UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ODONTOLOGIA DE BAURU

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Clinical pharmacogenetics of ibuprofen enantiomers after lower third molar surgeries

Farmacogenética clínica dos enantiômeros do ibuprofeno após exodontias de terceiros molares inferiores

Versão Corrigida

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

Orientador: Prof. Dr. Carlos Ferreira dos Santos
Co-orientador: Prof. Dr. Leonardo Rigoldi Bonjardim

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FOLHA DE APROVAÇÃO

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"O cientista não é o homem que fornece as verdadeiras respostas; é quem faz as verdadeiras perguntas".

Claude Lévi-Strauss

RESUMO

Os anti-inflamatórios não esteroidais estão disponíveis no mercado e são altamente consumidos pela população para o controle de processos inflamatórios dolorosos crônicos e agudos. A extração de terceiros molares inferiores é o modelo preconizado para a avaliação do efeito de fármacos, pois esse procedimento gera dor, edema e trismo. A família do citocromo P450 (CYP) principalmente os genes CYP2C8 e CYP2C9 são responsáveis pela metabolização dos AINES. Nesse contexto a farmacogenética, área da farmacologia que estuda a contribuição de polimorfismos e fatores genéticos para a variabilidade das respostas individuais ao metabolismo dos fármacos, vem crescendo e obtendo resultados com sua utilização clínica. Além da possível influência de biomarcadores genéticos e teciduais, o sistema inibitório descendente da dor também pode impactar na resposta e efeitos dos AINES, e uma maneira de verificar seu funcionamento é por meio da modulação da dor condicionada. Nesse aspecto, o receptor opióide OPRM1, tem sido vastamente estudado pela farmacogenética, devido à sua variação estrutural, e sua função em uma variedade de desordens dolorosas. O receptor opióide μ- (MOR), codificado pelo gene OPRM1, regula naturalmente a resposta analgésica à dor. Variabilidades genéticas no gene OPRM1, particularmente o SNP A118G, têm sido associados a um número de efeitos funcionais. O objetivo deste estudo foi avaliar o elo entre os diferentes haplótipos dos genes CYP2C8, CYP2C9, CYP1A2, CYP3A4 e CYP3A5 e a eficácia clínica do ibuprofeno, após exodontias de terceiros molares inferiores em relação à dor, edema e trismo, reações adversas, necessidade de utilização de medicação analgésica de socorro e satisfação do paciente em relação ao medicamento com a sua capacidade de modulação de dor condicionada pré-operatória. Avaliou-se também, a relação entre os diferentes haplótipos do gene OPRM1 e COMT as concentrações salivares das citocinas pró-inflamatórias (IL-2, IL-6, IFN-γ e TNF-α), e a modulação de dor condicionada pré-operatória. Foi feito o sequenciamento genético dos 200 pacientes brasileiros, com DNA genômico extraído de sua saliva, dos genes CYP2C8, CYP2C9 CYP1A2, CYP3A4, CYP3A5, OPRM1 e COMT, utilizando o instrumento MiSeq® System (Illumina®) com um comprimento de leitura de 2 x 78bp, para verificação de possíveis novas correlações destes genes com a dor pós-operatória e modulação de dor, na Kailos Genetics, Inc. em Huntsville, Alabama, Estados Unidos da América.

PALAVRAS-CHAVE: Terceiro molar. Ibuprofeno. Dor Facial. Farmacogenética. Cirurgia Bucal.

ABSTRACT

Clinical pharmacogenetics of ibuprofen enantiomers after lower third molar surgeries

Non-steroidal anti-inflammatory drugs (NSAIDs) are over-the-counter agents frequently consumed by the population in order to control chronic pain and also acute pain after inflammatory processes. Extraction of lower third molars is recommended for evaluation of the NSAIDs effect since it generates pain, swelling and trismus. Metabolism of NSAIDs is mainly dependent on the family of cytochrome P450 (CYP), more precisely CYP2C8 and CYP2C9 genes. In this context, pharmacogenetics, which studies the contribution of polymorphisms and genetic factors to the individual variability of responses to drug metabolism, is growing and starting to show results regarding the clinical use of drugs. In addition, the possible influence of genetic and tissue biomarkers at the descending inhibitory system of pain, may also impact the response and effects of NSAIDS, and this inhibitory system could be checked through the modulation of conditioned pain. In this aspect, the OPRM1 opioid receptor has been widely studied by pharmacogenetics, due to the structural variation, and its function in a variety of painful disorders. The μ-opioid receptor (MOR), encoded by the OPRM1 gene naturally regulates the analgesic response to pain. Genetic variabilities in the OPRM1 gene, particularly A118G SNP have been associated with a number of functional purposes. Thus, the aim of this study was assess the link between the different haplotypes of CYP2C8, CYP2C9 CYP1A2, CYP3A4 and CYP3A5 genes and the clinical efficacy of ibuprofen after lower third molar extractions regarding pain, swelling and trismus, adverse reactions, the amount of pain medication used, the patient's satisfaction with the drug and the influence of the ability on preoperative modulation of conditioned pain. The relationship between the different haplotypes of the OPRM1 and COMT gene was also evaluated, the salivary concentrations of the pro-inflammatory cytokines (IL-2, IL-6, IFN-γ and TNF-α), and the modulation of pre- operative. The genetic sequencing of 200 Brazilian patients was carried out, with genomic DNA extracted from their saliva, from the genes CYP2C8, CYP2C9 CYP1A2, CYP3A4, CYP3A5, OPRM1 and COMT, using MiSeq® System (Illumina®) instruments with a 2 x 78bp read length, to check for possible new correlations of these genes with postoperative pain and modulation of pain, at Kailos Genetics, Inc. in Huntsville, Alabama, United States of America.

Keywords: Third Molar. Ibuprofen. Facial Pain. Pharmacogenetics. Oral Surgery.

LIST OF FIGURES

LIST OF TABLES

	haplotype, pain modulation capacity (CPM), and pain catastrophizing scale (PCS) are independent variables	
Table S4	Multiple logistic regression model. Pain, 96 h after surgery, is the dependent variable and interferon (IFN)-γ, interleukin (IL)-2, (IL)-6, tumor necrosis factor (TNF)-α, body mass index (BMI), surgery difficulty and duration, opioid receptor (*OPRM1)* and catechol-O-methyltransferase (*COMT)* haplotype, pain modulation capacity (CPM), and pain catastrophizing scale (PCS) are independent variables	89
Table S5	Multiple logistic regression model. Trismus on the 2nd day after surgery is the dependent variable and interferon (IFN)-γ, interleukin (IL)-2, (IL)-6, tumor necrosis factor (TNF)-α, body mass index (BMI), surgery difficulty and duration, opioid receptor (*OPRM1)* and catechol-O-methyltransferase (*COMT)* haplotype, pain modulation capacity (CPM), and pain catastrophizing scale (PCS) are independent variables	90
Table S6	Multiple logistic regression model. Swelling on the 2nd day after surgery is the dependent variable and interferon (IFN)-γ, interleukin (IL)-2, (IL)-6, tumor necrosis factor (TNF)-α, body mass index (BMI), surgery difficulty and duration, opioid receptor (*OPRM1)* and catechol-O-methyltransferase (*COMT)* haplotype, pain modulation capacity (CPM), and pain catastrophizing scale (PCS) are independent variables	90

LIST OF ABBREVIATIONS AND ACRONYMS

Asn	asparagine
Asp	aspartic acid
BEPE	Research Internships Abroad
BMI	Body Mass Index
CPM	Pain modulation capacity
COX	Cyclooxygenase
COX 1	Cyclooxygenase 1
COX 2	Cyclooxygenase 2
CYP	Cytochrome P450
CYP1A2	Cytochrome P450 family 1 subfamily A polypeptide 2
CYP3A4	Cytochrome P450 family 3 subfamily A polypeptide 4
CYP2C8	Cytochrome P450 family 2 subfamily C polypeptide 8
CYP2C9	Cytochrome P450 family 2 subfamily C polypeptide 9
CYP2C19	Cytochrome P450 family 2 subfamily C polypeptide 19
CYP2D6	Cytochrome P450 family 2 subfamily D polypeptide 6
DNA	Deoxyribonucleic acid
DOR	δ-opioid receptor
et al	and collaborators
FAPESP	São Paulo Research Foundation
FOB	Bauru School of Dentistry
GMW	Giovana Maria Weckwerth
GPCRs	rhodopsin family of G protein-coupled receptors
IFN-γ	Interferon- γ
IQR	Interquartile range
IL- 2	Interleukin - 2
IL- 6	Interleukin - 6
KOR	κ-opioid receptor
MET	Methionine
MOR	receptor μ-opióide
NSAIDS	Non-steroidal anti-inflammatory drug
OPRM1	gene encoding the μ-opioid receptor
OPRD1	gene encoding the δ-opioide receptor

OPRK1	gene encoding the κ-opioide receptor
PGs	prostaglandins
PCS	pain catastrophizing scale
PCR	polymerase chain reaction
PhD	philosophiae doctor
R-ibuprofen	enantiomers R - (-) - ibuprofen
SNP	Single Nucleotide Polymorphism
SPID	Sum of the differences in pain intensity
S-ibuprofen	enantiomer S - (+) - ibuprofen
TNF-α	tumor necrosis factor-α
USA	United States of America
USP	University of São Paulo
VAL	Valine
VAS	visual analog scale

LIST OF SIMBOLS

%	percent
h	hour
ml	mililiter
min	minute
mg	miligrams
mm	milimeters
p	level of significance
SD	standard deviation of the mean
±	mean
>	bigger
<	smaller

TABLE OF CONTENTS

Introduction

1 INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) include a large number of diverse drugs which, although not chemically related, are widely available over the counter and consumed at high frequency by the general population to control painful inflammatory processes. (PERINI et al., 2005). Chronic and acute inflammatory painful manifestations are treated and controlled worldwide using NSAIDs (RODA et al., 1992; MICHAEL-HILL et al., 2006; KÖSEOĞLU et al., 2008; YOUNG et al., 2013).

In this context, lower third molar extraction is currently the study model recommended worldwide to evaluate and investigate the pharmacological and other effects of agents intended to treat acute postoperative pain, such as analgesics and anti-inflammatory drugs. (SISK; GROVER, 1990; MORRISON et al., 2000; MICHAEL-HILL et al., 2006; CALVO et al., 2006; CALVO et al., 2007; CALVO et al., 2012; BENETELLO et al., 2007; KÖSEOĞLU et al., 2008; VARNER et al., 2009; KARA et al., 2010; BJÖRNSSON; SIMONSSON, 2011; TRINDADE et al., 2011; TRINDADE et al., 2012; MOORE; HERSH, 2013; MORRE; DERRY, 2013; QUIDING et al., 2013; YOUNG et al., 2013; AKBULUT et al., 2014).

This surgical procedure is recommended for study, because some tissues are manipulated and traumatized in situ and such manipulations invariably result in functional and structural changes, including the release of inflammatory exudates and edema formation, with the subsequent appearance of trismus and pain. (SISK et al., 1986; SISK; GROVER, 1990; CALVO, et al., 2006; COLOMBINI, et al. 2006; CALVO, et al., 2007; KÖSEOĞLU et al., 2008; KARA et al., 2010; TRINDADE et al., 2011; TRINDADE et al., 2012; AKBULUT et al., 2014).

The pain that results from third-molar extraction is generally of short duration and moderate intensity, but very intense soon after surgery, and patients generally need postoperative analgesic drugs (McGRATH et al., 2003). Many studies have evaluated the needs of patients to supplement medications prescribed for pain relief (relief medication) with other analgesics such as paracetamol. Thus, the ability of medication to modulate pain can be evaluated according to the need for supplementary relief medication. (SINDET-PEDERSEN et al., 1986; MICHAEL-HILL et al., 2006; CALVO et al., 2006; CALVO et al., 2007; BENETELLO et al., 2007; VARNER et al., 2009; BJÖRNSSON; SIMONSSON, 2011; TRINDADE et al., 2011; TRINDADE et al., 2012; MARQUES et al., 2014).

Edema develops due to the surgical manipulation of tissues, and reaches maximum intensity at 48 hours after the procedure (TROULLOS et al. 1990). Trismus, or limited mouth opening, is closely linked to the amounts of edema and inflammation elicited by surgery, and it usually manifests with moderate intensity, although it can occasionally be severely painful (GRAZIANI et al., 2006).

To prevent the occurrence of postoperative complications such as acute pain, studies have indicated that physical procedures together with medications such as NSAIDs have been used extensively as adjuvants to reduce postoperative complications (SISK; GROVER, 1990; CALVO et al., 2006; CALVO et al., 2007; BENETELLO et al., 2007; KÖSEOĞLU et al., 2008; VARNER et al., 2009; KARA et al., 2010; TRINDADE et al., 2011; TRINDADE et al., 2012; POUCHAIN et al., 2015).

Both the adverse and anti-inflammatory properties of NSAIDs are executed by inhibiting cyclooxygenase, which has two isoforms, cyclooxygenase 1 and 2 (COX1 and COX 2). The COX 1 and 2 isoforms act on the inflammatory cascade via arachidonic acid, producing by-products associated with physiological protection and

inflammatory pain processes. Cyclooxygenases are responsible for the production of prostanoids, mainly thromboxanes, prostaglandins, and eucosanoids. (SMITH; WILLIS, 1971; VANE, 1971). Prostanoids and prostaglandins (PG) alone do not cause pain, but they potentiate the action of chemical mediators of inflammation (such as bradykinin) on nociceptive fibers, and consequently promote pain amplification (DIONNE; BERTHOLD, 2001).

The CYP2C9 gene among the cytochrome P450 (CYP) family is predominantly responsible for the metabolism, absorption and excretion of NSAIDs, and CYP1A2, CYP3A4 and CYP2C8 might also contribute to the hydroxylation of some NSAIDs (LEE; GOLDSTEIN; PIEPER, 2002; KIRCHHEINER; BROCKMÖLLER, 2005; MINERS et al., 1996; TANG et al., 2000; GARCIA-MARTIN et al., 2004).

Important pharmacogenetic findings have recently emerged through studies of the contribution of genetic factors to individual therapeutic or adverse responses to agents that are important for the further improvement and development of drugs in general. This branch of science implies variability in pharmacodynamics through the study of polymorphisms, for example, in genes that encode drug receptors, an area of pharmacology that is expanding and obtaining its first clinical results. (MARTÍNEZ et al., 2006; AGÚNDEZ et al., 2009; OCHOA et al 2015).

Adverse responses to NSAIDs are associated with individual variability and might be related to polymorphisms of genes encoding enzymes that metabolize NSAIDs. Enzymes that metabolize drugs are considered polymorphic due to mutations that occur in the genes that encode them. Such mutations can arise due to the absence of a gene, polymorphisms of single nucleotides (SNP), alleles or combinations and genetic duplications, which in turn can cause the absence of enzymatic activity, or a decrease, change or increase in such activity (MEYER, 2004; MARTÍNEZ et al., 2005).

Thus, plasma levels of a drug will be higher when individuals harboring mutations in such genes are treated with standard doses (MARTÍNEZ et al., 2005; GARCÍA-MARTÍN et al., 2004). Consequently, the frequency and severity of associated adverse reactions might increase with further intake (MARTÍNEZ et al., 2004; SANDERSON; EMERY; HIGGINS, 2005; AGÚNDEZ et al., 2009).

The cytochrome P450 2C8 (CYP2C8) and 2C9 (CYP2C9) enzymes belong to one of the main families of enzymes that are involved in drug metabolism. The genes that encode them, together with those that encode other components of CYP2C, are grouped in two consecutive clusters on chromosome 10 and have a high degree of association. Thus, mutations in one gene could coincide with mutations in other genes within the cluster (revised in GARCÍA-MARTÍN et al., 2006). The clinical importance of CYP2C8 and CYP2C9 polymorphisms mainly associates both enzymes with the metabolism of several prescribed drugs, some of which have a very limited therapeutic margin, and that a high ratio (%) of the population in some countries carry mutations in the genes that encode these enzymes.

The CYP2C9 genotype is likely to impact clearance of the NSAIDs, indomethacin, celecoxib, valdecoxib, lornoxicam, tenoxicam, meloxicam, piroxicam and ibuprofen, which are the targets of the present study (RODRIGUES et al., 2005). Martinez et al. (2004) genotyped CYP2C9 in individuals who were treated with these NSAIDs that are extensively metabolized by CYP2C9 and others that are not considered CYP2C9 substrates, such as salicylic acid and acetaminophen. The authors concluded that the CYP2C9 allele variant was gene and dose-dependently associated with risk of gastrointestinal bleeding, and such risk is high in patients treated with drugs that are metabolized mainly by CYP2C9. In conclusion, genotyping

CYP2C9 can identify subgroups of individuals who are potentially at increased risk of acute gastrointestinal bleeding (RODRIGUES et al., 2005; CALVO et al., 2017).

Ibuprofen is a chiral NSAID that is widely used worldwide, due to its proven analgesic and antipyretic properties. It exerts pharmacological effects through the non-selective inhibition of cyclooxygenase 1 and 2. In clinical practice, ibuprofen is administered as a racemic mixture of (s)-(+)-ibuprofen (S-ibuprofen) and (r)-(-)-ibuprofen (R-ibuprofen) enantiomers (DAVIES, 1998; HAO; WANG; SUN, 2005).

When R-ibuprofen exerts physiological effects, it undergoes unidirectional chiral inversion and is converted to S-ibuprofen (REICHEL et al., 1997). This is significant because the anti-inflammatory pharmacological activity of COX inhibition after ingesting ibuprofen, is largely attributed to the S-enantiomer (KAISER et al.,1976; LEE et al.,1985).

Ibuprofen metabolism has enantioselective complexity (OCHOA et al., 2015; LÓPEZ-RODRÍGUEZ et al., 2008). Thus, the CYP2C9 and CYP2C8 genes preferentially metabolize the S- and R-ibuprofen enantiomers, respectively (LEEMANN et al., 1993; HAMMAN et al., 1997). In addition, several polymorphisms have been found in Caucasians, for example, alleles *2 (R144C) and *3 (I359L) of the CYP2C9 gene (SOLUS et al., 2004; http://www.imm.ki.se/CYPalleles.htm). Some variants of CYP2C8 have been identified in Caucasians such as the *3 (two amino acid changes, R139K and K399R) and *4 (I264M) alleles (http://www.imm.ki.se/CYPalleles.htm; BAHADUR et al., 2002). The *2 allele (I269F) has been identified in eastern and Portuguese populations (http://www.imm.ki.se/CYPalleles.htm; CAVACO et al., 2006).

