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FACULDADE DE FARMÁCIA
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STUDY OF GENETIC FACTORS INVOLVED IN PAIN PERCEPTION AND MORPHINE ANALGESIA IN CANCER-RELATED PAIN

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Tese do 3º Ciclo de Estudos Conducente ao Grau de Doutor em Ciências Farmacêuticas – Especialidade: Toxicologia

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“Não sou nada.

Nunca serei nada.

Não posso querer ser nada.

À parte isso, ***tenho em mim todos os sonhos do mundo****.“*

(Álvaro de Campos)

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PUBLICATIONS

Articles in international peer-reviewed journals

1. Oliveira A, Dinis-Oliveira RJ, Nogueira A, Azevedo AS, Gonçalves F, Silva P, Carvalho F, Medeiros R. Genetic Profile and Cancer-Related Pain: A Tale from Two Outlier Cases with Bone Metastatic Disease. Pain Med. *In press*

2. Oliveira A, Dinis-Oliveira RJ, Nogueira A, Azevedo AS, Gonçalves F, Silva P, Carvalho F, Medeiros R. COMT genetic polymorphisms are associated with opioid dose requirements in cancer patients. *Submitted for publication*

3. Oliveira A, Dinis-Oliveira RJ, Nogueira A, Gonçalves F, Silva P, Vieira C, Silvestre R. Carvalho F, Medeiros R, Interleukin-1 Genotype and Circulating Levels in Cancer Patients: Metastatic Status and Pain Perception. *Submitted for publication*

4. Oliveira A, Carvalho F, Pinho PG, Remião F, Medeiros R, Dinis-Oliveira RJ. Quantification of morphine and its major metabolites M3G and M6G in *antemortem* and *postmortem* samples. *Submitted for publication*

5. Oliveira A, Pinho D, Albino-Teixeira A, Medeiros R, Dinis-Oliveira RJ, Carvalho F. Morphine glucuronidation increases its analgesic effect in guinea-pigs. *Submitted for publication*

ABSTRACT

Pain is one of the most persistent and incapacitating symptoms of cancer. In fact, unsatisfactory treatment of cancer-related pain or absence of analgesic response has an enormous impact on patients' quality of life. The World Health Organization treatment guidelines include opioid analgesics as the drugs of choice, with morphine as the first line option for moderate to severe pain. However, wide variations in dose requirement, pharmacological efficacy, tolerability and adverse effects have been observed. Age, gender, race/ethnicity, mood states and stress are known influencing factors but have failed to explain the high degree of interindividual variability. In the last decade, pharmacogenetic has been proposed to be an important and influent factor on opioids response, especially morphine. Polymorphisms in opioid receptors, transporters and metabolizing enzymes are under extensive evaluation, along with genetic variations in modulators/suppressors involved in pain perception and transmission.

The prevalence of cancer-related pain, the unsuccess of the analgesic treatment and the potential of tailored-pain treatment in a foreseeable future prompted us to study important genetic variations in genes involved in opioids and pain mechanisms, along with a more focused study in morphine metabolism. In order to fulfil all the objectives, a method for the quantification of morphine and its major metabolites, morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G), was initially developed. The method revealed to be simple, sensitive, precise and accurate to quantify the three compounds in several *antemortem* and *postmortem* matrices, during animal and human studies.

Concerning genetic variations studies, important genes related to opioids action were selected, as μ-opioid receptor (*OPRM1*); morphine major metabolizing enzyme UDP-Glucuronosyltransferase 2B7 (*UGT2B7*); transporters ATP binding cassette sub family B member 1 transporter (*ABCB1*); and organic anion-transporting polypeptides 1A2 (*OATP1A2*), along with pain and inflammation modulators, such as catechol-O-methyltransferase (*COMT*) and several cytokines. The first study of this thesis analyzed the influence of polymorphisms in *OPRM1*, *COMT* and *ABCB1* genes. The results suggested that *COMT* Val(108/158)Met polymorphism is associated with opioid requirements, with carriers of Met allele being significantly associated with higher opioid doses. Later, an individual approach was performed and the patients with the higher (Patient 1, 800 mg/day) and lower (Patient 2, 20 mg/day) morphine requirements were analyzed, as Patient 1 reported uncontrolled pain and higher pain intensity. Results of genetic analysis has shown that polymorphisms *OPRM1* A118G, *COMT* Val(108/158)Met and *UGT2B7* C802T and T801A seemed to influence the analgesic effect, with individuals

