sleep disruption on nociceptive processes. The authors observed reduced cutaneous thresholds for touch and pain in response to Von Frey hairs applied to multiple sites. They also noticed the greater the amount of sleep deprivation, the lower de PPT. It has been hypothesized that the relationship between sleep disturbance and chronic pain is due to a vicious cycle, with pain contributing to disturbed sleep and disturbed sleep

also contributing to pain sensitivity and negative mood states (MOLDOFSKY, SCARISBRICK, 1976). This hypothesis seems to be more reasonable when involving more diffuse nature of pain, like muscle pain, as stated by Vazquez-Delgado et al. (2004). In a prospective study, self-ratings of sleep and pain in patients with fibromyalgia showed that nights with poor sleep tended to be followed by days with greater pain, and days with greater pain were followed by nights with greater sleep disturbance (AFFLECK et al., 1996)

The causal relation of pain and sleep loss and its consequence is difficult to determine. Also, the source and etiology of the pain (peripheral versus central, inflammatory versus structural) may be very important with regard to the neurobiology of the pain-sleep interaction. The sleep disturbance expressed in chronic pain is likely due to hypothalamic-pituitary-adrenal axis (HPA) dysregulation. Its activation has antisleep, antireproductive, antigrowth, immunosuppressive and catabolic effects (ROEHRS, ROTH, 2005).

The power of this study was limited for several reasons. However, this study still is an important step forward in etiologic research about TMD pain. The heterogenous and reduced sample size is the main limitation. Also, part of our study consists of a cross-sectional study, but only cohort studies can directly address the time sequence between exposure and consequence. For now, part of our findings is consistent with previous studies showing the importance of psychological and behavioral influences on the incidence and maintenance of TMD. This study points out to sleep bruxism, anxiety and poor sleep as contributors to TMD, and endorses the importance of cognitive-behavioral therapies on TMD management.

The other part of the present study indicates that pain sensitivity of masticatory muscles and TMJ are differently associated with psychological and genetic factors. Our results points to the influence of SNPs of COMT Val^{158}Met (rs4680) and IL6-174 (rs:1800795) on lowered PPT of the TMJ; and the influence of bruxism and poor sleep on the PPT of masseter and anterior temporalis muscles. If these findings could be replicated in other populations, these genetic markers could help to identify patients most likely to experience TMJ pain. Furthermore, those results suggest that treatments focused on normalizing levels of COMT Val^{158}Met and IL6-174; and also methods to control sleep bruxism and poor sleep could be beneficial. Future case-control studies, comparing homogeneous subgroups of TMD and asymptomatic individuals are needed to confirm our results.

4 Conclusions

4 CONCLUSIONS

Based on the results presented in the present study, it can be concluded:

1. The PPT of TMJ was influenced by SNPs of COMT Val^{158}Met (rs4680) and IL6-174 (rs:1800795);

2. The PPT of masseter and anterior temporalis muscles were influenced by the presence of sleep bruxism and poor sleep quality;

3. Sleep bruxism, anxiety and poor sleep quality were associated with the presence of TMD;

4. SNPs of COMT Val^{158}Met (rs4680), MMP1-1607 (rs:1799750), IL-1β3954 (rs:1143634), IL6-174 (rs:1800795) and IL10-592 (rs:1800872) were not associated with the presence of TMD.

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Annexes

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Processo nº 118/2010

Bauru, 07 de julho de 2011.

Senhor Professor,

Informamos que após o envio da documentação solicitada, o projeto de pesquisa encaminhado a este Comitê de Ética em Pesquisa ***Características genéticas, ambientais e comportamentais nas disfunções temporomandibulares***, de autoria de Bruno D'Aurea Furquin, foi novamente analisado em reunião realizada no dia **06 de julho de 2011.**

O CEP-FOB/USP considerou o projeto APROVADO lembrando que a condição de aprovação da pesquisa propriamente dita exige o que segue:

- que sejam encaminhados ao CEP-FOB/USP relatórios anuais sobre o andamento da pesquisa (parciais e finais), conforme o cronograma apresentado;
- que sejam notificados ao CEP-FOB/USP, com a devida justificativa, qualquer modificação na metodologia e/ou título e a inclusão ou exclusão de autores;
- na apresentação do relatório final, incluir todos os TCLEs e/ou termos de doação de dentes devidamente assinados e rubricados.

Atenciosamente,

Prof. Dr. Flavio Augusto Cardoso de Faria
Coordenador

Prof. Dr. Paulo César Rodrigues Conti
Docente do Departamento de Prótese

Universidade de São Paulo
Faculdade de Odontologia de Bauru

Comitê de Ética em Pesquisa

50 ANOS 1962 - 2012 FOB USP

Proc. CEP nº 118/2010

Bauru, 3 de abril de 2013.

Senhor Professor,

Em atenção à solicitação de Vossa Senhoria para a inclusão da pesquisadora Lívia Maria Sales Pinto, como coautora do projeto de pesquisa **Características genéticas, ambientais e comportamentais nas disfunções temporomandibulares**, de autoria de Bruno D'Aurea Furquin, desenvolvido sob sua orientação, informamos que foi analisado por um relator e **APROVADO** em reunião deste Comitê, realizada no dia **20 de março de 2013.**

Informam os autores que a pesquisa será desenvolvida em conjunto, compartilhando os dados já coletados, que resultará em duas teses de doutorado, cujo outro título será "*Comorbidades e fatores genéticos como contribuintes na etiologia das Disfunções Temporomandibulares*", não ocorrendo alterações na metodologia inicialmente proposta.

Vale lembrar aos pesquisadores:

- que sejam encaminhados ao CEP-FOB/USP relatórios anuais sobre o andamento da pesquisa (parciais e finais), conforme o cronograma apresentado;
- que sejam notificados ao CEP-FOB/USP, com a devida justificativa, qualquer modificação na metodologia e/ou título e a inclusão ou exclusão de autores;
- na apresentação do relatório final, sejam incluídos todos os TCLEs devidamente assinados e rubricados, se for o caso.

Atenciosamente,

Izabel Regina F. Rubira Bullen

Profª Drª Izabel Regina Fischer Rubira de Bullen
Coordenadora

Prof. Dr. Paulo César Rodrigues Conti

Docente do Departamento de Prótese

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