About 22% and 31% of Caucasians have mutations in the CYP2C8 and CYP2C9 genes, respectively. The most frequent variations in the CYP2C8 gene are

the *2 and *3 genes, found in 0% – 5.3% and 9.5% – 17%, respectively, of the global population, and the *4 gene, found in 4% – 8% of Caucasian populations (OCHOA et al., 2015; CABALEIRO et al., 2013; PRIETO-PÉREZ et al., 2013; LÓPEZ-RODRÍGUEZ et al., 2008; MARTÍNEZ et al., 2005; DAI et al., 2001).

Common coding mutations in the CYP2C9 gene are CYP2C9*2 and CYP2C9*3 that are associated with reduced enzyme activity and clearance of NSAIDs (CALVO et al., 2017).

About 66% of Caucasians express the wild genotype (CYP2C9*1/*1), ~33% express both the CYP2C9*1/*2 or CYP2C9*1/*3 genotypes, and < 2.5% express CYP2C9*2/*2, CYP2C9*2/*3, and CYP2C9*3/*3 (LEE; GOLDSTEIN; PIEPER, 2002). Given the high frequency of variations in the CYP2C8 and CYP2C9 genes among Caucasian populations, understanding their roles could help to recognize variability in treatment responses and the adverse effects of ibuprofen (OCHOA et al., 2015).

Some agents such as NSAIDs are sold in Brazil without much control, yet CYP2C8 and CYP2C9 gene mutations predispose carriers to gastrointestinal bleeding when treated with some of them (MARTÍNEZ et al., 2004; CALVO et al., 2017). This indicates that understating genetic anomalies can improve the health of patients, which explains why the study of pharmacogenetics is becoming more important.

In addition to the possible influence of genetic and tissue biomarkers described above, a descending inhibitory system of pain could also impact the response and effects of NSAIDs (PORPORATTI et al., 2017). One way to verify the functioning of involved neurons is through the conditioned pain modulation paradigm (CPM), in which pain is applied to inhibit pain (YARNITSKY, 2015). This conditioned pain modulation paradigm (CPM) it is a test that consists in a combination of thresholds and tolerance

of pressure and thermal pain tests, and the result is obtained by a temporal summation of thresholds and tolerance of the thermal and ischemic pain (DIATCHENKO et al., 2005). Psychological profiles of patients can be analyzed using the pain catastrophizing scale (PCS) (SULLIVAN et al., 1995), in which patients respond to a questionnaire that measures catastrophic thoughts while in pain.

The classes of nociceptive pain are neuropathic, inflammatory, and pathological (MERSKEY; BOGDUK, 1994). Different painful stimuli elicit different physiological responses and the effects of genetic variability on thresholds and pain tolerance levels probably also differ among individuals (FILLINGIM et al., 2005; CRIST; BERRETTINI, 2014).

Painful stimuli cause the release of endogenous opioids such as endorphins, which activate different types of opioid receptors (OPRM1) causing analgesic responses. The three most prevalent types of opioid receptors are the μ-opioid receptor (MOR), the δ-opioid receptor (DOR), and the κ-opioid receptor (KOR), encoded by the OPRM1, OPRD1, and OPRK1 genes, respectively (CRIST; BERRETTINI, 2014).

Opioid receptors (OPRM1) belong to the rhodopsin family of G protein-coupled receptors (GPCR), which activate signaling through interactions with heterotrimeric G proteins. Each of the three types of receptors has seven transmembrane domains, three intracellular loops, three extracellular loops, an extracellular N-terminus, and an intracellular C-terminus. Variations in these loops regulate interactions between ligands and receptors, allowing different endogenous peptides to bind to the three types of opioid receptors. The opioid MOR receptor, for example, is activated by endorphins and β-endorphins, whereas the DOR receptor is activated by enkephalin and deltorphin, while dynorphin specifically activates the KOR

receptor. Many of these peptides have an affinity for more than one type of receptor (CRIST; BERRETTINI, 2014).

Despite similar mechanisms, differences in the intracellular domains of MOR, DOR, and KOR result in different phenotypes when receptors are activated by their ligands and this can cause analgesia, euphoria, and sedation (TRESCOT et al., 2008).

Sequencing several ethnic groups has led to the identification of 3,324 polymorphisms in the OPRM1 gene that occupies a 200-kb region in the long arm of chromosome 6 (http://www.1000genomes.org). The frequency of most of these polymorphisms is low and minimally relevant to the world population (CRIST; BERRETTINI, 2014). The most prevalent and studied SNP in the OPRM1 gene is rs1799971 (A118G) at position 118, which occurs at an overall allelic frequency of 19% (MATSUNAGA et al., 2009; CRIST; BERRETTINI, 2014). Several functional effects have been associated with the A118G polymorphism (CRIST; BERRETTINI, 2014).

This SNP encodes a change from asparagine (Asn) to aspartic acid (Asp) at position 40, resulting in 3-fold stronger binding of β-endorphin to opioid receptor 1 (BOND et al., 1998). Carriers of the G allele might respond differently to drugs mediated by β-endorphin, by developing analgesia, euphoria and sedation (CHOU et al., 2006; LÖTSCH et al., 2006; MATSUNAGA et al., 2009).

For example, the OPRM1 118G variant allele is associated with a piritramide-induced decrease in acute postoperative pain relief (BARTOŠOVÁ et al. 2015). This diminished efficacy leads to the increased consumption of opioid drugs that do not fully compensate for pain relief, but instead increase the incidence of adverse effects (BARTOŠOVÁ et al. 2015).

The secretion of pro-inflammatory cytokines from peripheral immune cells is also regulated by β-endorphin through G-opioid receptor-dependent mechanisms (MATSUNAGA et al., 2009). Matsunaga and collaborators observed a close link between β-endorphins and the G-opioid receptor in G allele carriers compared with individuals without the G allele (MATSUNAGA et al., 2009). Therefore, a polymorphism in the OPRM1 gene (SNP: A118G) might influence the levels of circulating peripheral proinflammatory cytokines associated with the perception of pain, inflammation and pain modulation.

The descending inhibitory system also acts by modulating pain, and it is partially controlled by the central catecholaminergic noradrenaline and dopamine systems (JENSEN et al., 2009). This system is influenced by the catecholamine enzyme, catechol-O-methyltransferase that is encoded by the COMT gene. This enzyme moderates pain signal transmission by removing catechols such as dopamine, epinephrine and norepinephrine (SENAGORE et al., 2017). An SNP in the coding region of the COMT gene (rs4680G4A or Val158Met) can result in three genotypes (Met/Met, Met/Val, and Val/Val) (JENSEN et al., 2009; SENAGORE et al., 2017). The prevalence of the COMT genotypes was AA, AG and GG in 22%, 46%, and 32% in one study of 50 patients (LIU; WANG, 2012). Reduced COMT activity is associated with increased pain sensitivity and proinflammatory cytokine production (SENAGORE et al., 2017).

Therefore, an evaluation of the relationship between opioid receptor 1 (OPRM1) and COMT polymorphisms, concentrations of the pro-inflammatory cytokines, IL-2, IL-6, IFN-γ and TNF-α, and pain modulatory capacity that can be tested in patients, can help to determine the capacity of patients with these genotypes to modulate pain.

The patients recruited for this study had their lower third molars extracted at the Laboratory of Clinical Pharmacology at the Bauru School of Dentistry, University of São Paulo (FOB-USP). The patients were then medicated with ibuprofen 600 mg every 8 hours for 4 days. Data regarding pain, edema, trismus, amount of medication required by the patients for analgesic relief, global assessment outcomes and satisfaction regarding the medication taken were collected. In addition, sensory pain modulation was tested and saliva samples were collected before surgery for genotyping and phenotyping polymorphisms of the CYP2C8, CYP2C9, CYP3A4, CYP3A5, CYP1A2, OPRM1 and COMT genes.

The CYP2C8, CYP2C9, CYP3A4, CYP3A5, CYP1A2, OPRM1 and COMT genes were sequenced at the laboratory of Kailos Genetics Inc. (Huntsville, AL, USA) using lyophilized DNA extracted from the saliva of the patients. Correlations between these genes and postoperative pain and the modulation of preoperative pain were assessed.

A global clinical assessment of the effects of ibuprofen, the capacity to modulatory pain, genotyping and phenotyping can determine influences on adverse and therapeutic effects. Such clinical pharmacogenetic knowledge is scant and would be of great help when deciding to prescribe ibuprofen to control pain and inflammation in a Brazilian population.

Articles

2. ARTICLE

2.1. ARTICLE 1

The article presented in this Thesis was written according to the **British Journal of Clinical Pharmacology** instructions and guidelines for article submission.

CYP450 polymorphisms and clinical pharmacogenetics of ibuprofen after lower third molar extraction

Running title: CYP450 polymorphisms and ibuprofen

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"The authors confirm that the Principal Investigator for this paper is Giovana M. Weckwerth and that she had direct clinical responsibility for patients."

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Keywords

Cytochrome P450 Enzymes; clincal trials; pain; NSAIDs, polymorphisms

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What is already known about this subject

- NSAIDs are metabolized by CYP450 enzymes and widely studied for the control of postoperative acute pain after dental extractions.
- CYP450 polymorphic individuals keep higher plasma NSAID levels, and consequently, there may be an increase in the frequency and severity of adverse reactions.

What this study adds

- CYP450 polymorphisms, salivary cytokines, and other surgical parameters do not influence postoperative inflammatory symptoms in the ibuprofen-treated Brazilian population.
- In medical and dental environments, these data aid in individual medical records complementation with NSAID metabolization information, to establish a personalized and safe prescription, with attenuation of adverse effects.

Abstract

Aim:

This study hypothesized that drugs accumulate in the bloodstream of slow-metabolizing patients and may have more adverse effects and different pain perceptions, and aimed to investigate the influence of CYP450 polymorphisms and saliva cytokines on acute postoperative pain, swelling, and trismus managed by ibuprofen (600 mg) in 200 volunteers after dental extraction. In addition, saliva cytokine levels can determine pain, edema, and trismus and can indicate and exacerbate inflammatory reactions after oral surgeries. Therefore, we aimed to evaluate the level of salivary cytokines, IL-2, IL-6, IFN-γ, and TNF-α, before surgeries.

Methods:

Genetic sequencing was performed to identify CYP450 polymorphisms and the surgical parameters evaluated: pre- and postoperative swelling, trismus, temperature; self-reported postoperative pain with (VAS); rescue medication consumed; incidence, type, and severity of adverse reactions. The correlations with salivary proinflammatory cytokines were also evaluated.

Results:

A multiple linear regression model (reliability r^2, 0.97), with independent variables (SNPs, cytokines, sex, age, IBM, and difficult of surgery) and dependent variables (postoperative pain by SPID, trismus, and swelling), was used for analysis. No statistically significant difference was observed in the parameters evaluated; thus, CYP450 polymorphisms and cytokine concentration did not affect inflammation, and the adverse reactions were minimal. When evaluating CYP2C8 and C9 genotyped SNPs, we observed that normal metabolizers showed higher pain levels than the intermediate/poor metabolizers, at the postoperative periods than at time 0 h.

Conclusions:

Ibuprofen 600 mg was very effective in controlling inflammatory pain after lower third molar surgeries, without relevant adverse reactions, independent of the CYP450 polymorphisms.

Introduction

The cytochrome P450 (CYP) family is responsible for the metabolism, absorption, and excretion of most non-steroidal anti-inflammatory drugs (NSAIDs), predominantly *CYP2C9, CYP1A2, CYP3A4,* and *CYP2C8,* which function in the hydroxylation of some NSAIDs [1-5]. Cytochrome P450 2C8 (CYP2C8) and 2C9 enzymes are involved in drug metabolism [5] and the clinical importance of polymorphisms in these genes mainly are two-fold: both are associated with the metabolism of several drugs, some with very limited therapeutic margin, and a high percentage of the population carry mutations in the genes that encode them [2]. NSAIDs are extensively used globally to control chronic and acute painful inflammatory processes [6-8]. Lower third molar extraction is the recommended model for evaluating the drug effect, as it generates postoperative pain, generally reported to be short-term with mild intensity, followed by greater pain intensity 2 to 4 hours post-surgery [9,10], swelling, and trismus [8]. Adverse reactions after the use of NSAIDs depend on individual variability in response to the medication and sometimes can be related to these specific gene polymorphisms.

Drugs are metabolized by polymorphic enzymes due to mutations. These genetic mutations could be the deletion of the gene, single nucleotide polymorphisms (SNP), allele or genetic combinations, and duplications, which can cause absence, reduction, alteration or increasing of enzymatic activity.[1,2] Thus, individuals who have these mutations, when treated with usual doses of a drug metabolized by this polymorphic enzyme, will have higher plasma levels of this drug.[2,3] Consequently, there may be an increase in the frequency and severity of adverse reactions associated with its use.[4,5,6]

It is in this aspect that pharmacogenetics has contributed to personalized medicine, in which the assessment of patients' genetic profile contributes to improving personalized prescriptions for the different metabolizers. International literature contains reports such as that of Senagore and collaborators, who began guiding their prescriptions with a multigene panel.[7]

Calvo and collaborators [8] assumed that individuals with CY2C8*3 and CYP2C9 mutant alleles might have lesser pain or more side effects, than the wild type individuals. However, after therapy with piroxicam (20 mg) daily for 4 days, post lower third molar extraction, postoperative pain scores between volunteers were not different when comparing the CYP2C8*3 and CYP2C9 groups. Thus, it is very important to understand the genetic profile of patients to interpret how these mutations can impact their postoperative response.

Polymorphisms in other genes such as the G-opioid receptor (OPRM1) can affect the levels of circulating peripheral pro-inflammatory cytokines induced by β-endorphin such as interleukin-2 (IL-2), IL-6, interferon-γ

(IFN-γ), and tumor necrosis factor-α (TNF-α), and correlates with pain perception, inflammation and, pain modulation [9]. Therefore, they may have some effect on acute postoperative pain control.

Given the present study's hypothesis that drugs stay longer in the bloodstream of slow metabolizers and consequently, may have more adverse effects and different pain perceptions, the aim of this study was to investigate the influence of CYP450 polymorphisms and saliva cytokines on acute postoperative pain, swelling, and trismus managed by ibuprofen (600 mg) every 8 hours for 4 days, in 200 volunteers who extracted one lower third molar. It was also hypothesized that cytokines levels in saliva can determine pain, edema, and post-surgical trismus since, without clinical signs of oral infection, these cytokines can indicate and exacerbate inflammatory reactions after oral surgeries. Therefore, the objective of this study was to evaluate the levels of salivary cytokines, IL-2, IL-6, IFN-γ, and TNF-α, before the surgeries.

Methods

Study Design

This study was performed, per the Helsinki Declaration, analyzed, and approved by the Institutional Ethics Committees of the Bauru School of Dentistry (FOB), University of São Paulo (USP) Bauru, SP, Brazil and by the National Commission of Ethics Research (CONEP), Brazil National Research Ethics System (CAAE number:59807716.9.0000.5417), due to human genetics. The study was held, per resolution 466/12 of the National Council of Health / Ministry of Health and registered with ClinicalTrials.gov ID (NCT03169127). Before the study, all volunteers completed an informed consent form during screening.

Adults (≥18 years old) requiring unilateral lower third molar extraction, based on panoramic radiographs from FOB-USP archives, and were screened for participation, based on previously published studies on sample size determination [10]. Inclusion criteria: the absence of inflammation or infection at the extraction sites and systemic diseases that could interfere with the study. Furthermore, extractions were, among other dental indications, based on orthodontic, periodontic, and endodontic indications [11,12].

Exclusion criteria included: a history of allergy to local anesthetics or inability to receive articaine; a history of bleeding or gastrointestinal ulcers, kidney disease, asthma, or allergic sensitivity to ibuprofen or any other NSAIDs; pregnant or breastfeeding; use of antidepressants within one year before the research; use of anticoagulants, diuretics, and/or antibiotics within two months before surgery; and hepatic, kidney, intestinal, cardiac, pulmonary, circulatory, and/or brain dysfunction [12,13]. Furthermore, volunteers were excluded if they had adverse drug reactions or required different doses of local anesthetics during surgery [14].

DNA sample collection for Genetic Sequencing

Before the surgeries, 5 mL of non-stimulated saliva was collected from all volunteers in a microtube for the genetic tests. DNA was extracted and processed from the saliva at the Clinical Pharmacology and Physiology Laboratory (LAFFIC) at FOB-USP (Bauru, SP, Brazil), with QIAamp DNA Mini Kit (250), (Cat No./ID: 51306) (QIAGEN®), after which it was lyophilized, sequenced, and analyzed for the CYP2C8, CYP2C9, CYP1A2, CYP3A4, and CYP3A5 genes, and the control of pain, using the Kailos PGX-Pannel. Genetic sequencing was conducted at the Kailos Genetics, Inc.® in association with HudsonAlpha, Institute for Biotechnology, Huntsville, Alabama, USA.

Gene Sequencing and Analyses

The MiSeq® System (Illumina®), with a 2 x 78bp read length, was used in sequencing the CYP2C8, CYP2C9, CYP1A2, CYP3A4, and CYP3A5 gene. The proprietary system TargetRich was used to capture specific genome regions that were relevant to pharmacogenetics. Once captured and enriched, 98% of the resulting sequences were aligned to the target regions. Initially, the regions flanking the regions of interest were targeted with a type II restriction enzyme tethered to a guide oligonucleotide sequence, enabling precision cutting of the genome. Once released, patch oligonucleotides, a two-part oligo that was complementary to the cut ends of the target region and to a universal PCR primer. Additionally, each universal PCR primer annealed to the target sequence possessed a chemical protective group. This allowed the digestion of background genomic DNA without harming the target-patched regions. This method enables multiplexing of patient samples (up to 48 per run) and includes unique molecular identifiers (UMI). UMIs tag each starting DNA template molecule, allowing for counting of starting molecules, error correction, and increased variant detection sensitivity. Once sequenced, Kailos, the cloud-based analysis system, performed: (i) sample demultiplexing, (ii) quality assessment, (iii) alignment to the genome, (iv) variant calling, and (v) report generation.