GA, Val/Met and T801C802 being related with less morphine efficacy and higher doses. Also, differences in plasma concentrations of metabolites and metabolic *ratios* were found and correlated with the genetic variances. These observations confirmed the previous result but also highlighted the importance of case series analysis. Polymorphisms in inflammatory mediators were subsequently analyzed (interleukin (IL) 1α, IL-1β, IL-1 receptor antagonist (IL-1Ra), IL-2, IL4 receptor (IL-4R), IL-6, IL-10, tumor necrosis α and interferon γ). In this study, carriers of TT genotype of the C3954T polymorphism in IL-1β were associated with lower levels of IL1-β and lower levels of pain. Also, IL1-β levels were related with cancer onset status and metastatic disease. This result pointed out another non-opioid system that might be involved in pain sensitivity in cancer pain patients.

Finally, a relevant animal model was established to study morphine metabolism and its influence in the analgesic effect. Guinea pig revealed to be an adequate model, with morphine metabolic *ratios* close to humans. The obtained results showed that morphine metabolism induction leads to higher metabolic *ratios* (M3G/morphine and M6G/morphine) and faster and better analgesic effect, after a single morphine intraperitoneal administration. On the other hand, opposite results were observed during metabolism inhibition. These results demonstrated the importance of morphine pharmacokinetics in its final analgesic effect and the animal model developed seems promising for future studies concerning morphine metabolism and its implication in clinical practice.

In conclusion, the results of this thesis suggest that genetic variants in opioid and non-opioid systems can affect opioids analgesic effect, especially by influencing opioids requirements and pain perception. Additionally, further studies on the modulation of morphine metabolism might contribute to an improved analgesic effect of morphine, increasing patients' life quality.

Keywords: cancer pain, morphine, morphine-6-glucuronide, morphine-3-glucuronide, pharmacogenetic.

RESUMO

A dor é um dos sintomas mais persistentes e incapacitantes do cancro. De facto, o seu tratamento insatisfatório ou ausência de resposta analgésica têm um enorme impacto na qualidade de vida dos doentes. As diretrizes de tratamento da Organização Mundial de Saúde incluem os analgésicos opioides como os fármacos de escolha, com a morfina como opção de primeira linha para a dor moderada a grave. No entanto, têm sido observadas grandes variações na dose de opioide necessária, na sua eficácia farmacológica, tolerabilidade e efeitos adversos. Alguns fatores que podem contribuir para esta variabilidade são a idade, sexo, raça/etnia, estados de humor e stress. Apesar da sua influência conhecida, não conseguem explicar o alto grau de variabilidade interindividual. Na última década, a farmacogenética tem sido apontada como um fator importante e influente na resposta aos opioides, principalmente à morfina, em polimorfismos em recetores opioides, transportadores e enzimas de metabolismo, assim como em moduladores/supressores envolvidos na perceção da informação nociceptiva .

A prevalência de dor relacionada com o cancro, o insucesso do tratamento analgésico e o potencial desenvolvimento de um tratamento individualizado para a dor num futuro próximo motivaram o estudo de variações importantes em genes envolvidos nos mecanismos de ação dos opioides e da transmissão/modulação da dor, integrando também um estudo mais focado no metabolismo da morfina. Para cumprir todos os objetivos foi inicialmente desenvolvido um método de quantificação da morfina e seus principais metabolitos, morfina-3-glucoronídeo (M3G) e morfina-6-glucoronídeo (M6G). O método revelou ser simples, sensível, preciso e exacto para o doseamento dos três compostos em diversas matrizes *antemortem* e *postmortem*, e apropriado para aplicação durante os estudos em animais e humanos.