ELISA MILLIPLEX® detection of proinflammatory cytokines in saliva

The presence of 4 proinflammatory cytokines (IL-2, IL-6, INF-γ, and TNF-α) in the LAFFIC saliva were detected using an ELISA MILLIPLEX®, with the HCYTOMAG-60K ELISA MILLIPLEX kit (Cat. # HCYTOMAG-60K, Millipore, Merck), according to the manufacturer's protocol. The results were utilized as independent variables in multiple linear regression analysis models in this study.

Surgery Intervention and Assessments

After saliva collection, the patients were subjected to a one lower third molar extraction. All surgeries were performed by the same dental surgeon (GMW), using a standard protocol [12,10,13,15], from December 2016 to April 2018 at LAFFIC. All surgical procedures and drugs provided to the patients were funded by the São Paulo Research Foundation (FAPESP). The extraction site (right or left) was determined randomly (http://www.randomization.com, number: 20434).

All surgical interventions were performed with the local anesthetic blockade of the buccal, lingual, and inferior alveolar nerves, with 4% articaine with 1:200,000 epinephrine [10,16,17,14]. After the lower third molar extraction, using a standard protocol [8,11,12,18], the surgical cavity was thoroughly cleaned, which removed alveolar bone spicules and curettage, irrigated with saline solution, aspirated, and followed finally by the closure suture of the incision. Postoperative care included a 48-hour rest period, application of ice packs, oral cleaning, and soft and cold food consumption.

Immediately after the surgery, patients consumed one tablet of ibuprofen (600 mg) every 8 hours for 4 days. The medication was consumed at the following times: 0, 8, 16, 24, 32, 40, 48, 56, 64, 72, 80, 88 and 96 h. Patients were instructed to record their postoperative pain using a Visual Analogue Scale (VAS) (0–100 mm), and were also provided with rescue medication (acetaminophen 750 mg) to be consumed every 8 h if necessary; another VAS form was provided for recording the amount of pain experienced when consuming the rescue medication. In addition to the forms, a single researcher (GMW) evaluated all the pre, intra, and postoperative periods (Table 1). Antibiotics were prescribed only in two cases where local oral infections were observed during follow-up visits [17].

Collected data and surgical outcomes

During this research, the following parameters were evaluated and analyzed in the pre, trans, and postoperative periods: weight, height, lower third molar position, the total volume of local anesthetic, onset of anesthetic agent action, surgery duration, quality of anesthesia, surgery difficulty, intraoperative bleeding, quality of wound healing, adverse reactions, body temperature, mouth opening, facial swelling, subjective evaluation of postoperative pain, the total amount of rescue medication, and overall surgery experience. All these surgical parameters evaluated and their units of measurements are detailed in Table 1, and the analysis results obtained from the patients recovering after the surgeries are described in Table 2.

Postoperative pain, swelling, and trismus (mouth opening), which are the main dependent variables evaluated in this research, could be influenced by the independent variables SNPs (*CYP2C8, CYP2C9, CYP1A2,*

CYP3A4, and *CYP3A5*), cytokines IL-2, IL-6, IFN-γ and TNF-α, sex, age, surgical difficulty, surgery duration, and BMI (Body Mass Index) and were analyzed as described below.

Postoperative pain was evaluated as described in previous studies [11, 13], using a VAS (0–100 mm) with 0 mm indicating no pain and 100 mm, worst possible pain. Additionally, postoperative pain was recorded after surgery at specific time points: 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 5, 6, 7, 8, 10, 12, 18, 24, 48, 72, and 96 h (the end of surgery was considered time 0 h) (Table 1).

Facial swelling was measured using the method by Üstün and colleagues (2003) (obtained with flexible measuring tape), which takes into account the sum of the following measures: A) distance between the lateral corner of the eye and the gonion, B) distance from the tragus to the labial commissure, and C) distance from the tragus to the soft tissue of the pogonion. The preoperative sum of the three measurements was considered the baseline value. The difference between the values obtained in the postoperative period and the baseline indicated facial swelling on the 2nd and 7th postoperative days [11,12,18] are shown in Table 1.

Mouth opening to evaluate the quantity of postoperative trismus, also obtained with a flexible measuring tape, was considered by measuring the distance (in mm) between the edges of the upper and lower incisors during the maximum opening achieved by the patient before surgery on the 2nd and 7th postoperative days [11,12,18]. The postoperative ability to open the mouth was expressed as a percentage of the preoperative measurement (Table 1).

Statistical Analyses

A multiple linear regression model was used in this study to evaluate the results, taking as independent variables all SNPs of the tested genotypes (*CYP2C8*, *CYP2C9*, *CYP1A2*, *CYP3A4*, and *CYP3A5*), 4 proinflammatory cytokines detected in saliva (IL-2, IL-6, IFN-γ and TNF-α), sex, age, the difficulty of surgery, and BMI (Body Mass Index). Similarly, dependent variables were also analyzed: postoperative pain, analyzed by the time variable, Sum of the differences in pain intensity (SPID), mouth opening (difference on 2nd postoperative day and preoperative period), and swelling (2nd postoperative day). The SPID test, used in pain analyses, was obtained by summing the scores of individual pain intensity differences over the postoperative moments analyzed in the research [19]. For the analysis of the influence of CYP2C8 and CYP2C9 polymorphisms on postoperative pain, a two-way repeated measures ANOVA was used.

Results

Initially, 300 patients were screened based on tooth position, classified by panoramic radiography [17], according to the Pell and Gregory's classification. Of these, 90 patients were excluded because they did not meet all the research criteria, and thus, 210 patients were recruited for this research and 10 were excluded after the surgeries. Specifically, eight patients had much longer surgeries with big postoperative trauma; they were excluded and received a different NSAID prescription for more than 4 days. The other two patients dropped out of the study after the surgeries (Figure 1).

A total of 200 volunteers (200 molars) were studied, of which 133 (66,5%) were women and 67 (33.5%) men, with an average age of 26 years (ages ranging from 18 to 44 years) (Table 2).

After all the analyses made between preoperative, intraoperative, and postoperative surgical parameters (total volume of local anesthetic, the onset of anesthetic agent action, surgery duration, quality of anesthesia, surgery difficulty, intraoperative bleeding, and quality of wound healing), the median and interquartile range (IQR) were obtained, and the results are shown in Table 2.

Adverse reactions in this research were minimal and did not affect the recuperation of the patients. The mean quantity of rescue medication used by patients after surgery was 2.5 pills of acetaminophen (750 mg), and in general, they used the first rescue medication about 6 hours after the surgery (Table 2).

After the surgeries, patients received therapy with ibuprofen 600 mg for the control of pain, swelling, and trismus. The overall experience, after the surgeries, of 93.50 % of the patients determined was that ibuprofen was “excellent,” “very good,” and “good” and only 6.50 % of patients classified ibuprofen treatment as “fair” and “poor” (Figure 3).

According to the frequencies of the tested SNP genotypes in the present study, 68.5% were wild-type CYP*2C8,* 67.5% CYP2C9, 45.5% CYP1A2, 92.5% CYP3A4, and 3.5% CYP3A5. Regarding allelic variant carriers, 30.5% were CYP2C8 carriers, 30.5% CYP2C9, 47% CYP1A2, 5.5% CYP3A4, and 95.5% CYP3A5 (Table 1, Supplementary).

A multiple linear regression model was used to evaluate the results, with SNPs (*CYP2C8*, *CYP2C9*, *CYP1A2*, *CYP3A4*, and *CYP3A5*), IL-2, IL-6, IFN-γ and TNF-α, sex, age, the difficulty of surgery, and BMI as independent variables. The dependent variables analyzed were: postoperative pain, trismus, and swelling. However, this model with all these variables tested showed a 0.33 r^2 value, which is not very reliable. Therefore, a second multiple linear regression analysis was performed, using data from all the SNPs and interleukins as independent variables, postoperative pain at three different time points (8, 48, and 96 h on 100 mm-VAS), trismus, and swelling, thus, reaching reliability in the r^2 test (0.97). When SPID was used as a dependent variable, and SNPs and cytokines as independent variables, the r^2 was equivalent to 0.97. When we assumed swelling and trismus as

independent variables, that is being able to influence pain levels, the multiple linear regression model presented an r^2 of 0.97. However, none of the variables proved to be significant.

With no statistical results, the research group was curious about the individual SNP and their influence on postoperative pain, based on CYP2C8 and CYP2C9 polymorphisms, which were the only genes that showed relevant results. Patients were divided into two large groups of normal metabolizers (NM) and intermediate and/or poor metabolizers (IPM) (Table 3).

A total of 200 patients were separated into two large groups of wild-type, with a normal metabolism (for CYP2C8 and/or CYP2C9 and the other genes) and carriers of the allelic variants, with an intermediate and/or poor metabolism (for CYP2C8 and/or CYP2C9 and the other genes); there were differences in the postoperative pain (Two-way repeated measures ANOVA). As shown in Figure 2-a, in a more targeted analysis of acute pain, patients considered NM for CYP2C8 showed statistically significant differences when comparing the VAS pain values from 0 h to 4, 5, 6, 7, 8, 18, 24, 48, 72, and 96 h after surgery. IPM, on the contrary, showed statistically significant differences only with pain in the periods of 6 and 8 hours after surgery, compared to the 0 h time. There were no significant differences between pain levels when comparing NM with IPM to the CYP2C8 or CYP2C9 gene.

Regarding the CYP2C9 gene, patients with NM showed statistically significant differences in pain levels when the time between 0 and 4, 5, 6, 7, 8, 18, 24, and 72 h after surgery were compared. The IPM, however, showed significant differences only when the times 7 and 8 h after surgery were compared with the time 0 h (Figure 2-b).

Discussion

The present study evaluated the influence of the variables (*CYP2C8*, *CYP2C9*, *CYP1A2*, *CYP3A4*, and *CYP3A5* SNPs, IL-2, IL-6, IFN-γ and TNF-α concentrations, sex, age, difficult of surgery, and BMI) on controlling postoperative acute pain, trismus, and swelling, on inflammatory process managed by ibuprofen (600 mg), after dental surgeries. For this analysis, a multiple linear regression model was used with an 0.97 r^2 of reliability. The main findings were: 1) No statistically significant difference was observed in all parameters evaluated, which demonstrates that polymorphisms in the CYP450 superfamily and the concentration of proinflammatory cytokines (IL-2, IL-6, IFN-γ, and TNF-α) do not affect postoperative pain, swelling, and trismus. These findings are supported by other results from the present study, such as lower rescue medication consumption, low incidence of adverse effects, and the positive global evaluation of ibuprofen effectiveness made by patients. Therefore, we can conclude that study models that aim to evaluate acute postoperative pain may not be ideal for identifying the influence of genetic mutations on metabolism. 2) Although the following results are not clinically relevant,

considering that the pain scores were less than 20 mm on VAS (Figure 2-a,b), the SNPs of both CYP2C8 and C9 genes were evaluated using ANOVA and it was observed that the normal metabolizers (NM) showed more pain levels during the postoperative periods, compared with the time 0 h, than the intermediate/poor metabolizers IPM.

This study was based on the fact that patients with CYP450 mutations present differences in NSAID metabolism such as differences in clearance and drug plasma peak. About 10% of the Caucasian population with mutations in cytochrome P450 superfamily genes, mainly CYP2C8 and 2C9, can present some peculiarities with their clearance; that is having difficulties in fully metabolizing NSAIDs [20], reaching a different plasma peak and modifying the effectiveness of NSAIDs consumed [21, 3, 8]. Therefore, in this regard, patients who are considered to have mutations in these specific genes can keep the medication in their bodies for a longer period because they are intermediate or poor metabolizers, and these NSAIDs can remain at plasma peak concentrations for longer. Additionally, NSAIDs can remain on cell receptors for a longer time; thus, enabling the variant allele carrier to feel less pain than patients considered wild type [3, 8]. Several studies have tested whether the *CYP2C8*3 and CYP2C9 *2* and **3* allelic variants could reduce ibuprofen metabolism and/or clearance [22,23], predisposing the carriers of these allele variants to a higher risk of adverse reactions. In this context, CYP genotyping may identify patients with increased risk and can help clinicians make personalized prescriptions, adjust doses, or prescribe other NSAIDs for them [24, 3]. Disseminating this idea in the medical and dental environments, aiming at complementing individual medical records with information on NSAID metabolism, can determine safe prescription and lower adverse effects.

In the present study, the experimental model of lower third molar surgery is one of the most common clinical trials used to evaluate acute postoperative pain, as this procedure generates moderate (40%) to severe pain (60%) on patients.[25, 26] However, this study model does not seem to be ideal for assessing the influence of CYP mutations in the management of post-surgical pain, due to the short period of post-surgical follow-up. Therefore, it is possible to conclude that experimental models that assess chronic pain would be more appropriate to test the hypothesis of the present study.

Despite the polymorphisms in the studied genes *(CYP2C8, CYP2C9, CYP1A2, CYP3A4,* and *CYP3A5)*, the concentration of saliva proinflammatory cytokines (IL-2, IL-6, IFN-γ, and TNF-α), and the other surgical outcomes evaluated did not confirm ibuprofen (600 mg) every 8 h for 4 days as very effective in controlling inflammatory symptomatology after lower third molar surgeries in these 200 Brazilian patients, without relevant adverse reactions; hence, the need for little rescue medication and good general assessment of the medication by patients.

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Author contribution

G. M. Weckwerth performed all surgeries, all laboratory experiments, genetic sequencing, and analyzed the data. T. J. Dionísio and Y. M. Costa provided and analyzed the primary data. B. L. Colombini-Ishiquiriama, G. M. Oliveira, and E. A. Torres were essential in the execution of all surgeries and data collections during the patient's treatment and provided primary data. T. Moore and D. M. Absher developed and supervised all genetic sequencing performed at Kailos Genetics Inc. in association with HudsonAlpha Institute for Biotechnology. A.M. Calvo, L. R. Bonjardim, and C. F. Santos contributed to the study design, supervised the experiments, reviewed, and edited the manuscript. All authors approved the final version of the article, including the authorship list.

Declaration of interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Data availability statement

Data supporting the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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Table 1. Study parameters evaluated

Parameter	**Unit**
Weight	kg
Height	m
Lower third molar position	Pell & Gregory Classification (IA, IB, IC, IIA, IIB, IIC, IIIA, IIIB, IIIC)
Total volume of local anesthetic	mL
Onset of anesthetic agent action	min
Surgery duration	min
Quality of anesthesia	3-point scale: 1) no discomfort during surgery; 2) discomfort without anesthesia required; and 3) any discomfort with anesthesia.
Surgery difficulty	3-point scale: 1) no need for osteotomies without tooth sectioning; 2) need for osteotomies without tooth sectioning; and 3) need for osteotomies and tooth sectioning (complicated).
Intraoperative bleeding	3-point scale: 1) minimal bleeding; 2) normal bleeding; and 3) excessive bleeding.
Quality of wound healing	7^{th} day; 3-point scale: 1) normal healing without inflammation; 2) delayed healing; and 3) healing complicated by inflammation or local infection with or without purulent material.
Adverse reactions	Observed by the surgeon or reported by the volunteer, during surgery in the first postoperative hour and the 2 and 7 days after surgery (craving, vomiting diarrhea, headache, and stomach pain).
Body temperature	Preoperative period; 2^{nd} and 7^{th} postoperative days (^{o}C)
Mouth opening	Preoperative period; 2^{nd} and 7^{th} postoperative days (mm)
Facial swelling	Preoperative period; 2^{nd} and 7^{th} postoperative days (mm)
Subjective evaluation of postoperative pain	Visual analog scale (VAS, 0 to 100 mm)
Total amount of rescue medication	Number of acetominophen tablets
Overall experience of surgery reported by volunteer on the 7^{th} postoperative day	5-point scale: 1) “poor”; 2) “fair”; 3) “good”; 4) “very good”; and 5) “excellent”

Table 2. Preoperative, intraoperative, and postoperative parameters

	All (n=200)	
Age (mean years. SD)	26.5 (6.5)	
Lower third molar position (Pell & Gregory Classification; n. %)		
IA	92.0	46.0
IB	27.0	13.5
IC	5.0	2.5
IIA	33.0	6.0
IIB	11.0	16.5
IIC	12.0	5.5
IIIA	1.0	7.5
IIIB	4.0	2.0
IIIC	15.0	0.5
Surgery (median. IQR)		
duration (min)	20.0	9.0
difficulty	2.0	1.0
intraoperative bleeding	1.0	0.0
quality of wound healing	1.0	0.0
quality of surgery	4.0	2.0
Local anesthetic (median. IQR)		
quantity (mL)	1.5	0.5
onset (min)	64.0	60.0
quality	2.0	2.0
Mouth Opening (median mm. IQR)		
preoperative measurement	50.0	15.0
2 day postoperative measurement	25.0	10.0
7 day postoperative measurement	34.0	15.0
Facial Swelling (mean mm. SD)		
preoperative measurement	370.0	30.0
2 day postoperative measurement	387.0	30.0
7 day postoperative measurement	380.0	30.0
Under-arm temperature (median ºC. IQR)		
preoperative measurement	36.1	0.6
2 day postoperative measurement	36.0	1.0
7 day postoperative measurement	36.0	0.8
Rescue medication (median. IQR)		
time between first consumption & surgery (hr)	6.0	15.5
VAS during first consumption (mm)	20.0	18.0
total quantity consumed (mg)	2.250	2.250

Table 3. Distribution of normal and intermediate/poor metabolizers

Allele	**Normal Metabolizers**		**Intermediate/Poor Metabolizers**		**Undetermined**	
	n	%	n	%	n	%
CYP2C8	137	68.50	61	30.50	2	1.00
CYP2C9	135	67.50	61	30.50	4	2.00
CYP1A2	90	45.00	94	47.00	16	8.00
CYP3A4	184	92.00	11	5.50	5	2.50
CYP3A5	7	3.50	190	95.00	3	1.50

* NM: Normal Metabolizers; IPM: Intermediate/Poor Metabolizers

Table 1 (Supplementary). Distribution of CYP2C8, CYP2C9, CYP1A2, CYP3A4 and CYP3A5 genotypes

	CYP2C8		CYP2C9		CYP1A2		CYP3A4		CYP3A5	
Polymorphisms	**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**
***1/*1**	137	68,5	135	67,5	91	45,5	185	92,5	7	3,5
***1/*2**	6	3	37	18,5						
***1/*3**	30	15	20	10					63	31,5
***1/*4**	20	10	0	0						
***2/*3**	0	0	1	0,5						
***2/*3**	3	1,5	2	1						
***3/*3**	0	0	1	0,5					128	64
***3/*4**	2	1	0	0						
***8**					94	47				
***1/*22**							11	5,5		
UNDETERMINED	2	1	4	2	16	8	5	2,5	3	1,5

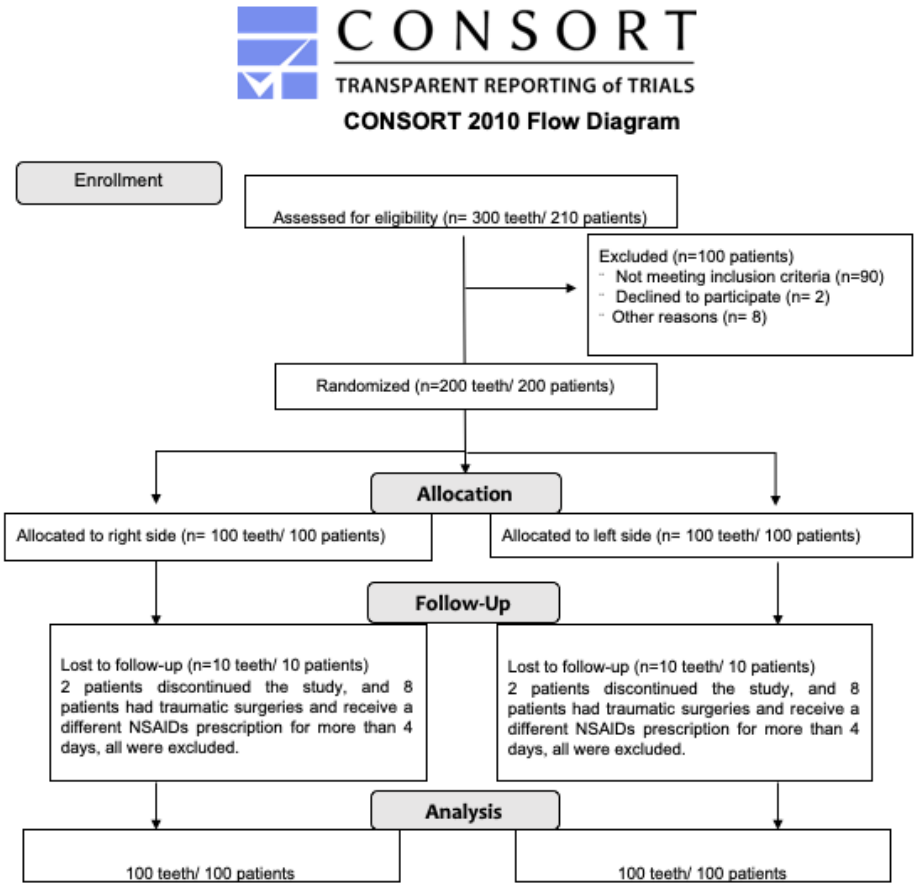

Figure 1 – CONSORT flow diagram of the study design.

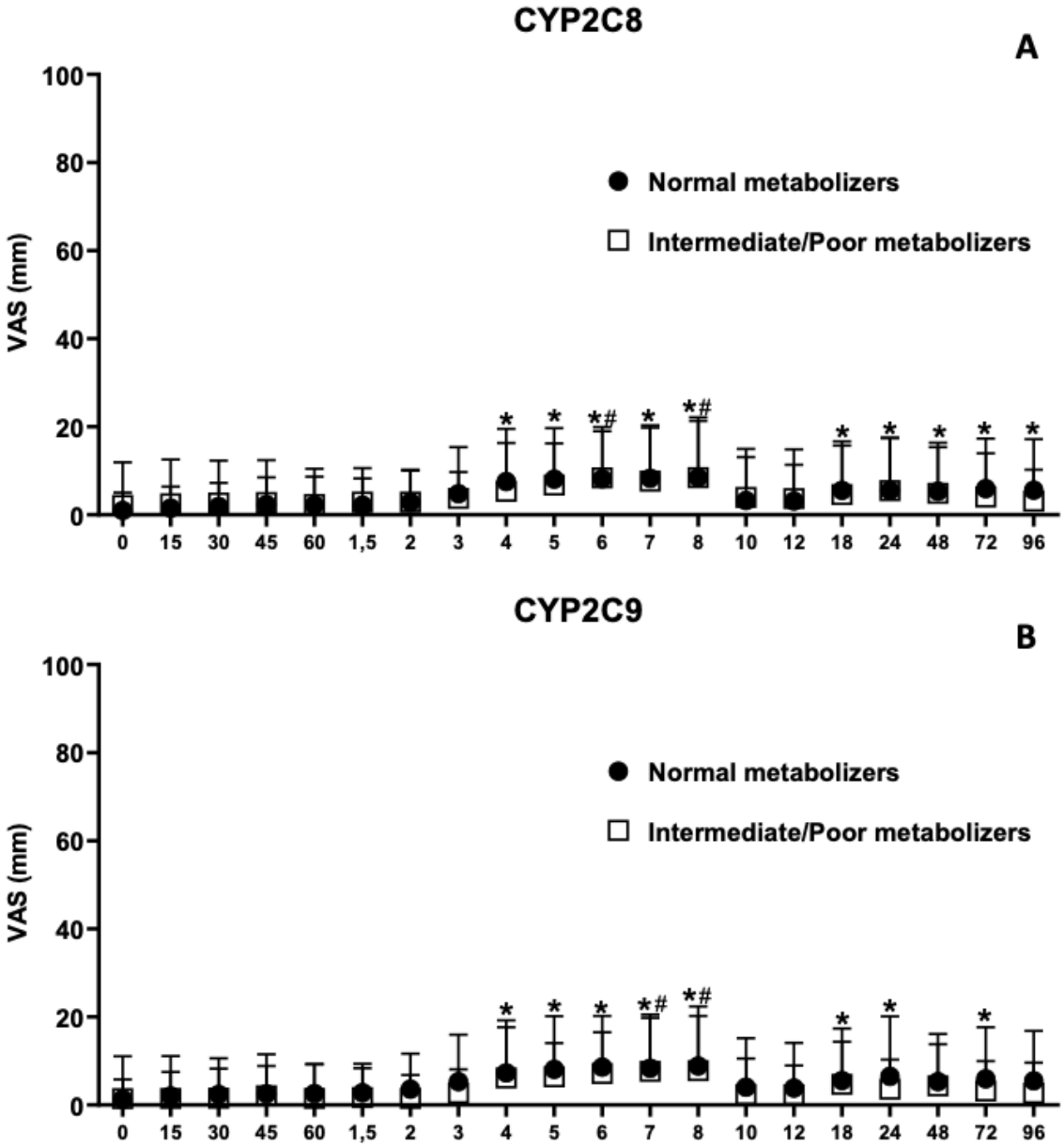

Figure 2- a and b – Postoperative pain scores, with ibuprofen (600 mg), in volunteers. The visual analog scale (VAS) of self-reported postoperative pain scores after lower third molar surgeries assessed at 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 5, 6, 7, 8, 10, 12, 18, 24, 48, 72, and 96 h. Scores ranged from 0 to 100 mm with larger scores indicating increased pain.

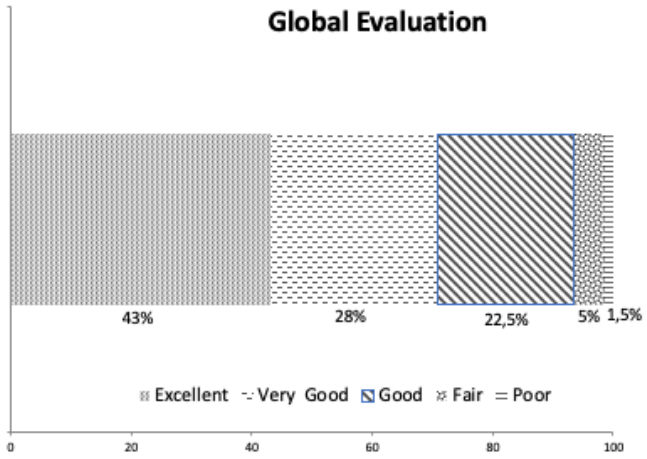

Figure 3. Global evaluation

2.2. ARTICLE 2

The second article presented in this Thesis was written according to the **European Journal of Pain** instructions and guidelines for article submission.

Opioid receptor and catechol-O-methyltransferase polymorphisms associated with patient's endogenous pain modulation when undergoing ibuprofen therapy after dental surgery

Running Title: *OPRM1*, *COMT*, and ibuprofen therapy pain modulation

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Declaration of interest: The authors declare that there are no conflicts of interest regarding the publication of this paper.

Significance: This study was developed to understand whether the profile of the modulation capacity of pre and postoperative pain, swelling, and trismus was affected by psychological and clinical parameters after dental surgery. *COMT* polymorphism, IL-2, IFN-γ, BMI, and duration of surgery influenced postoperative inflammation in this ibuprofen-treated Brazilian population. For future treatments, these data were added to patient medical records as a personalized and safe prescription, based on their profile of pain modulation capacity with attenuation of adverse effects.

Abstract

Background: To analyze the pain modulation capacity profile in a Brazilian population, the relationship between opioid receptor (*OPRM1*) and catechol-O-methyltransferase (*COMT)* polymorphisms with patient pain modulation capacity was determined through preoperative pain modulation tests and acute postoperative pain control evaluation, swelling, and trismus in 200 volunteers undergoing lower third molar extraction with ibuprofen therapy.

Material: Psychologic and clinical parameters were measured. Patient DNA was sequenced for single nucleotide polymorphisms in *OPRM1* and *COMT*, and the salivary concentration of interleukin (IL)-2, (IL)-6, interferon (IFN)-γ and tumor necrosis (TNF)-α was evaluated. Primary outcomes were the influence of all predictors on the fluctuation of pain intensity using a visual analog scale (VAS), and swelling and trismus on the 2nd and 7th postoperative days. Preoperative pain modulation capacity (CPM), pain catastrophizing scale (PCS), body mass index (BMI), and surgery duration and difficulty were evaluated.

Results: The concentration of IFN-γ, IL-2, and the duration of surgery influenced the fluctuation of postoperative pain in the VAS, and in the sum of the differences in pain intensity test at 8, 48, and 96 h. BMI influenced swelling, while both BMI and the *COMT* haplotype influenced trismus on the 2nd postoperative day. A positive global evaluation of ibuprofen effectiveness was observed.

Conclusions: Polymorphisms in *COMT*, concentrations of IL-2 and IFN-γ, BMI, and duration of surgery were predictors for pain fluctuation, swelling, and trismus on the 2nd postoperative day. This therapy was effective in controlling inflammatory symptomatology after surgery and ibuprofen was well tolerated by patients.

Introduction

Classes of nociceptive pain include neuropathic, inflammatory and pathological (Merksey and Bogduk, 1994). Different stimuli induce diverse physiological responses, and differences in genetic variability likely effect pain tolerance thresholds and levels (Crist and Berrettini, 2014; Fillingim et al., 2005).

Painful stimuli induce release of endogenous opioids, including endorphins, that activate opioid receptors (OPRM1) causing analgesic responses (Crist and Berrettini, 2014). The three most common opioid receptors are receptor μ-opioid (MOR), δ-opioid (DOR) and κ-opioid (KOR), encoded by the *OPRM1*, *OPRD1* and *OPRK1* genes, respectively (Crist and Berrettini, 2014). The μ-opioid (MOR) is activated by endomorphins and β-endorphins (Crist and Berrettini, 2014).

Sequencing of several ethnic groups identified 3,324 polymorphisms in *OPRM1*. The most common single nucleotide polymorphism (SNP) in *OPRM1* is rs1799971 ($Asn^{40}Asp$), referred to as A118G. The A118G polymorphism has an overall frequency of 19% (Crist and Berrettini, 2014; Matsunaga et al., 2009) and is associated with functional effects (Crist and Berrettini, 2014). G allele carriers show behavioral differences in β-endorphin-mediated responses, including analgesia, euphoria, and sedation, due to their 3-fold stronger binding (Bond et al., 1998; Chou et al., 2006; Lötsch et al., 2006; Matsunaga et al., 2009). A prevalence of 31.3% AA, 58.3% AG and 10.4% GG genotypes has been identified (Liu and Wang, 2012).

Investigators observed that serum concentrations of interleukin (IL)-6, tumor necrosis factor (TNF)-α and interferon (IFN)-γ are significantly lower and a quality of life health score is significantly higher in G allele carriers compared to subjects without allele G (Matsunaga et al., 2009). The endogenous opioid system is important in these carriers and may suppress proinflammatory cytokine secretion, influencing their health perception (Matsunaga et al., 2009).

The underpinnings of the descending pain inhibition also involve the central catecholaminergic systems, noradrenaline and dopamine (Jensen et al., 2009). This system is influenced by the catecholamine enzyme, encoded by catechol-O-methyltransferase (*COMT*), which moderates pain signal transmission through removal of catechols (dopamine, epinephrine, and norepinephrine) (Senagore et al., 2017). A highly-studied SNP occurs in the coding region (rs4680G4A or $Val^{158}Met$), and results in three possible genotypes (Met/Met, Met/Val, and Val/Val) (Jensen et al., 2009; Senagore et al., 2017). Reduction of *COMT* activity is related to increased pain sensitivity and the production of proinflammatory cytokines (Senagore et al., 2017). A recent systematic review

showed that genetic polymorphisms of the catecholaminergic pathways are associated with thermal and blunt pressure sensitivity (SOARES et al., 2020). Finally, the conditioned pain modulation (CPM) paradigm is a valid and reliable psychophysical approach to evaluate the efficacy of the descending pain inhibition (Costa et al., 2017; Yarnitsky, 2015).

The lower third molar extraction model evaluates a drug's efficacy on acute postoperative pain and inflammation, according to soft tissue and bone trauma during surgery (Akbulut et al., 2014; Degirmenci and Yalcin, 2019; Silva de Oliveira et al., 2016).

A drug class that controls inflammation is the non-steroidal anti-inflammatory drugs (NSAIDs) (Köseoğlu et al., 2008; Michael Hill et al., 2006; Young et al., 2013), mediated by cyclooxygenase (COX) inhibition. Their isoforms, COX-1 and COX-2, play important roles in inflammation, pain, and fever via prostaglandins (PGs) and thromboxane production (Vane, 1971). Ibuprofen is a COX-1 and -2 inhibitor, and is effective in the control of acute postoperative pain, and inflammation.

That being said, the primary aim of this study was to evaluate the predictive value of *OPRM1* and *COMT* polymorphisms and preoperative proinflammatory cytokines on postoperative pain intensity, swelling and trismus in individuals who underwent lower third molar extraction and undergoing ibuprofen 600 mg every 8 hours for 4 days. We hypothesized that these variables would be associated with either postoperative pain, swelling or trismus after controlling the possible effect of clinical, perioperative and pain catastrophizing variables.

Methods

Registration and Study Design

This study was performed in accordance with the Declaration of Helsinki and since this project involved human genetics, this study was evaluated and approved by the Institutional Ethics Committee of the Bauru School of Dentistry, University of São Paulo, and by the National Commission of Ethics Research (CONEP), Brazil National Research Ethics System (CAAE number: 59807716.9.0000.5417), in accordance with resolution 466/12 of the National Council of Health/Ministry of Health, and registered with ClinicalTrials.gov ID (NCT03169127). All volunteers completed an Informed Consent form during screening prior to carrying out any study procedures. The sample size comprised 200 volunteers, and was determined based on previous studies (Gregorio et al., 2008).

Briefly, 210 adults (≥18 years old) were selected to participate in this study of unilateral lower third molar extraction after analysis of their panoramic radiograph (Simoneti et al., 2018; Weckwerth et al., 2017), according to dental indications, such as orthodontic, endodontic and periodontic problems. All volunteers should

have a similar lower third molar position, according to the Pell and Gregory's classification, to provide similar tissue trauma during surgery. Additionally, per the inclusion criteria, volunteers could not have systemic diseases, which could interfere in the study, and the extraction sites could not have inflammation or infection.

Volunteers were excluded from the study if they presented a history of allergy to local anesthetics, kidney disease, history of bleeding or gastrointestinal ulcers, asthma, hepatic, kidney, intestinal, cardiac, pulmonary, circulatory and/or brain dysfunction or allergic sensitivity to any NSAIDs. Volunteers that were pregnant or breast-feeding, and individuals that used antidepressants, anticoagulants, diuretics and/or antibiotics within two months before surgery were also excluded (Calvo et al., 2017; Santos et al., 2007; Zupelari-Goncalves et al., 2017). Volunteers who had very traumatic surgeries and who needed to use higher doses of local anesthetics and NSAIDs, or who had adverse drug reactions, were also excluded from the study (Senes et al., 2015).

Surgery Intervention and Assessments

At the end of this study a total of 200 extracted lower third molars from 200 volunteers, were analyzed. Initially, 210 volunteers with the dental indication to remove at least one lower third molar were screened for participation, after completing all inclusion criteria. Of these 210 volunteers, ten were excluded from the study. Two dropped out of the study and the other eight volunteers experienced very traumatic surgeries, and required increased NSAID dosage. Of the eight excluded volunteers, two experienced postoperative infection on the extraction site and received antibiotic therapy.

All costs of this research were funded by the São Paulo Research Foundation (FAPESP). Dental surgeries were performed from December 2016 to July 2018 at the Clinical Pharmacology and Physiology Laboratory (LAFFIC) at the Bauru School of Dentistry (FOB), University of São Paulo (USP), Bauru, SP, Brazil, by the same dental surgeon (GMW), using a worldwide standardized (Calvo et al., 2012; Trindade et al., 2011; Weckwerth et al., 2016; Zupelari-Goncalves et al., 2017), in which the amount of anesthetic used, the duration and the surgical trauma of the operated volunteers was standardized (data not shown). The side to be operated (right or left) was determined randomly (http://www.randomization.com, number: 20434). Surgical parameters evaluated during and after the surgeries analyzed in this study are described in Table 1.

One tablet of 600 mg ibuprofen was consumed by volunteers every 8 h for 4 days; the first tablet was taken immediately after the procedure. Rescue medication (750 mg acetaminophen tablets) was also provided; volunteers could consume this medication to supplement the ibuprofen effect every 8 h, if they fell the necessity.