No estudo das variações genéticas, foram selecionados genes envolvidos no mecanismo opioide, como recetor opioide μ (*OPRM1*); UDP-Glucuronosiltransferase 2B7 (*UGT2B7*), a enzima maioritariamente responsável pelo metabolismo da morfina; transportadores, como a glicoproteína P (*ABCB1*) e o transportador de aniões orgânicos 1A2 (*OATP1A2*). Adicionalmente foram também selecionados polimorfismos em moduladores de dor e inflamação, como catecol-O-metiltransferase (*COMT*) e várias citoquinas. O primeiro estudo desta tese analisou a influência da variação genética nos genes *OPRM1*, *COMT* e *ABCB1*. Os resultados sugeriram que o polimorfismo *COMT* Val(108/158)Met está associado ao requerimento total de opioides, em que os portadores do alelo Met foram significativamente associados com doses mais elevadas. Seguidamente, uma abordagem individual foi realizada e foram analisados os doentes com a dose mais alta (Doente 1, 800 mg/dia) e mais baixa (Doente 2, 20 mg/dia) de morfina, tendo em conta que o Doente

1 descrevera falhas no alívio da dor e maior intensidade da dor. Os resultados da análise genética revelaram que polimorfismos A118G do *OPRM1*, Val(108/ 158)Met da *COMT* e C802T e T801A da UGT2B7 parecem influenciar o efeito analgésico, com indivíduos portadores do genótipo GA, Val/Met e T801C802 relacionados com menor eficácia e consumo superior de morfina. Adicionalmente, foram encontradas diferenças nas concentrações plasmáticas dos metabolitos e respetivos índices metabólicos e correlacionados com as variações genéticas. Estas observações confirmaram o resultado previamente encontrado, mas também destacaram a importância da análise de casos de estudo. Posteriormente foram também analisados polimorfismos em mediadores inflamatórios (interleucina (IL) 1α, IL-1β, antagonista do recetor da IL-1 (IL-1Ra), IL-2, recetor de IL-4 (IL-4R), IL-6, IL-10, fator de necrose tumoral-α e interferão γ). Neste estudo, os portadores do genótipo TT do polimorfismo C3954T da IL-1β foram associados a níveis mais baixos de IL-1β e menor intensidade de dor. Além disso, os níveis de IL-1β foram também relacionados com o cancro e doença metastática. Estes resultados sugerem o envolvimento de um outro sistema não-opioide na sensibilidade à dor, em doentes com dor relacionada com o cancro.

Por último, foi desenvolvido um modelo animal relevante para o estudo do metabolismo da morfina e a sua influência no efeito analgésico. Os cobaios revelaram ser um modelo adequado, com rácios metabólicos de morfina e metabolitos próximos aos humanos. Os resultados obtidos durante o estudo demonstraram que a indução do metabolismo da morfina resulta em concentrações mais elevadas dos seus metabolitos e rácios metabólicos (M3G/morfina e M6G/morfina), assim como num aumento do efeito analgésico, após uma única administração intraperitoneal de morfina. Por outro lado, foram observados resultados opostos durante a inibição do metabolismo. Estes resultados demonstram a importância da farmacocinética da morfina no efeito final analgésico e a potencialidade do modelo animal desenvolvido para futuros estudos do metabolismo da morfina e da sua implicação na prática clínica.

Em conclusão, os resultados desta dissertação sugerem que a variação em genes envolvidos nos sistemas opioides e não-opioides podem afetar o efeito analgésico, especialmente ao influenciar a dose necessária e a perceção da dor. Adicionalmente, estudos sobre a modulação do metabolismo de morfina parecem contribuir para a compreensão da relação da farmacocinética e efeito analgésico da morfina, aumentando o seu efeito melhorando a qualidade de vida dos doentes.

Palavras-chave: dor relacionada com o cancro, morfina, morfina-6-glucuronídeo, morfina-3-glucuronídeo, farmacogenética.

OUTLINE OF THE THESIS

The thesis is organized in 6 chapters.

Chapter I is an introduction to contextualize the state of art of the key topics within the thesis. Aspects of pain categories, perception and transmission are addressed, as well as the main treatments for cancer-related pain and major polymorphisms implicated in pain sensitivity and morphine analgesia.

Chapter II comprises the aims of the thesis and explains how these articulate with the subsequent experimental results presented.

Chapter III contain the main studies performed, including materials, methods, results and discussion which are presented in the form of manuscripts published or under submission in peer-reviewed journals. For each study, information concerning the journal and date of publication (for published papers) / co-authors is provided.

Chapters IV to VI include a general discussion and main conclusions of the thesis, highlighting the most relevant achievements and also the presenting prospects for future work.

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LIST OF ABBREVIATIONS

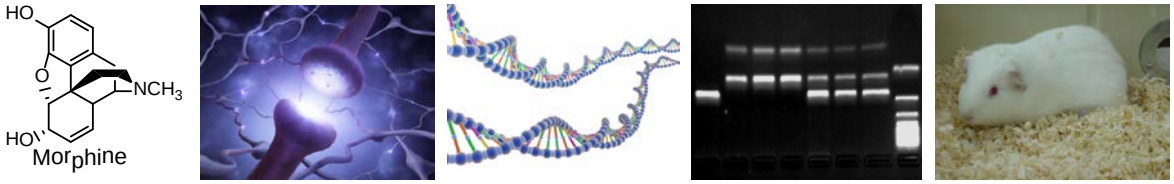
5-HT	5-hydroxytryptamine / serotonin
5-HTP	5-hydroxytryptophan
5-HTTLPR	Serotonin-transporter-linked polymorphic region
ABCB1	ATP-binding cassette B_1
AC	Adenylyl cyclase
ACC	Anterior cingulated cortex
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate
Arg	Arginine
ARRB2	β-arrestin 2 gene
ASA	Acetylsalicylic acid
ASIC	Acid-sensing receptors
BH2	Dihydrobiopterin
BH4	Tetrahydrobiopterin
bp	base pair
CAM	Complementary and alternative medicine
cAMP	Cyclic adenosine monophosphate
CB	Cannabinoid receptors
CCL3	Chemokine ligand 3
CGRP	Calcitonin gene-related peptide
COMT	Catechol-O-methyltransferase
COX	Cyclooxygenase
CRP	C-reactive protein
CYP2D6	Cytocrome P450 2D6
DAT	Dopamine transporter
DRD_4	Dopamine receptor 4
DRG	Dorsal root ganglion
ECOG	Eastern cooperative oncology group
ERK	Extracellular signal-related kinases
FAAH	Fatty acid amide hydrolase
FDA	Food and drug administration
FPS-R	Face pain scale revised
GABA	γ-aminobutyric acid
GCH1	Guanosine triphosphate cyclohydrolase
GDNF	Glial cell-derived neurotrophic factor
GIRK	G-protein-activated inwardly rectifying potassium
GPCR	G-protein coupled receptor
His	Histidine
HPLC	High performance liquid chromatography