Evaluation of postoperative pain was determined using a visual analogue scale (VAS, 0 to 100 mm) with 0 mm indicating no pain and 100 mm indicating the worst possible pain, as previously described (Simoneti et al., 2018; Trindade et al., 2012). Volunteers recorded their postoperative pain using the VAS at the following time points after the surgical procedure: 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 5, 6, 7, 8, 10, 12, 18, 24, 48, 72 and 96 h (0 =

immediately after the surgery). Volunteers also received a VAS form to record postoperative pain experienced when they used the rescue medication. The principal investigator (GMW) evaluated the swelling, trismus and temperature in pre, intra and postoperative periods.

Using the method proposed by Üstün and colleagues (2003), three facial measures (A, B and C) were obtained with flexible measuring tape. Preoperative sum of the three measurements was considered baseline value. Differences obtained between baseline and postoperative values indicated facial swelling on the 2nd and 7th postoperative days (Simoneti et al., 2018; Weckwerth et al., 2016; Zupelari-Goncalves et al., 2017).

Quantity of mouth opening (trismus), also obtained with a flexible tape, was considered by measuring the distance (in mm) between the edges of incisors during mouth opening before, on 2nd and 7th postoperative days (Simoneti et al., 2018; Weckwerth et al., 2016; Zupelari-Goncalves et al., 2017).

Genetic Sequencing and Analyses

Briefly, the saliva of these 200 volunteers were previously collected and processed at the LAFFIC, genomic DNA was extracted using the QIAamp DNA Mini Kit (Cat No./ID: 51306, QIAGEN® Hilden, Germany) and lyophilized with the FreeZone 4.5 Liter Benchtop Freeze Dry System (Catalog #: 7750020, Labconco®, Kansas City, MO, United States). Lyophilized DNA was utilized for genetic sequencing of *OPRM1* rs1799971 and *COMT* rs4680 using MiSeq® System (Illumina®, San Diego, CA, United States) instruments with a 2 × 78 bp read length, and the protocol of Kailos Genetics Inc. (Huntsville, AL, United States).

MILLIPLEX® Enzyme-Linked Immunosorbent assay (ELISA) Detection of Proinflammatory Cytokines in Saliva

The saliva of these 200 volunteers were previously processed at LAFFIC, and the MILLIPLEX® ELISA was used to detect the presence of four proinflammatory cytokines (IL-2, IL-6, IFN-γ and TNF-α) using the HCYTOMAG-60K MILLIPLEX ELISA kit (Milliplex MAP Human Cytokine/Chemokine Kit; Millipore, Billerica, MA, United States), according to the manufacturer's protocol.

CPM and PCS

In addition to the possible influence of genetic and tissue biomarkers already presented, the descending pain inhibitory system also impacts the NSAID response and effects. To check the functioning of this group of neurons, the conditioned pain modulation paradigm (CPM) was utilized, in which the concept that pain inhibits pain applies (Costa et al., 2017; Yarnitsky, 2015). Prior to surgery, all volunteers underwent the CPM test. Briefly, the pressure pain threshold (PPT) was utilized as a test stimulus (TS) and the conditioning stimulus (CS) was submersion of the non-dominant hand in a 46 ºC water bath for 1 min. The PPT was measured by means of a 1 cm² flat circular tip algometer with constant and increasing pressure application of 0.5 kg/cm²/sec in the anterior temporal muscle region of the dominant side. The TS was measured before and after the CS. The PPT was

calculated by subtracting the absolute values of the TS after the CS, minus the TS before the CS; the relative change was computed as a percentage (Porporatti et al., 2017; Yarnitsky, 2015).

Volunteers also answered a questionnaire that measures catastrophic thoughts in pain, the PCS (Sullivan et al., 1995), that has been translated and validated in Portuguese (Sehn et al., 2012). Before the surgery, volunteers answered 13 questions that indicated the frequency of catastrophic thoughts when they feel strong pain. This frequency scale ranged from 0–5 (0 = almost never and 5 = almost always), and the total score was calculated by summing all items, ranging from 0 to 52 points (Sehn et al., 2012).

Statistical Analysis

Collected data and surgical outcomes

Data were analyzed using Microsoft® Excel 2002 (version 10.6871.6870) and IBM® SPSS® statistics software (version 20.0.0). The description of quantitative data is presented as the mean and standard deviation (SD), while qualitative variables are presented as a percentage. For the inferential analysis, the Shapiro-Wilk test was performed to verify the normality in the distribution of quantitative variables.

Multiple linear regression models were used to determine the main factors associated with the postoperative pain intensity, swelling and trismus. In this sense, the dependent variables analyzed were: pain intensity at times 8, 48, and 96 h after surgery, and swelling and trismus. This study used a time variable, the sum of the differences in pain intensity (SPID). The SPID value is obtained by summing the scores of individual pain intensity differences from the baseline at each assessment time (Cooper et al., 2016). The independent variables were: CPM magnitude, PCS, surgery difficulty and duration, IL-2, IL-6, INF-γ, and TNF-α concentrations, body mass index (BMI), and the genetic profile of *OPRM1* and *COMT* haplotypes. The SPID test was used in pain analyses, and an ANOVA was used to verify the relevance of the SPID test. Statistical significance was set at 0.05.

Results

A total of 200 volunteers (200 molars) were studied, of which 133 (66.5%) were from female volunteers and 67 (33.5 %) were from male volunteers (Table 2). The mean age (SD) of the volunteers was 24 years, with an age range of 18–57 years. BMI was calculated by the division of kilograms by squared height of each volunteer; the mean of all volunteers was 25.17 kg/m^2 (5.57 SD) (Table 2).

According to the CPM test, the magnitude of the preoperative CPM was -67,97 % (SD). The average frequency of catastrophic thoughts was 21.31 points in the 200 volunteers, after answering the questions from the PCS (Table 2).

Genetic sequencing results related to *COMT* and *OPRM1* haplotypes, obtained using the MiSeq® Illumina® instrument, are described in Table 3. For the *OPRM1* haplotypes, 69.5% of volunteers were A/A, characterized as a normal metabolizer, 25.5% were identified as A/G, reduced metabolizers, and 2.5% were G/G, characterized as poor metabolizers. For the *COMT* haplotypes, 31% of volunteers were VAL/VAL, characterized as normal metabolizers, 38% were VAL/MET, reduced metabolizers, and 16% were identified as MET/MET, poor metabolizers. During genetic sequencing, the *OPRM1* and *COMT* haplotypes of 2.5% and 15% of this population could not be determined, respectively (Table 3).

In the multiple linear regressions performed with all studied variables, the *OPRM1* and *COMT* haplotypes were divided in two groups of Mutated and Ancestral, thus patients that were considered reduced and poor metabolizers were grouped and analyzed as Mutated, and the normal metabolizers were analyzed as Ancestral (Table 2).

The SPID test was performed to evaluate what type of variable influenced the fluctuation of reported postoperative pain scores in the VAS during all analyzed periods. A multiple linear regression was used to predict SPID from the concentration of IL-2, IL-6, IFN-γ and TNF-α, the *OPRM1* and *COMT* haplotypes, BMI, CPM, PCS, and surgery duration and difficulty. The multiple regression model was significant ($p = 0.2617$) with an R^2 of = 0.08072. Two predictors contributed significantly to this result, IL-2 ($p = 0.046$) and duration of surgery ($p = 0.0157$) (Table S1). Likewise, considering pain at the period of 8 hours after surgery as the dependent variable and using the same eleven independent variables, there were three variables that added significantly to the prediction ($p = 0.1736$): the presence of IFN-γ ($p = 0.0248$), IL-2 ($p = 0.0176$) and the duration of surgery ($p = 0.0228$) (R^2= 0.09092). Regression coefficients and standard errors are found in Table S2.

Another multiple linear regression was used to predict the pain at 48 h after surgery, using the same independent variables ($p = 0.1597$), and again, the predictors IL-2 ($p = 0.0107$) and duration of surgery ($p = 0.0142$) influenced the pain fluctuation in the VAS ($R^2 = 0.09223$) (Table S3). Using the same model, at 96 h after the procedure, just duration of surgery ($p = 0.007$) was a predictor of pain fluctuation, with $R^2 = 0.09198$, and a significance of ($p = 0.1615$) (Table S4).

This regression model also evaluated what variables influenced the quantity of swelling on the 2nd postoperative day, and between all variables analyzed (IL-2, IL-6, INF-γ, and TNF-α, the *OPRM1* and *COMT* haplotypes, BMI, CPM, PCS, and surgery duration and difficulty), only the model containing BMI was a predictor for swelling on the 2nd postoperative day ($p = 0.0177$, $R^2 = 0.0855$) (Table S5). An ANOVA test confirmed this.

One last linear regression analysis was performed to evaluate the influence of these variables on the presence of trismus (related to the mouth opening) on the 2nd postoperative day. When all variables were analyzed, the presence of the *COMT* haplotype ($p = 0.0119$) and BMI ($p = 0.029$) predicted the presence of trismus ($p = 0.0385$, $R^2 = 0.1202$); an ANOVA test confirmed the relevance of this model (Table S6).

The overall surgery experience reported by volunteers on the 7th postoperative day, after postoperative control of inflammation symptoms mediated by 600 mg ibuprofen, demonstrated that 93.50% of the researched patients determined, in their Global evaluation, that ibuprofen was "excellent", "very good" and "good" and only 6.50% of then classified treatment with ibuprofen as "fair" and "poor" (Figure 1).

Discussion

In this study multiple linear regression was performed using data from 200 patients who underwent a dental extraction with ibuprofen therapy, to evaluate if the fluctuation of postoperative pain (determined by the VAS) and the quantity of swelling and trismus on the 2nd postoperative day was influenced or predicted by the following variables: concentration of IL-2, IL-6, IFN-γ, and TNF-α, *OPRM1* and *COMT* haplotypes, BMI, CPM, PCS, surgery duration, and difficulty. The main findings were: 1) The concentration of two proinflammatory cytokines IFN-γ and IL-2, and the duration of surgery were the principal predictors that influenced the fluctuation of postoperative pain in the VAS and in the SPID test, at 8, 48, and 96 h. 2) BMI influenced both the presence of swelling and trismus on the 2nd postoperative day. 3) The *COMT* haplotype also influenced the presence of trismus on the 2nd postoperative day. 4) A positive global evaluation of ibuprofen effectiveness was reported by patients after surgery.

This study is corroborated by the hypothesis that people with polymorphisms in *OPRM1* and *COMT* have differences in the levels of perception and modulation of pain, since people with these polymorphisms exhibit different affinities for the binding sites of their receptors, which determines different analgesic capacities (BARTOŠOVÁ et al., 2015), according to the endogenous ligand that is coupled to these receptors. Therefore, people with these polymorphisms may have different perceptions of pain when treated with opioids. The most common SNP studied in *OPRM1* is rs1799971, referred to as A118G (Crist and Berrettini, 2014; Matsunaga et al., 2009). When treated with opioids, or rescue medication, individuals with the G allele have more pain symptoms than individuals without the G allele, and thus have increased adverse effects, such as those demonstrated in studies evaluating orthopedic surgeries (Bartošová et al., 2015; Bond et al., 1998). In our study, the frequency of *OPRM1* haplotypes identified was similar to the ratio reported previously, where, among the 200 volunteers, 69.5% were A/A, 25.5% were A/G and 2.5% were G/G (Kopecky, 2019; Liu and Wang, 2012). However, in this study, we did not observe a relationship in the effectiveness of a NSAID with the rs1799971 genotype and control of acute postoperative pain by the VAS. These results contrast with other studies that found a relationship among *OPRM1* polymorphisms, mainly in carriers of the variant 118G allele, and postoperative pain after opioids treatment

(Bartošová et al., 2015), because these patients required higher opioid doses for pain relief (Zhang et al., 2005), (Chou et al., 2006).

The presence of the proinflammatory cytokines IFN-γ and IL-2, and the duration of surgery were important predictors for the fluctuation of postoperative pain in the VAS and the SPID test at 8, 48, and 96 h after surgery (Table S2). The endogenous opioid peptide β-endorphin is known to regulate secretion of proinflammatory cytokines from peripheral immune cells through mechanisms dependent on the μ-opioid receptor, including IL-2, IL-6, TNF-α, and IFN-γ. Matsunaga and collaborators find a strong relationship between decreased concentrations of cytokines and a higher quality of life assessed in G allele carriers compared to individuals without the G allele (Matsunaga et al., 2009). Thus, it is expected that G allele carriers have a different amount of circulating cytokines, which influences their perception and postoperative pain modulation (Matsunaga et al., 2009). In the present study, it was observed that individuals with increased salivary concentration of IL-2 and IFN-γ demonstrated increased pain fluctuation in the SPID test, 8 and 48 h after surgery than individuals with decreased concentrations. Thus, we infer that the concentration of IL-2 and IFN-γ influenced the fluctuation of acute postoperative pain in this Brazilian population.

The duration of surgery was a significant predictor in the multiple linear regression analysis and influenced the fluctuation of postoperative pain in the SPID test, at 8, 48, and 96 h after surgery (Tables S1, S2, S3 and S4). An important finding of this research was that the duration of surgery probably affected tissue trauma, suggesting that a longer time of tissue manipulation generates increased tissue trauma (Troullos et al., 1990), (Graziani et al., 2006), which influences the quantity of swelling and inflammation after surgery. Consequently, there were increased levels of inflammatory infiltrates containing proinflamatory cytokines around the manipulated area after surgery, which presents a potential for pain that influences the amount of postoperative pain. The duration of surgery is strongly correlated with tooth position, the degree of surgical trauma, the need for removing bone tissue, complications in the transoperative period, the extension of the flap, and periosteum displacement around the surgical site, which influences postoperative pain (Antunes et al., 2011).

Another predictor that influenced both swelling and trismus on the 2nd postoperative day in the present study was BMI (Tables S5 and S6). Volunteers with lower BMI showed increased mouth opening limitation (trismus) while swelling was greater in volunteers with higher BMI. This index is measured by the correlation between weight and height that determines body fat, and has been amply described in previously studies (Pérez-González et al., 2018; de Santana-Santos et al., 2013). Pérez-González and collaborators found in their research that BMI and other predictors (gender, relation to lingual and buccal walls, and age) are determinants in

explaining swelling; specifically, BMI has a low influence on swelling in their patients, and trismus is not influenced by these predictors (Pérez-González et al., 2018).

The catechol-O-methyltransferase encoded by *COMT* is another important and well-studied enzyme related to transmission of pain signals in the body (Liu and Wang, 2012; Senagore et al., 2017), and is correlated with variations of pain sensitivity in experimental models of noxious stimuli (Kim et al., 2006; Lee et al., 2011). The most studied SNP is rs4680G4A, recognized as $Val^{158}Met$ (Kim et al., 2006; Liu and Wang, 2012; Senagore et al., 2017). Increased pain sensitivity has been correlated with the production of proinflammatory cytokines in patients with *COMT* haplotypes with reduced enzymatic activity (Senagore et al., 2017). In our 200 patients, the prevalence was 31% VAL/VAL (AA), 38% VAL/MET (AG) and 16% MET/MET (GG), similar to that found in the literature (Liu and Wang, 2012).

We did not find an association between *COMT* SNP rs4680 and acute postoperative pain after third molar extraction, as observed in another survey, albeit with a weak relationship (Kim et al., 2006). An important finding of the present study was that the *COMT* mutated allele (AG and GG genotypes) influenced trismus on the 2nd postoperative day (Table S6). Patients with these genotypes showed greater mouth opening limitation (trismus) on the 2nd postoperative day compared to that observed before surgery.

A limitation of the study is that most studies discuss chronic and acute pain, including osteoarthritis of the knee (Martire et al., 2016), laparoscopic cholecystectomy pain (Jensen et al., 2005), and surgeries, such as total knee arthroplasty and others (Chou et al., 2006; Senagore et al., 2017) In addition, patients received opioids, such as morphine, piritramide, and fentanyl (Bartošová et al., 2015; Chou et al., 2006; Zhang et al., 2005). In our model, we evaluated patients after lower third molar extraction under NSAID therapy. In the present study, postoperative cytokine concentrations were not evaluated and there was no control group that did not use NSAIDs but only analgesics.

Conclusion

Polymorphisms in the *COMT* gene, the concentration of saliva proinflammatory cytokines IL-2 and IFN-γ, BMI, and the duration of surgery were predictors for the fluctuation of pain, and the presence of swelling and trismus on the 2nd postoperative day. Therefore, these results confirmed that 600 mg ibuprofen every 8 h for 4 days was effective in controlling inflammatory symptomatology after lower third molar surgery in these 200 Brazilian patients, without relevant adverse reactions; additionally, the medication was well tolerated, in general, by patients.

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Author contributions

G. M. Weckwerth performed all surgeries, all laboratory experiments, genetic sequencing, and analyzed the data. T. J. Dionísio and Y. M. Costa provided and analyzed the primary data. P. Zupelari-Gonçalves, G. M. Oliveira and E. A. Torres were essential in the execution of all surgeries and data collection during patient treatment and provided primary data. T. Moore and D. M. Absher developed and supervised all genetic sequencing performed at Kailos Genetics Inc., in association with HudsonAlpha Institute for Biotechnology. A.M. Calvo, L. R. Bonjardim, F.A.C. Faria and C. F. Santos contributed to the study design, supervised experiments, reviewed, and edited the manuscript. All authors approved the final version of the article, including the authorship list, discussed the results and commented on the manuscript.

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Figure Legends

Figure 1. Global evaluation of patient surgery experience.

Table Legends

Table 1 Evaluated study parameters.

Table 2 Preoperative, intraoperative and postoperative parameters.

Table 3 Percentage and number of volunteers that presented mutations in the opioid receptor (*OPRM1)* and catechol-O-methyltransferase (*COMT)* haplotypes.

Table S1 Multiple logistic regression model. The sum of the differences in pain intensity (SPID) test (fluctuation of pain) is the dependent variable and interferon (IFN)-γ, interleukin (IL)-2, (IL)-6, tumor necrosis factor (TNF)-α, body mass index (BMI), surgery difficulty and duration, opioid receptor (*OPRM1)* and catechol-O-methyltransferase (*COMT)* haplotype, pain modulation capacity (CPM), and pain catastrophizing scale (PCS) are independent variables.

Table S2 Multiple logistic regression model. Pain, 8 h after surgery, is the dependent variable and interferon (IFN)-γ, interleukin (IL)-2, (IL)-6, tumor necrosis factor (TNF)-α, body mass index (BMI), surgery difficulty and duration, opioid receptor (*OPRM1)* and catechol-O-methyltransferase (*COMT)* haplotype, pain modulation capacity (CPM), and pain catastrophizing scale (PCS) are independent variables.