I_2	Imidazoline
IASP	International association for the study of pain
IFN-γ	Interferon-γ
IL	Interleukin
IL-1Ra	Interleukin 1 receptor antagonist
K_V	Potassium voltage-gated channel
KCNS1	Potassium voltage-gated channel subfamily S member 1
LOX	Lipoxygenase
MAO	Monoamine oxidase
M3G	Morphine-3-glucuronide
M6G	Morphine-6-glucuronide
MCR1	Melanocortin-1 receptor
Met	Methionine
MRP	Multidrug resistance-associated protein
NA	Noradrenaline
Na_V	Sodium voltage-gated channel
NAT	Noradrenaline transporter
NGF	Nerve growth factor
NK-1	Neurokinin 1
NKA	Neurokinin A
NMDA	*N*-Methyl-D-Aspartate
NO	Nitric oxide
N/OFQ	Nociceptin / orphanin FQ peptide
NOP	Nociceptin / orphanin FQ peptide receptor
NOS	Nitric oxide synthase
NRS	Numerical rating scale
NSAIDs	Nonsteroidal anti-inflammatory drugs
OATP	Organic anion-transporting polypeptides
OMEQ	Oral morphine equivalents
OPG	Osteoprotegerin
OPGL	Osteoprotegerin ligand
OpR	Opioid receptor
OPRM1	μ-opioid receptor gene
PAG	Periaqueductal gray
PCA	Patient-controlled analgesia
PGH	Prostaglandin H
PGE2	Prostaglandin E2
Pgp	P-glycoprotein
Phe	Phenylalanine
PTPS	6-pyruvoyl tetrahydropterin synthase

RANKL	Receptor activator of nuclear factor kappa-B ligand
RVM	Rostral ventromedial medulla
SERT	Serotonin transporter
SG	Substantia gelatinosa
SLC6A2	Solute carrier family 6 member 2
SLC6A3	Solute carrier family 6 member 3
SLC6A4	Solute carrier family 6 member 4
SNP	Single nucleotide polymorphism
SP	Substance P
SSNRI	Selective serotonin and noradrenaline reuptake inhibitor
SSRI	Selective serotonin reuptake inhibitor
Stat6	Signal transducer and activator of transcription 6
STin2	Second intron
T	Transmission cell
TCA	Tricyclic antidepressants
TCDD	2,3,7,8-tetraclorodibenzo-p-dioxina
TGFβ	Transforming growth factor β
TLR4	Toll-like receptor 4
TNF-α	Tumor necrosis factor-α
TREK-1	TWIK-related potassium channel 1
TRP	Transient receptor potential
Trp	Tryptophan
Tyr	Tyrosine
TRPA1	Transient receptor potential cation channel subfamily A member 1
TRPM8	Transient receptor potential cation channel subfamily M member 8
TRPV	Transient receptor potential vanilloid
UGT2B7	Uridine 5'-diphospho-glucuronosyltransferase 2B7
Val	Valine
VAS	Visual analogue scale
VNTR	Variable number tandem repeat
VRS	Verbal rating scale
WHO	World health organization

CHAPTER I

INTRODUCTION



1.1 Pain classification and general concepts

Pain is an unpleasant feeling and one of the most common reasons for patients to seek health care (Fishbain *et al.*, 2010). A high number of people suffer from chronic pain, often in multiple anatomic locations simultaneously, and complain of lack of efficacy in the treatments prescribed. The World Health Organization (WHO) estimates that the prevalence of chronic pain is about 37.3 % in developed countries and 41.4 % in developing countries (Tsang *et al.*, 2008). In Portugal the prevalence is around 37 %, with 68 % of people with chronic pain complaining of moderate-to-severe intensity (Azevedo *et al.*, 2012). This leads to a high degree of dissatisfaction and high economic costs in the health sector, increasing the need to study and identify the problems related to pain, its treatments, and more recently, the genetic influence.

Pain is defined by the International Association for the Study of Pain (IASP) as an "unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (IASP, 1994). With this definition, IASP recognizes pain as a subjective phenomenon and that tissue damage is not essential for pain to be felt.

Pain can be categorized in different ways (Figure 1), based in several criteria, as time, initiating conditions, underlying mechanisms, location and tissue damage, among others (Goucke, 2003; Nicholson, 2006; Kumar and Saha, 2011; Xu and Yaksh, 2011). However, there are common concepts in all the classification systems, which are essential to understand due to their importance to the evaluation and treatment of pain.