Table S3 Multiple logistic regression model. Pain, 48 h after surgery, is the dependent variable and interferon (IFN)-γ, interleukin (IL)-2, (IL)-6, tumor necrosis factor (TNF)-α, body mass index (BMI), surgery difficulty and duration, opioid receptor (*OPRM1)* and catechol-O-methyltransferase (*COMT)* haplotype, pain modulation capacity (CPM), and pain catastrophizing scale (PCS) are independent variables.

Table S4 Multiple logistic regression model. Pain, 96 h after surgery, is the dependent variable and interferon (IFN)-γ, interleukin (IL)-2, (IL)-6, tumor necrosis factor (TNF)-α, body mass index (BMI), surgery difficulty and duration, opioid receptor (*OPRM1)* and catechol-O-methyltransferase (*COMT)* haplotype, pain modulation capacity (CPM), and pain catastrophizing scale (PCS) are independent variables.

Table S5 Multiple logistic regression model. Trismus on the 2nd day after surgery is the dependent variable and interferon (IFN)-γ, interleukin (IL)-2, (IL)-6, tumor necrosis factor (TNF)-α, body mass index (BMI), surgery difficulty and duration, opioid receptor (*OPRM1)* and catechol-O-methyltransferase (*COMT)* haplotype, pain modulation capacity (CPM), and pain catastrophizing scale (PCS) are independent variables.

Table S6 Multiple logistic regression model. Swelling on the 2nd day after surgery is the dependent variable and interferon (IFN)-γ, interleukin (IL)-2, (IL)-6, tumor necrosis factor (TNF)-α, body mass index (BMI), surgery difficulty and duration, opioid receptor (*OPRM1)* and catechol-O-methyltransferase (*COMT)* haplotype, pain modulation capacity (CPM), and pain catastrophizing scale (PCS) are independent variables.

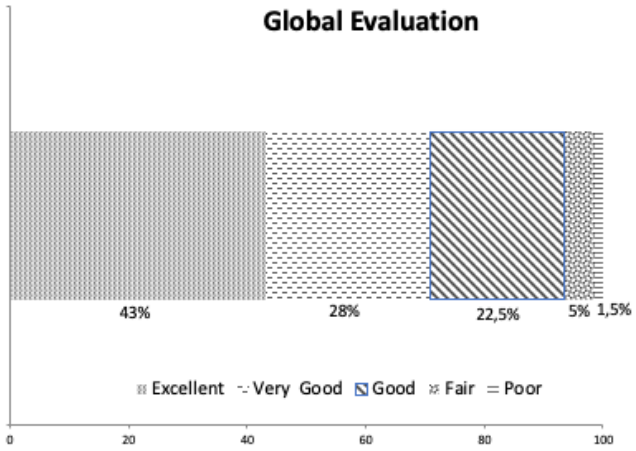

Figure 1. Global evaluation of patient surgery experience.

Table 1 - Evaluated study parameters

Parameter	Unit
Gender	N and %
Age	(mean years. SD)
BMI	Kg/Height^2
OPRM1 Haplotypes	Haplotype mutated or ancestral
COMT Haplotypes	Haplotype mutated or ancestral
Conditioned Pain Modulation (CPM)	Pressure Pain Threshold (PPT) - %
Pain Catastrophizing Scale (PCD)	Range from 0-52 points
Surgery difficulty - (score assessed by surgeon)	3-point scale: 1) no need for osteotomies without tooth sectioning; 2) need for osteotomies without tooth sectioning; 3) need for osteotomies and tooth sectioning complicated
Mouth opening	Preoperative period, 2nd, 7th postoperative day (mm)
Facial swelling	Preoperative period, 2nd, 7th postoperative day (mm)
Subjective evaluation of postoperative pain	Visual analog scale (VAS, 0 to 100 mm)
Overall experience of surgery reported by volunteer in the 7th postoperative day	5-point scale: 1) “poor”; 2) “fair”; 3) “good”; 4) “very good”; 5) “excellent”

Table 2 - Preoperative, intraoperative and postoperative parameters

Parameters Evaluated	**All (n = 200)**	
Parameter (n, %)	**n**	**%**
Male (n, %)	67	33.5
Female (n, %)	133	66.5
Parameter (mean, SD)	**mean**	**SD**
Age (mean years. SD)	24	6.5
BMI (mean Kg/m^2. SD)	25.17	5.57
Gene Haplotypes (n)	**Ancestral**	**Mutated**
OPRM1 (5 undetermined)	139	56
COMT (30 undetermined)	62	108
Modulation of pain tests (mean, SD)	**mean**	**SD**
Conditioned Pain Modulation (CPM) (mean CPM. SD)	-67.97	45.3
Pain Catastrophizing Scale (PCD) (mean points.SD)	21.31	11.41
Surgery difficulty - (score assessed by surgeon) (n, %)	**n**	**%**
1) no need for osteotomies without tooth sectioning;	5	2.5
2) need for osteotomies without tooth sectioning;	101	50.5
3) need for osteotomies and tooth sectioning complicated	94	47.0
Mouth Opening (median mm. IQR)	**median**	**IQR**
preoperative measurement	50.0	15.0
2nd day postoperative measurement	25.0	10.0
7nd day postoperative measurement	34.0	15.0
Facial Swelling (mean mm. SD)	**mean**	**SD**
preoperative measurement	370.0	30.0
2nd day postoperative measurement	387.0	30.0
7nd day postoperative measurement	380.0	30.0

Table 3 - Percentage and number of volunteers that presented mutations in the opioid receptor (OPRM1) and catechol-O-methyltransferase (COMT) haplotypes.

OPRM1 Haplotypes	**% (n)**	**Type of Metabolizer**	**COMT Haplotypes**	**% (n)**	**Type of Metabolizer**
A/A	69.5% (139)	Normal	**VAL/VAL**	31% (62)	Normal
A/G	25.5% (51)	Reduced	**VAL/MET**	38% (76)	Reduced
G/G	2.5% (5)	Poor	**MET/MET**	16% (32)	Poor
INDETERMINED	2.5% (5)	-	**INDETERMINED**	15% (30)	-

Supplementary Material

Table S1 - Multiple logistic regression model. The sum of the differences in pain intensity (SPID) test (fluctuation of pain) is the dependent variable and interferon (IFN)-γ, interleukin (IL)-2, (IL)-6, tumor necrosis factor (TNF)-α, body mass index (BMI), surgery difficulty and duration, opioid receptor (*OPRM1)* and catechol-O-methyltransferase (*COMT)* haplotype, pain modulation capacity (CPM), and pain catastrophizing scale (PCS) are independent variables.

	SPID			
Variable	**β**	**S. E**	**P value**	**β - 95% CI**
Intercept	-70	66.83	0.2965	-202.0 to 62.00
IFN-γ	41.53	21.51	0.0553	-0.9544 to 84.01
IL-2	-105.7	52.52	**0.046**	-209.4 to -1.922
IL-6	-6.007	3.774	0.1135	-13.46 to 1.448
TNF-α	3.01	2.931	0.3061	-2.780 to 8.799
BMI	-0.07904	1.716	0.9633	-3.469 to 3.311
Surg. Difficult	37.94	22.28	0.0907	-6.080 to 81.96
Surg. Duration	-3.798	1.555	**0.0157**	-6.869 to -0.7265
OPMR1	6.886	22.16	0.7564	-36.88 to 50.65
COMT	-23.95	20.46	0.2435	-64.37 to 16.46
CPM	-3.459	20.61	0.8669	-44.16 to 37.25
PCS	0.7847	0.8617	0.3639	-0.9173 to 2.487

Sum of the differences in pain intensity (SPID),interferon (IFN)-γ, interleukin (IL)-2, (IL)-6, tumor necrosis factor (TNF)-α, body mass index (BMI), opioid receptor (*OPRM1)* and catechol-O-methyltransferase (*COMT)* haplotype, pain modulation capacity (CPM), pain catastrophizing scale (PCS), standard errors (S.E), confidence interval (CI).

Table S2 - Multiple logistic regression model. Pain, 8 h after surgery, is the dependent variable and interferon (IFN)-γ, interleukin (IL)-2, (IL)-6, tumor necrosis factor (TNF)-α, body mass index (BMI), surgery difficulty and duration, opioid receptor (*OPRM1)* and catechol-O-methyltransferase (*COMT)* haplotype, pain modulation capacity (CPM), and pain catastrophizing scale (PCS) are independent variables.

	Pain after 8h			
Variable	**β**	**S. E**	**P value**	**β - 95% CI**
Intercept	10.65	6.861	0.1225	-2.899 to 24.21
IFN-γ	-4.998	2.206	**0.0248**	-9.356 to -0.6406
IL-2	12.92	5.385	**0.0176**	2.282 to 23.56
IL-6	0.5392	0.3871	0.1656	-0.2254 to 1.304
TNF-α	-0.2003	0.3005	0.506	-0.7940 to 0.3933
BMI	-0.2537	0.1762	0.152	-0.6017 to 0.09440
Surg. Difficult	-2.196	2.289	0.3387	-6.717 to 2.324
Surg. Duration	0.3667	0.1594	**0.0228**	0.05181 to 0.6816
OPMR1	1.52	2.276	0.5052	-2.976 to 6.016
COMT	2.674	2.099	0.2047	-1.473 to 6.821
CPM	-0.9902	2.113	0.64	-5.164 to 3.183
PCS	-0.02906	0.08877	0.7439	-0.2044 to 0.1463

Sum of the differences in pain intensity (SPID),interferon (IFN)-γ, interleukin (IL)-2, (IL)-6, tumor necrosis factor (TNF)-α, body mass index (BMI), opioid receptor (*OPRM1)* and catechol-O-methyltransferase (*COMT)* haplotype, pain modulation capacity (CPM), pain catastrophizing scale (PCS), standard errors (S.E), confidence interval (CI).

Table S3- Multiple logistic regression model. Pain, 48 h after surgery, is the dependent variable and interferon (IFN)-γ, interleukin (IL)-2, (IL)-6, tumor necrosis factor (TNF)-α, body mass index (BMI), surgery difficulty and duration, opioid receptor (*OPRM1)* and catechol-O-methyltransferase (*COMT)* haplotype, pain modulation capacity (CPM), and pain catastrophizing scale (PCS) are independent variables

	Pain after 48h			
Variable	**β**	**S. E**	**P value**	**β - 95% CI**
Intercept	2.211	5.431	0.6845	-8.516 to 12.94
IFN-γ	-3.289	1.748	0.0617	-6.741 to 0.1627
IL-2	11.03	4.267	**0.0107**	2.597 to 19.46
IL-6	0.1277	0.3067	0.6777	-0.4781 to 0.7335
TNF-α	-0.02416	0.2382	0.9193	-0.4946 to 0.4463
BMI	-0.04694	0.1395	0.7369	-0.3224 to 0.2285
Surg. Difficult	-1.385	1.811	0.4457	-4.962 to 2.192
Surg. Duration	0.3133	0.1263	**0.0142**	0.06369 to 0.5628
OPMR1	-2.34	1.8	0.1956	-5.896 to 1.216
COMT	0.9383	1.663	0.5733	-2.346 to 4.222
CPM	0.9278	1.675	0.5803	-2.380 to 4.235
PCS	-0.01545	0.07002	0.8256	-0.1538 to 0.1229

Sum of the differences in pain intensity (SPID),interferon (IFN)-γ, interleukin (IL)-2, (IL)-6, tumor necrosis factor (TNF)-α, body mass index (BMI), opioid receptor (*OPRM1)* and catechol-O-methyltransferase (*COMT)* haplotype, pain modulation capacity (CPM), pain catastrophizing scale (PCS), standard errors (S.E), confidence interval (CI).

Table S4 – Multiple logistic regression model. Pain, 96 h after surgery, is the dependent variable and interferon (IFN)-γ, interleukin (IL)-2, (IL)-6, tumor necrosis factor (TNF)-α, body mass index (BMI), surgery difficulty and duration, opioid receptor (*OPRM1)* and catechol-O-methyltransferase (*COMT)* haplotype, pain modulation capacity (CPM), and pain catastrophizing scale (PCS) are independent variables

	Pain after 96 h			
Variable	**β**	**S. E**	**P value**	**β - 95% CI**
Intercept	-0.1211	5.467	0.9824	-10.92 to 10.68
IFN-γ	-2.746	1.759	0.1206	-6.222 to 0.7292
IL-2	7.048	4.296	0.1029	-1.439 to 15.53
IL-6	0.2103	0.3088	0.4968	-0.3996 to 0.8202
TNF-α	-0.06569	0.2398	0.7845	-0.5394 to 0.4080
BMI	0.02317	0.1404	0.8691	-0.2542 to 0.3005
Surg. Difficult	-1.835	1.823	0.3158	-5.436 to 1.767
Surg. Duration	0.3476	0.1272	**0.007**	0.09633 to 0.5988
OPMR1	-3.405	1.813	0.0622	-6.985 to 0.1758
COMT	2.153	1.674	0.2003	-1.153 to 5.459
CPM	-0.4819	1.686	0.7754	-3.812 to 2.848
PCS	0.0408	0.07049	0.5636	-0.09844 to 0.1800

Sum of the differences in pain intensity (SPID),interferon (IFN)-γ, interleukin (IL)-2, (IL)-6, tumor necrosis factor (TNF)-α, body mass index (BMI), opioid receptor (*OPRM1)* and catechol-O-methyltransferase (*COMT)* haplotype, pain modulation capacity (CPM), pain catastrophizing scale (PCS), standard errors (S.E), confidence interval (CI).

Table S5 – Multiple logistic regression model. Trismus on the 2nd day after surgery is the dependent variable and interferon (IFN)-γ, interleukin (IL)-2, (IL)-6, tumor necrosis factor (TNF)-α, body mass index (BMI), surgery difficulty and duration, opioid receptor (*OPRM1)* and catechol-O-methyltransferase (*COMT)* haplotype, pain modulation capacity (CPM), and pain catastrophizing scale (PCS) are independent variables

	Trismus			
Variable	**β**	**S. E**	**P value**	**β - 95% CI**
Intercept	-23.23	4.631	<0.0001	-32.38 to -14.08
IFN-γ	0.3566	1.49	0.8112	-2.587 to 3.300
IL-2	-2.438	3.639	0.5039	-9.627 to 4.751
IL-6	-0.1072	0.2615	0.6824	-0.6238 to 0.4094
TNF-α	0.1867	0.2031	0.3594	-0.2145 to 0.5879
BMI	0.2621	0.1189	**0.029**	0.02717 to 0.4970
Surg. Difficult	0.1575	1.544	0.9189	-2.893 to 3.208
Surg. Duration	-0.08231	0.1077	0.4461	-0.2951 to 0.1305
OPMR1	-1.148	1.535	0.4559	-4.180 to 1.885
COMT	-3.606	1.418	**0.0119**	-6.407 to -0.8058
CPM	2.67	1.428	0.0634	-0.1511 to 5.490
PCS	-0.09572	0.05971	0.1109	-0.2137 to 0.02223

Sum of the differences in pain intensity (SPID),interferon (IFN)-γ, interleukin (IL)-2, (IL)-6, tumor necrosis factor (TNF)-α, body mass index (BMI), opioid receptor (*OPRM1)* and catechol-O-methyltransferase (*COMT)* haplotype, pain modulation capacity (CPM), pain catastrophizing scale (PCS), standard errors (S.E), confidence interval (CI).

Table S6 – Multiple logistic regression model. Swelling on the 2nd day after surgery is the dependent variable and interferon (IFN)-γ, interleukin (IL)-2, (IL)-6, tumor necrosis factor (TNF)-α, body mass index (BMI), surgery difficulty and duration, opioid receptor (*OPRM1)* and catechol-O-methyltransferase (*COMT)* haplotype, pain modulation capacity (CPM), and pain catastrophizing scale (PCS) are independent variables

	Swelling			
Variable	**β**	**S. E**	**P value**	**β - 95% CI**
Intercept	-32.47	16.26	0.0476	-64.58 to -0.3508
IFN-γ	-4.421	5.232	0.3994	-14.76 to 5.914
IL-2	11.36	12.78	0.3755	-13.88 to 36.59
IL-6	0.4319	0.9182	0.6388	-1.382 to 2.246
TNF-α	-0.2114	0.7131	0.7673	-1.620 to 1.197
BMI	1.001	0.4175	**0.0177**	0.1765 to 1.826
Surg. Difficult	4.965	5.422	0.3612	-5.744 to 15.67
Surg. Duration	0.1861	0.3783	0.6234	-0.5611 to 0.9333
OPMR1	-2.55	5.39	0.6368	-13.20 to 8.097
COMT	4.411	4.978	0.3769	-5.421 to 14.24
CPM	4.915	5.013	0.3284	-4.988 to 14.82
PCS	0.3863	0.2096	0.0673	-0.02778 to 0.8004

Sum of the differences in pain intensity (SPID),interferon (IFN)-γ, interleukin (IL)-2, (IL)-6, tumor necrosis factor (TNF)-α, body mass index (BMI), opioid receptor (*OPRM1)* and catechol-O-methyltransferase (*COMT)* haplotype, pain modulation capacity (CPM), pain catastrophizing scale (PCS), standard errors (S.E), confidence interval (CI).

Discussion

3. DISCUSSION

Discussion of the article: **CYP450 polymorphisms and clinical pharmacogenetics of ibuprofen 600mg after lower third molar extraction**

One lower third molar was extracted from each of 200 volunteers, then postoperative pain was managed with ibuprofen 600 mg every 8 hours for 4 days. Hemodynamic parameters, postoperative pain, swelling, trismus, total amount of rescue medication required, concentration of salivary pro-inflammatory cytokines and their genetic profiles, were analyzed after gene sequencing using the Illumina® MiSeq® System (Illumina Inc., San Diego, CA, USA)

A multiple linear regression model included SNP of *CYP2C8, CYP2C9, CYP1A2, CYP3A4* and *CYP3A5*, concentrations of IL-2, IL-6, IFN-γ and TNF-α, gender, age, difficult surgery, duration of surgery, and Body Mass Index (IBM) as independent variables, and postoperative summed pain intensity difference (SPID), mouth opening and swelling as dependent variables. Reliability (r^2) was 0.97, indicated that polymorphisms in the CYP450 superfamily and interleukin concentrations did not affect postoperative pain, swelling and trismus in patients treated with ibuprofen. Adverse reactions were minimal and did not impact postoperative outcomes.