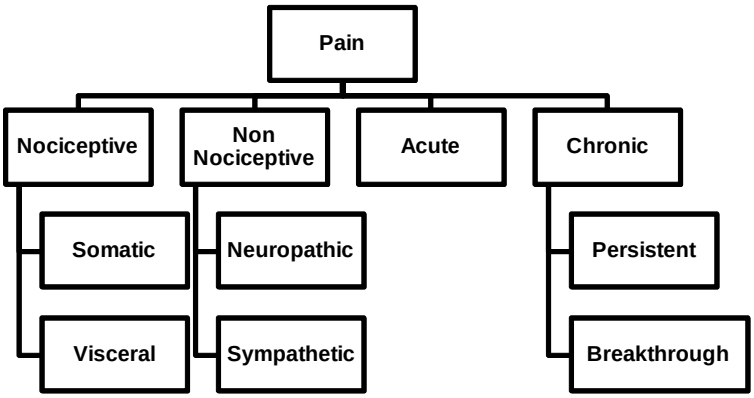


Figure 1. Schematic representation of pain categories.

Regarding duration, there are essentially two types: acute and chronic pain. Acute pain is defined as a “normal, predicted physiological response to noxious chemical, thermal or mechanical stimulus, typically associated with invasive procedures, trauma and disease and it is generally time-limited” (FSMB, 2005). Briefly, this kind of pain is characterized by a recent onset, short-lasting sensation and identifiable cause, with a variety of current therapies available (Friedrich, 2012). Usually, acute pain occurs intermittently or last up to several days (Fink and Mata, 2008) and it is considered critical for healthy survival, triggering an individual response to potentially harmful stimuli (Fink and Mata, 2008; Mata *et al.*, 2008).

On the other hand, chronic pain is defined as “a state in which pain persists beyond the usual course of an acute disease or healing of an injury, or that may or may not be associated with an acute or chronic pathologic process that causes continuous or intermittent pain over months or years” (FSMB, 2005). Chronic pain is essentially characterized by its persistence (minimum of three months) (Fink and Mata, 2008), suffering and complicated pathways, involving neurotransmission and electrophysiological alterations (peripheral and central sensitization), being considered a major cause of morbidity and decreased life quality (Niv and Devor, 2004; Fink and Mata, 2008; Mata *et al.*, 2008; Huang *et al.*, 2011; Friedrich, 2012). A usual pain condition in chronic cancer pain patients is breakthrough pain, a transitory flare of severe or excruciating pain, over a well-controlled baseline pain (Mercadante *et al.*, 2002; Caraceni *et al.*, 2004). This kind of pain is usually described in cancer pain patients when interrupts a background pain well controlled by opioids (Portenoy *et al.*, 1999). However, its implication in chronic non cancer pain has also been described (Manchikanti *et al.*, 2011).

Besides the temporal characteristics, an important clinical division concerning its causal factor classifies pain in nociceptive, non-nociceptive and mixed (both nociceptive and non-nociceptive pain). Nociceptive pain is defined as “pain that arises from actual or threatened damage to non-neural tissue and is due to the activation of nociceptors” (IASP, 1994). This concept was designed to contrast with neuropathic pain (normal somatosensory nervous system *vs.* abnormal function) (IASP, 1994), referring to a sharp and well localized pain after mechanical, chemical or thermal irritation of peripheral sensory nerves (Goucke, 2003). Examples of nociceptive pain include pain after surgery, arthritis pain, mechanical low back pain, and pain associated with sports injuries (Goucke, 2003; Nicholson, 2006). Nociceptive pain can be divided in somatic and visceral pain, especially when referring to cancer-pain patients (Carver and Foley, 2000). Somatic pain is characterized as well localized, intermittent or constant, and results from the activation of peripheral nociceptors. Common causes include bone metastasis and postsurgical pain (Carver and Foley, 2000). On the other hand, visceral pain refers to a deep, squeezing, or colicky pain, caused by the activation of nociceptors in cardiovascular, respiratory, gastrointestinal and genitourinary system. This activation is a result of infiltration, compression, extension, or stretching of the thoracic (chest), abdominal, or pelvic viscera (Carver and Foley, 2000).