Ibuprofen is an important NSAID comprising a racemic mixture of (s)-(+)- and (r)-(-)- enantiomers that can be administered in different doses and formulations [20]. The CYP2C8 and CYP2C9 genes mainly metabolize the r-(-)- and s-(+)- enantiomers, respectively, in the human liver (DAVIES et al., 1998; NEUNZIG et al., 2012). The 2C8 and 2C9 enzymes are encoded by polymorphic genes, and about 10% of the Caucasian population has difficulties with fully metabolizing NSAIDs (PREISSNER et al., 2013). Understanding the genetic profiles of these polymorphic

enzymes can help clinicians to predict the metabolism and possible harmful side effects in their patients, rendering personalized prescriptions possible (PREISSNER et al., 2013).

The frequencies of the tested genotypes in this Brazilian population showed that 68.5% and 30.5% carried wild-type *2C8 (*1/*1*) and allelic *2C8* (**1/*2; *1/*3; *1/*4; *2/*3;*2/*4;*3/*3;*3/*4*) variant combinations, respectively, and that 67.5% and 30.5% carried wild-type 2C9, *(*1/*1)* and allelic *2C9 (*1/*2; *1/*3; *2/*2; *2/*3*) variant combinations, respectively (Supplementary Table 1). Others have also identified these genotypes in various populations (GARCÍA-MARTÍN et al., 2004; AGÚNDEZ et al., 2009; VIANNA-JORGE et al., 2004).

Clinical studies are underway to improve the quality of information about the impact of these CYP450 polymorphisms on metabolism, clearance and drug effectiveness. Many authors have emphasized the importance of *CYP2C8*3, CYP2C9*2, and *3* allelic variants because of their relevance to the effectiveness and adverse effects of numerous NSAIDs, such as ibuprofen and diclofenac, antidiabetic drugs, and oral anticoagulants such as warfarin, and to their clearance and efficacy (KRASNIQI et al., 2016; ZHOU et al., 2010). Studying these polymorphisms is important to help physicians understand how medications are metabolized and how this can affect their patients. Thus, optimal patient outcomes can be achieved and information about their effectiveness and safety can be improved, particularly for drugs with a narrow therapeutic index, such as some NSAIDs (SENAGORE et al., 2017; GREGORIO et al., 2008; JENSEN et al., 2005; DEMIRBAS et al., 2019). Although CYP450 polymorphism tests before treatment with some medications are important for clinicians, they are not yet clinically routine. The effectiveness of this strategy in improving treatment requires further investigation (KRASNIQI et al., 2016; ZHOU et

al., 2010). Therefore, one focus of this study is to disseminate this type of data in medical and dental environments to complement individual medical records with information about NSAID metabolism. This should facilitate the establishment of personalized treatments, and therefore safer drug prescriptions, which might attenuate possible adverse effects.

Postoperative pain, swelling and trismus were managed with ibuprofen 600 mg in the present study. The results of the global evaluation showed that 93.5% of patients classified the treatment with ibuprofen as “excellent”, “very good” and “good”, and only 6.5% classified it as “fair” or “poor” (Figure 3). These results showed that ibuprofen controlled acute postoperative pain and inflammatory manifestations in this study population. Ibuprofen/caffeine (400/100 mg) was clinically superior to ibuprofen monotherapy (400 mg) every 8 hours for 5 days in 562 patients with acute postoperative dental pain, thus indicating the effectiveness of caffeine as an analgesic adjuvant. Most patients rated tolerability as “very good” or “excellent” (WEISER et al., 2018). A study of pre-emptive intravenously (i.v.) administered ibuprofen (800 mg) in 75 healthy volunteers resulted in significantly reduced postoperative pain and a decreased requirement for rescue medication during the first 24 hours after lower third molar surgery, compared with the postoperative administration i.v. of ibuprofen 800 mg or a placebo ($P < .05$) (DEMIRBAS et al., 2019).

The present study found that pain levels significantly differed between 0 and 4, 5, 6, 7, 8, 18, 24, 48, 72 and 96 h postoperatively, in patients who were considered normal metabolizers (NM), and between 0 h and 6 and 8 h postoperatively, in those considered intermediate or poor metabolizers (IPM) of the CYP2C8 gene. Pain levels significantly differed between 0 and 4, 5, 6, 7, 8, 18, 24 and 72 h postoperatively in

patients considered NM, and between 0 and 7 and 8 h postoperatively in those considered IPM for the *CYP2C9* gene.

Patients with mutations in cytochrome P450 superfamily genes that are responsible for NSAID metabolism, mainly CYP2C8 and CYPC9, can have some peculiarities associated with NSAID clearance, such as different plasma peak values and modified NSAID effectiveness (VIANNA-JORGE et al., 2004). Therefore, patients who are considered to have mutations of these specific genes can retain NSAIDs longer in their circulation because they are IPM. Therefore, NSAIDs can remain at peak levels for longer periods in plasma. Furthermore, NSAIDs can remain on cell receptors longer, thus variant allele carriers feel less pain than patients considered wild type (GARCÍA-MARTÍN et al., 2004). Whether the *CYP2C8*3 and CYP2C9 *2, *3*, allelic variants could reduce ibuprofen metabolism and/or clearance has been investigated (WU et al., 2013; LÓPEZ-RODRÍGUEZ et al., 2008), as this could predispose carriers of these allelic variants to higher risk of adverse reactions. In this sense CYP genotyping might identify patients at increased risk, and help clinicians adjust the doses or prescribe other NSAIDs (KRASNIQI et al., 2016). GARCÍA-MARTÍN et al. found lower ibuprofen metabolism in carriers of *2C9*2* and **3* and *2C8*3* allelic variants compared with other genotypes, after a single oral dose of a racemic solution of ibuprofen (400 mg). The authors concluded that variant allele carriers maintained S-(+)-ibuprofen plasma levels for much longer, which led to a 40% higher area under the curve (AUC). Pharmacokinetic tests showed that plasma levels of ibuprofen metabolites also differed between variant and wild-type carriers (IBP-OH *vs.* IBP-COOH C_{max}: 1.53 *vs.* 2.71 and 1.66 *vs.* 4.52 mg L^{-1}, respectively) (KARAŹNIEWICZ-ŁADA, et al. 2009). That study also found reduced clearance and a longer half-life of ibuprofen (KARAŹNIEWICZ-ŁADA, et al. 2009).

A study of 47 healthy volunteers given a single oral dose of racemic ibuprofen (600 mg) revealed reduced metabolism in carriers of the *2C9*3* allele variant, resulting in a significantly higher AUC and lower clearance than in carriers of the *2C9*1* allele variant ($p < 0.05$). In terms of safety, the *CYP2C8**3 carriers had fewer adverse events (LÓPEZ-RODRÍGUEZ et al., 2008). Carriers of the *CYP2C8*3 and CYP2C9*2 or CYP2C9*3* variants manifested increased risk of gastrointestinal bleeding after the administration of ibuprofen and diclofenac; but whether the parent drug or its metabolites generated *via* alternative metabolic pathways caused the bleeds could not be determined (AGÚNDEZ et al., 2009). Adverse reactions were minimal in the present study, despite the polymorphisms on specific genes related to drug metabolism, and did not impact patient recovery. Adverse events were infrequent and mostly mild or moderate across treatment groups, and the median time to meaningful pain relief was shorter for a group given ibuprofen/caffeine compared with ibuprofen (1.13 *vs.* 1.78 h; $p = 0.0001$) as in a previous study (WEISER et al., 2018)

One study genotyped CYP2C9 in participants who received various doses and types of NSAIDs that are “extensively” metabolized by CYP2C9, or salicylic acid and acetaminophen, which are NSAIDs but not considered substrates of CYP2C9. The authors concluded that the effects of a combination of a *CYP2C9* allele variant and risk of gastrointestinal bleeding was gene-dose dependent, and high in patients given NSAIDs that are metabolized mainly by CYP2C9. The authors concluded that genotyping CYP2C9 can identify subgroups of individuals who are potentially prone to risk of acute gastrointestinal bleeding (MARTINEZ et al., 2004).

Lower third molar surgery is a prevalent type of clinical trial used to evaluate acute postoperative pain, as the procedure generates moderate (40%) to severe pain (60%) in patients (LUSTENBERGER et al., 2011; ALBUQUERQUE et al., 2017).

Although the present study also found postoperative differences, the level of pain measured on the visual analog scale (VAS) averaged < 20 mm among 200 patients (Figure 2a, b). This value is considered to reflect mild pain (JENSEN et al., 2005). Patients with post-surgical or chronic pain at intensity levels < 44 mm on a 100-mm VAS tend to describe their pain as “mild”, or report that it minimally impacts their daily activities (JENSEN et al., 2005). Therefore, ibuprofen must be considered effective in controlling postoperative pain in patients considered NM or IPM.

The mean number of rescue medications taken by patients in the present study was 2.5 pills of acetaminophen (750 mg), and the first dose was general taken ~6 hours postoperatively (Table 2). A recent study found that the average doses of acetaminophen administered within the first 24 hours after surgery to a group given ibuprofen 60 minutes before, and a placebo after surgery was 640 mg *vs*. 1,240 mg in a group given a placebo before, and ibuprofen 60 minutes after surgery and 1,840 mg in a group given a placebo 60 minutes before and after surgery) ($p < .001$) (DEMIRBAS et al., 2019).

The hypotheses of that study have not been confirmed, since the general genetic profile, concentrations of salivary proinflammatory cytokines (IL-2, IL-6, IFN-γ and TNF-α), and other parameters analyzed after the multiple linear regression model did not influence pain, swelling and trismus. In addition, the surgical outcomes of the patients did not significantly differ. Despite the CYP450 polymorphisms in the *CYP2C8, CYP2C9, CYP1A2, CYP3A4* and *CYP3A5* genes, the concentrations of salivary pro-inflammatory cytokines (IL-2, IL-6, IFN-γ and TNF-α), and surgical outcomes did not correlate. Ibuprofen 600 mg every 8 hours for 4 days effectively controlled inflammatory symptomatology after lower third molar surgery in 200 Brazilian patients, without any major adverse reactions.

3. DISCUSSION

Discussion of the article: **Opioid receptor and catechol-O-methyltransferase polymorphisms associated with patient's endogenous pain modulation when undergoing ibuprofen therapy after dental surgery**

Multiple linear regression was performed in this research to evaluate if the fluctuation of postoperative pain determined by the (VAS) and the quantity of swelling and trismus on the 2nd postoperative day was influenced or predicted by the following analyzed variables: concentration of IL-2, IL-6, IFN-γ, and TNF-α, *OPRM1* and *COMT* haplotypes, BMI, conditioned pain modulation (CPM), pain catastrophizing scale (PCS), surgery duration, and difficulty. This study used data derived from 200 patients who underwent a lower third molar extraction with 600 mg ibuprofen therapy every 8 h for 4 days.

At the end of the study, a total of 200 volunteers were studied through the extraction of 200 lower third molars, of which 133 (66.5%) were from female and 67 (33.5 %) were from male volunteers with a mean age (SD) of 24 years. (Table 2). Gender and age have been reported as a predictive factor for swelling (PÉREZ-GONZÁLEZ et al., 2018; DE SANTANA-SANTOS et al., 2013). According the analysis made in the present study, gender and age did not influenced any parameter evaluated. However, Pérez-González and colaborators reported an important influence of gender and age in facial swelling, in which in their 45 volunteers studied, swelling was higher in males than in females and older volunteers had a decreased swelling (PÉREZ-GONZÁLEZ et al., 2018). In other study of 80 patients, de Santana-

Santos et al. found higher swelling in females volunteers than in males, showing an influence of gender in prediction of swelling (DE SANTANA-SANTOS et al., 2013).

The BMI is an important variable measured by the correlation between weight and height that determines body fat, and has been amply described in previously studies (PÉREZ-GONZÁLEZ et al., 2018; DE SANTANA-SANTOS et al., 2013). In our study, the BMI mean of all volunteers was 25.17 kg/m2 (5.57 SD), like observed on Table 2, and BMI was a predictor that influenced both swelling and trismus on the 2nd postoperative day (Tables S5 and S6). Volunteers with lower BMI showed increased mouth opening limitation (trismus) while swelling was greater in volunteers with higher BMI. Pérez-González and collaborators reported that BMI and other predictors (male gender, relation to lingual and buccal walls, and age) are determinants in explaining swelling; specifically, the BMI has a low influence on swelling in their patients, and trismus was not influenced by these predictors (PÉREZ-GONZÁLEZ et al., 2018). On the other hand, de Santana-Santos et al. did not find an association with BMI and the prediction of swelling in their study (DE SANTANA-SANTOS et al., 2013).

We hypothesized that people with polymorphisms in *OPRM1* and *COMT* may have differences in their levels of pain perception and modulation. We evaluated the influence of these polymorphisms on CPM in a Brazilian population after the literature showed that people with these polymorphisms exhibit different affinities for the binding sites of their receptors when treated with opioids, which determines different analgesic capacities and different perceptions of pain (BARTOŠOVÁ et al., 2015), according to the endogenous ligand that is coupled to these receptors.

OPRM1 is an important gene correlated with pain perception and modulation that is activated by endomorphins and β-endorphins (CRIST; BERRETTINI, 2013); the

most common SNP studied from this gene is rs1799971, referred to as A118G (BOND, et al., 1998; MATSUNAGA et al., 2009; BARTOŠOVÁ et al., 2015). When treated with opioids or rescue medication, individuals with the G allele have increased pain symptoms than individuals without the G allele, and thus have increased adverse effects, such as those demonstrated in studies evaluating acute and chronic pain modulation after surgeries (CHOU et al., 2006; BARTOŠOVÁ et al., 2015; MARTIRE et al., 2015).

In their study, Liu & Wang identified a genotypic *OPRM1* prevalence of 31.3% AA, 58.3% AG and 10.4% GG in a population with adenocarcinoma of the colon or rectum (84 patients) or of the stomach (12 patients) (LIU et al., 2012). In our study, the frequency of *OPRM1* haplotypes identified was similar to the ratio reported previously, where, among the 200 volunteers, 69.5% were A/A, 25.5% were A/G and 2.5% were G/G (TRESCOT et al., 2008; LIU et al., 2012).

Many studies found a relationship among *OPRM1* polymorphisms, mainly in carriers of the variant 118G allele in the SNP rs1799971, and postoperative pain perception after opioid treatment (BARTOŠOVÁ et al., 2015), because these patients required higher opioid doses for pain relief (ZHANG et al., 2005; CHOU et al., 2006; BARTOŠOVÁ et al., 2015). However, in contrast to other studies (ZHANG et al., 2005; CHOU et al., 2006), we did not observe a relationship in the effectiveness of a NSAID, 600 mg ibuprofen every 8 h for 4 days, with the rs1799971 genotype and control of acute postoperative pain, as self-reported by the VAS.

Bartošová and collaborators, observed in their research that OPRM1 118G allele carriers had decreased acute postoperative pain relief after elective inguinal hernioplasty under piritramide treatment, which was associated with increased drug consumption and incidence of adverse effects. In addition, in his study there was no

control for confounding factors as robust as in the present study, which is fundamental, and a small sample size was analyzed in which only differences between groups were evaluated and not the relationship between them (BARTOŠOVÁ et al., 2015).

The endogenous opioid peptide β-endorphin is known to regulate the secretion of proinflammatory cytokines from peripheral immune cells through mechanisms dependent on the μ-opioid receptor, including IL-2, IL-6, TNF-α, and IFN-γ; according to the different activation of the receptors, the presence of analgesia, euphoria, and sedation is expected (BOND et al., 1998; CHOU et al., 2006; LÖTSCH et al., 2006; MATSUNAGA et al., 2009). In this study, three important predictors for the fluctuation of postoperative pain in the VAS and the SPID test, at 8, 48, and 96 h after surgery, were the presence of the proinflammatory cytokines IFN-γ and IL-2, and the duration of surgery. It was observed that individuals with increased salivary concentration of IL-2 and IFN-γ demonstrated increased pain fluctuation in the SPID test, 8 and 48 h after surgery. than individuals with decreased concentrations. Thus, we infer that the concentration of IL-2 and IFN-γ influenced the fluctuation of acute postoperative pain in this Brazilian population.

Matsunaga and collaborators found in their research a strong relationship between a decreased presence of cytokines and a higher quality of life assessed in G allele carriers. This group observed that the serum concentration of IL-6, TNF-α, and IFN-γ were significantly lower and an overall health score, on quality of life assessed by the patients, was significantly higher in the G allele carriers, who had a strong β-endorphin binding to the G-opioid receptor compared to subjects without allele G. Additionally, the correlation analysis indicated that health rate was negatively correlated with IL-6 concentration. It has been suggested that the endogenous opioid system is important in G allele carriers, and may suppress proinflammatory cytokine

secretion from peripheral immune cells, thus influencing their health perception (MATSUNAGA et al., 2009).

An important finding of this research was the influence of surgery duration on the fluctuation of postoperative pain in the SPID test, at 8, 48, and 96 h after surgery. It has been demonstrated that the duration of surgery affects tissue trauma, suggesting that a longer time of tissue manipulation generates increased trauma (TROULLOS et al., 1990; GRAZIANI et al., 2006), influencing the amount of swelling and inflammation after surgery. Consequently, in this area there are increased levels of inflammatory infiltrates containing proinflammatory cytokines and prostaglandins, which enhance the amount of edema and postoperative pain (TROULLOS et al., 1990). The duration of surgery is strongly correlated with tooth position, the degree of surgical trauma, the need for removing bone tissue, complications in the transoperative period, the extension of the flap, and periosteum displacement around the surgical site, which influences postoperative pain (ANTUNES et al., 2011; PÉREZ-GONZÁLEZ et al., 2018).

The catechol-O-methyltransferase encoded by *COMT* is another important and well-studied enzyme related to pain signal transmission in the body (LIU et al., 2012; SENAGORE et al., 2016), and is correlated with variations of pain sensitivity in experimental models of noxious stimuli (KIM et al., 2006; LEE et al., 2011). The most studied SNP is rs4680G4A, recognized as $Val^{158}Met$ (KIM et al., 2006; LIU et al., 2012; SENAGORE et al., 2016).

Increased pain sensitivity has been correlated with the production of proinflammatory cytokines in patients with *COMT* haplotypes with reduced enzymatic activity (SENAGORE et al., 2016). A prevalence of *COMT* genotypes of 22% AA, 46% AG and 32% GG has been identified in 50 patients (Liu et al, 2012), similar to that

found in our 200 patients, with a prevalence of 31% VAL/VAL (AA), 38% VAL/MET (AG) and 16% MET/MET (GG).

Besides the possible influence of genetic polymorphisms and cytokines already presented, the descending pain inhibitory system also impacts the NSAID response and effects on acute postoperative pain, swelling and trismus. To analyze this system, the CPM was utilized, where the concept that pain inhibits pain was tested in volunteers (YARNITSKY, 2015, COSTA et al., 2017). Costa and collaborators proved that the conditioned pain modulation (CPM) paradigm is a valid and reliable psychophysical approach to evaluate the efficacy of the descending pain inhibition COSTA et al., 2017). After undergoing CPM test, the magnitude of preoperative capacity of modulating pain after test stimulus before surgery was -67.97%, which demonstrated that this Brazilian population was able to modulate pain (Table 2). Analyzing the answered questions from the PCS, which takes in account the psychological profile of volunteers, the average frequency of catastrophic thoughts was 21.31 points (Table 2).

An association between *COMT* and experimental and chronic pain was reported by Diatchenko et al. in their sample (85% European Americans and 15% other ethnic populations). Similarly, to experimental tests applied in the current research, Diatchenko and colleagues, combined pressure and thermal pain thresholds and tolerance, temporal summation of thermal and ischemic pain threshold and tolerance to obtained a pain phenotype (DIATCHENKO et al., 2005). A recent systematic review showed that genetic polymorphisms of the catecholaminergic pathways are associated with thermal and blunt pressure sensitivity (SOARES et al., 2020).

We did not find an association between *COMT* SNP rs4680 and acute postoperative pain after third molar extraction, as observed in another survey, albeit

with a weak relationship showed no significant association with clinically induced acute pain responses in their sample (KIM et al., 2006). Contrasting with Diatchenko and collaborators who reported significant associations for *COMT* SNP8 (rs6269) and SNP10 (rs4818) and pain in their sample (DIATCHENKO et al., 2005). An important finding of the present study was that the *COMT* mutated allele (AG and GG genotypes) influenced trismus on the 2nd postoperative day (Table S6). Patients with these genotypes showed decreased mouth opening (trismus) on the 2nd postoperative day compared to that observed before surgery.

The overall surgery experience reported by volunteers on the 7th postoperative day, after postoperative control of inflammation symptoms mediated by 600 mg ibuprofen, demonstrated that 93.50% of the researched patients determined, in their Global evaluation, that ibuprofen was “excellent”, “very good” and “good” and only 6.50% of then classified treatment with ibuprofen as “fair” and “poor” (Figure 1).These results showed that ibuprofen was effectiveness controlling acute postoperative pain and inflammatory manifestations in this study population, like reported previously (WEISER et al., 2018; DEMIRBAS et al., 2019).

A limitation of the study is that most studies discuss chronic and acute pain, including osteoarthritis of the knee, laparoscopic cholecystectomy pain, major abdominal surgery, and total knee arthroplasty (JENSEN et al., 2005; LÖTSCH et al., 2006; AKBULUT et al., 2014; SENAGORE et al., 2016; MARTIRE et al., 2016). In addition, patients received opioids, such as morphine, piritramide, and fentanyl (CHOU et al., 2006; BARTOŠOVÁ et al., 2015). In our model, we evaluated patients after lower third molar extraction under NSAID therapy. The concentration of pro-inflammatory cytokines in the postoperative period was not assessed and there was no control group without the consumption of NSAIDs were other limitations of the present study

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This study was developed to understand whether the profile of the modulation capacity of pre and postoperative pain, swelling and trismus after dental surgery was affected by psychological and clinical parameters. Polymorphisms in the *COMT* gene, the concentration of saliva proinflammatory cytokines IL-2 and IFN-γ, BMI, and the duration of surgery were predictors for the fluctuation of pain, and the presence of swelling and trismus on the 2nd postoperative day. Therefore, these results confirmed that 600 mg ibuprofen every 8 h for 4 days was effective in controlling inflammatory symptomatology after lower third molar surgery in these 200 Brazilian patients, without relevant adverse reactions; additionally, the medication was well tolerated by patients. For future treatments, these data were added to patient medical records as a personalized and safe prescription, based on their profile of pain modulation capacity with attenuation of adverse effects.

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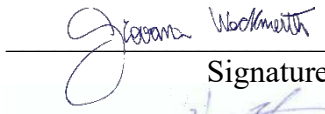
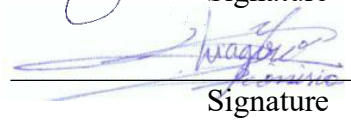
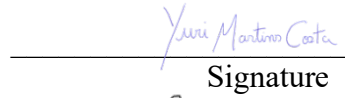
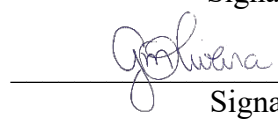
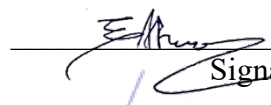
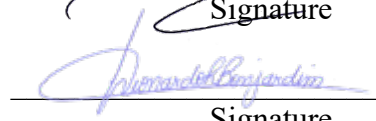
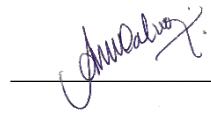
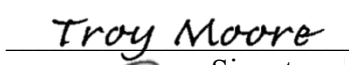
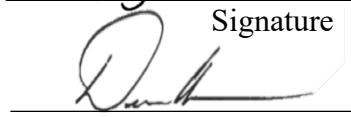
Appendixes

Appendix 1 – DECLARATION OF EXCLUSIVE USE OF THE ARTICLE IN THESIS

DECLARATION OF EXCLUSIVE USE OF THE ARTICLE IN DISSERTATION/THESIS
We hereby declare that we are aware of the article CYP450 polymorphisms and clinical pharmacogenetics of ibuprofen after lower third molar extraction will be included in Thesis of the student Giovana Maria Weckwerth was not used and may not be used in other works of Graduate Programs at the Bauru School of Dentistry, University of São Paulo.

Bauru, June 10th of 2020.

G M Weckwerth	 Signature
T J Dionísio	 Signature
Y M Costa	 Signature
B L Colombini-Ishiquiriama	 Signature
G M Oliveira	 Signature
E A Torres	 Signature
L R Bonjardim	 Signature
A M Calvo	 Signature
T Moore	 Signature
D M Absher	 Signature
C F Santos	 Signature

Appendix 2 – DECLARATION OF EXCLUSIVE USE OF THE ARTICLE IN THESIS

DECLARATION OF EXCLUSIVE USE OF THE ARTICLE IN DISSERTATION/THESIS
We hereby declare that we are aware of the article OPRM1 and COMT polymorphisms associated with patient's pain modulation capacity undergoing ibuprofen therapy after lower third molar surgery will be included in Thesis of the student Giovana Maria Weckwerth was not used and may not be used in other works of Graduate Programs at the Bauru School of Dentistry, University of São Paulo.

Bauru, June 10th of 2020.

G M Weckwerth	Signature
T J Dionísio	Signature
Y M Costa	Signature
P. Zupelari-Gonçalves	Signature
G M Oliveira	Signature
E A Torres	Signature
L R Bonjardim	Signature
F A C Faria	Signature
A M Calvo	Signature
T Moore	Signature
D M Absher	Signature
C F Santos	Signature

Appendix 3 – SURGERY ROOM FORM

DADOS A SEREM COLETADOS NA SALA DE CIRURGIA

NOME DO PACIENTE: _____ GÊNERO: M / F IDADE:_____ DATA: ___/___/_____ POSIÇÃO:_____
dd / mm / aaaa

NOME DO CIRURGIÃO: _____ NOME DO PESSOA QUE TRANSCREVEU DADOS: _____

LADO OPERADO: E / D SALIVA: _____

1) ABERTURA DE BOCA PRÉ-OPERATÓRIA: _____mm +2 DIAS: _____mm +7 DIAS: _____mm

2) TEMPERATURA: PRÉ-OPERATÓRIA: _____ °C +2 DIAS: _____ °C +7 DIAS: _____ °C

3) EDEMA:

BASAL						+2 DIAS						+7 DIAS					
A		B		C		A		B		C		A		B		C	
	mm		mm		mm		mm		mm		mm		mm		mm		mm
	mm		mm		mm		mm		mm		mm		mm		mm		mm
	mm		mm		mm		mm		mm		mm		mm		mm		mm

4) PARÂMETROS

			PRESSÃO ARTERIAL					
		HORARIO	PS	PD	PM	SpO_2	PULSO	Indice de Sangramento
4a)	Antes da cirurgia:		mmHg	mmHg	mmHg	%	bpm	X
4b)	1° tubete:		mmHg	mmHg	mmHg	%	bpm	1 2 3
4c)	Incisão:		mmHg	mmHg	mmHg	%	bpm	1 2 3
4d)	Retalho:		mmHg	mmHg	mmHg	%	bpm	1 2 3
4e)	Osteotomia:		mmHg	mmHg	mmHg	%	bpm	1 2 3
4f)	Extração:		mmHg	mmHg	mmHg	%	bpm	1 2 3
4g)	Limpeza:		mmHg	mmHg	mmHg	%	bpm	1 2 3
4h)	Sutura:		mmHg	mmHg	mmHg	%	bpm	1 2 3
4i)	Fim da cirurgia		mmHg	mmHg	mmHg	%	bpm	X

5) HORÁRIO DO INÍCIO DA AÇÃO DO ANESTÉSICO LOCAL: _____

6) TOTAL DE TUBETES PARA ANESTESIA LOCAL: _____

7) FOI REALIZADA OSTEOTOMIA: SIM / NÃO

8) FOI REALIZADA ODONTOSECÇÃO: SIM / NÃO

9) TOTAL DE TUBETES PARA ANESTESIA LOCAL: _____

10) QUALIDADE DA ANESTESIA: 1 2 3

11) DIFICULDADE DA CIRURGIA: 1 2 3

12) INCIDÊNCIA, TIPO E GRAVIDADE DAS REAÇÕES ADVERSAS: _____

13) QUALIDADE DA CICATRIZAÇÃO NO MOMENTO DA RETIRADA DE PONTOS (APÓS 7 DIAS): 1 2 3

14) AVALIAÇÃO GLOBAL: EXCELENTE MUITO BOM BOM RAZOÁVEL PÉSSIMO

15) INCIDÊNCIA, TIPO E GRAVIDADE DAS REAÇÕES ADVERSAS AO REMÉDIO: _____

Appendix 4 – VISUAL ANALOGUE SCALE

2 dias: _____ - _____
7 dias _____ - _____

Paciente: _____

REMÉDIO DO SAQUINHO **(8 horas)** **Cirurgia:** _____ **Data:** ___/___/___
Serão tomados por 4 dias (8h/8h) no seguinte horário: _____
Fazer um "X" na escala em todos os horários anotados nesta ficha

Após Dia:__/__ |_____|
Horário:_____ Ausência de dor Pior dor possível

15min Dia:__/__ |_____|
Horário:_____ Ausência de dor Pior dor possível

30min Dia:__/__ |_____|
Horário:_____ Ausência de dor Pior dor possível

45min Dia:__/__ |_____|
Horário:_____ Ausência de dor Pior dor possível

1h Dia:__/__ |_____|
Horário:_____ Ausência de dor Pior dor possível

1,30h Dia:__/__ |_____|
Horário:_____ Ausência de dor Pior dor possível

2h Dia:__/__ |_____|
Horário:_____ Ausência de dor Pior dor possível

3h Dia:__/__ |_____|
Horário:_____ Ausência de dor Pior dor possível

4h Dia:__/__ |_____|
Horário:_____ Ausência de dor Pior dor possível

7h Dia:__/__ |_____|
Horário:_____ Ausência de dor Pior dor possível

8h Dia:__/__ |_____|
Horário:_____ Ausência de dor Pior dor possível

10h Dia:__/__ |_____|
Horário:_____ Ausência de dor Pior dor possível

12h Dia:__/__ |_____|
Horário:_____ Ausência de dor Pior dor possível

16h Dia:__/__ |_____|
Horário:_____ Ausência de dor Pior dor possível

24h Dia:__/__ |_____|
Horário:_____ Ausência de dor Pior dor possível

48h Dia:__/__ |_____|
Horário:_____ Ausência de dor Pior dor possível

72h Dia:__/__ |_____|
Horário:_____ Ausência de dor Pior dor possível

96h Dia:__/__ |_____|
Horário:_____ Ausência de dor Pior dor possível

5h Dia:__/__ |_____|
Horário:_____ Ausência de dor Pior dor possível

6h Dia:__/__ |_____|
Horário:_____ Ausência de dor Pior dor possível

OBS: se precisar tomar o outro remédio (da cartela – SOCORRO), anotar o horário e fazer um “X” na escala da outra folha.
Qualquer dúvida ou se precisar mais remédios ligar p/ Dra. Giovana (14) 99782-7828 ou no departamento de Farmacologia 32358276.

Appendix 5 – CATASTROPHIZING PAIN SCALE

Escala de Pensamento Catastrófico sobre a Dor (B-PCS)

Nome: Idade: Sexo: ☐ M ☐ F Data: /
Escolaridade (anos completos de estudo, excluir mobral):

Instruções:
Listamos 13 declarações que descrevem diferentes pensamentos e sentimentos que podem lhe aparecer na cabeça quando sente dor. Indique o *GRAU destes pensamentos e sentimentos quando está com dor*

1	A preocupação durante todo o tempo com a duração da dor é	0 Mínima	1 leve	2 Moderada	3 Intensa	4 Muito intensa
2	O sentimento de não poder prosseguir (continuar) é	0 Mínimo	1 leve	2 Moderado	3 Intenso	4 Muito intenso
3	O sentimento que a dor é terrível e que não vai melhorar é	0 Mínimo	1 leve	2 Moderado	3 Intenso	4 Muito intenso
4	O sentimento que a dor é horrível e que você não vai resistir é	0 Mínimo	1 leve	2 Moderado	3 Intenso	4 Muito intenso
5	O pensamento de não poder mais estar com alguém é	0 Mínimo	1 leve	2 Moderado	3 Intenso	4 Muito intenso
6	O medo que a dor pode se tornar ainda pior é	0 Mínimo	1 leve	2 Moderado	3 Intenso	4 Muito intenso
7	O pensamento sobre outros episódios de dor é	0 Mínimo	1 leve	2 Moderado	3 Intenso	4 Muito intenso
8	O desejo profundo que a dor desapareça é	0 Mínimo	1 leve	2 Moderado	3 Intenso	4 Muito intenso
9	O sentimento de não conseguir tirar a dor do pensamento é	0 Mínimo	1 leve	2 Moderado	3 Intenso	4 Muito intenso
10	O pensamento que ainda poderá doer mais é	0 Mínimo	1 leve	2 Moderado	3 Intenso	4 Muito intenso
11	O pensamento que a dor é grave porque ela não quer parar é	0 Mínimo	1 leve	2 Moderado	3 Intenso	4 Muito intenso
12	O pensamento de que não há nada para fazer para diminuir a intensidade da dor é	0 Mínimo	1 leve	2 Moderado	3 Intenso	4 Muito intenso
13	A preocupação que alguma coisa ruim pode acontecer por causa da dor é	0 Mínima	1 leve	2 Moderado	3 Intenso	4 Muito intenso

Annexes

Annex 1 – COMPROVANT OF MANUSCRIPT SUBMITION

Giovana Maria Weckwerth <giovana.weckwerth@usp.br>

Manuscript submitted to British Journal of Clinical Pharmacology (MP-00295-20)

1 mensagem

Alice Kallaway <onbehalfof@manuscriptcentral.com> 1 de abril de 2020 18:56
Responder a: BJCPedoffice@wiley.com
Para: giovana.weckwerth@usp.br, giovana.weck@hotmail.com
Cc: giovana.weckwerth@usp.br, giovana.weck@hotmail.com, thiagoj@usp.br, yuricosta@fop.unicamp.br, bellacolombini@usp.br, gab.moraes@usp.br, elzatorres@usp.br, lbonjardim@fob.usp.br, dricalvo@usp.br, troy@kailosgenetics.com, dabsher@hudsonalpha.org, cfsantos@fob.usp.br

Dear Ms Giovana Weckwerth,

Your manuscript entitled "CYP450 polymorphisms and clinical pharmacogenetics of ibuprofen after lower third molar extraction" (MP-00295-20) has been received in the British Journal of Clinical Pharmacology's Editorial Office and is currently being reviewed. You can check the progress of manuscript by logging into your Author Centre at https://mc.manuscriptcentral.com/bcp.

The following persons have been listed as authors for this manuscript: Weckwerth, Giovana; Dionísio, Thiago; Costa, Yuri; Colombini- Ishiquiriama, Bella Luna; Oliveira, Gabriela; Torres, Elza ; Bonjardim, Leonardo; Calvo, Adriana; Moore, Troy; Absher, Devin; Santos, Carlos. Any co-authors have been cc'd to this email. Co-authors: Please contact the Editorial Office (BJCPedoffice@wiley.com) as soon as possible if you disagree with being listed as a co-author for this manuscript. Otherwise, no further action is required from you.

If you have any queries please contact the Editorial Office (BJCPedoffice@wiley.com). Please mention your manuscript reference number in all future correspondence and keep the Editorial Office informed of any changes to your contact details.

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Best wishes,

Alice Kallaway
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Annex 2 – COMPROVANT OF MANUSCRIPT SUBMITION

Giovana Maria Weckwerth <giovana.weckwerth@usp.br>

Fwd: Submission Confirmation
1 mensagem

Carlos Ferreira dos Santos <cfsantos@fob.usp.br> 14 de junho de 2020 21:58
Para: giovana weckwerth <giovana.weckwerth@usp.br>

-------- Forwarded message --------
De: **European Journal of Pain** <em@editorialmanager.com>
Date: dom, 14 de jun de 2020 21:45
Subject: Submission Confirmation
To: Carlos Ferreira dos Santos <cfsantos@fob.usp.br>

Dear Dr. dos Santos,

Your submission entitled "Opioid receptor and catechol-O-methyltransferase polymorphisms associated with patient's endogenous pain modulation when undergoing ibuprofen therapy after dental surgery" has been received by journal European Journal of Pain

You will be able to check on the progress of your paper by logging on to Editorial Manager as an author. The URL is https://www.editorialmanager.com/eurjpain/.

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Kind regards,

Dr. Bettina Haake-Weber
Editorial Assistant
European Journal of Pain

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