Non-nociceptive pain is essentially characterized by neuropathic pain. This pain category is defined by IASP as “pain caused by a lesion or disease of the somatosensory nervous system” (IASP, 1994) and is more considered as a clinical description and not a diagnosis. The sensation is generally described as burning, squeezing and shock-like, resulting from demonstrable lesion (abnormality or trauma) or a disease (diabetes mellitus, vasculites, stroke) (IASP, 1994; Carver and Foley, 2000). In fact, neuropathic pain is characterized by spontaneous and induced pain (Figure 2), generally causing allodynia, hyperalgesia and hyperpathia (Goucke, 2003) and its features are very different from nociceptive pain. Also, neuropathic pain patients usually have higher average pain scores, lack of good analgesic efficacy and lower quality of life comparing with non-neuropathic chronic pain patients (Smith *et al.*, 2007; Torrance *et al.*, 2007; Park and Moon, 2010).

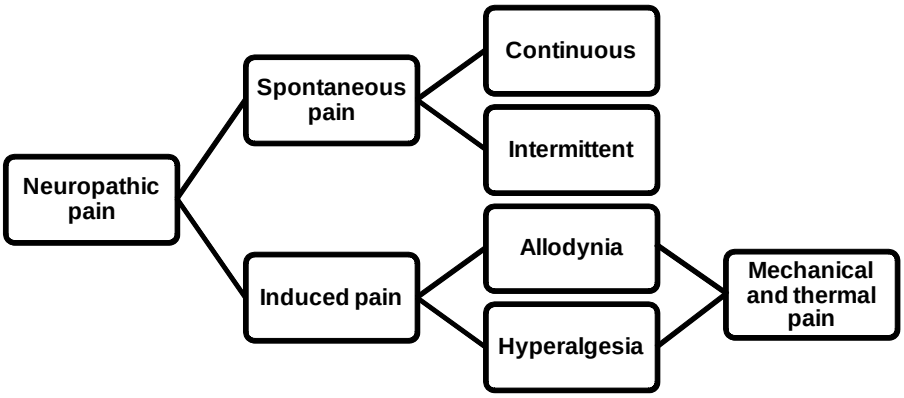


Figure 2. Characterization of neuropathic pain.

Sympathetic nervous system may also be involved in pain pathogenesis, especially in chronic pain syndromes characterized by severe pain, yielding the concept of sympathetically maintained pain (Baron *et al.*, 1999; Martinez-Lavin, 2004). This concept, which may be considered a subset of neuropathic pain (Gibbs *et al.*, 2008), is based in the identification of signs of autonomic dysfunction, as edema, sweating and changes in blood flow, and the efficacy of sympatholytic strategies in pain relief (Baron *et al.*, 1999). The influence of sympathetic nervous system in pain syndromes has been investigated, especially in some neuropathic pain patients (Kingery, 1997; Martinez-Lavin, 2004; Gibbs *et al.*, 2008), fibromyalgia (Martinez-Lavin, 2004) and complex regional pain syndromes Type I and II (Baron and Maier, 1996; Kingery, 1997; Baron *et al.*, 1999), although clinical sympathetically maintained pain model is still a controversial subject (Ochoa and Verdugo, 1995; Baron *et al.*, 1999; Martinez-Lavin, 2004).

Other terms that can be associated to pain division is inflammatory, functional, somatoform or existential (Fishbain *et al.*, 2010). Inflammatory pain involves a response to inflammatory mediators (Fishbain *et al.*, 2010), while functional pain is related to pain during dynamic functional activities as mobility tasks (Vincent *et al.*, 2013). The concept of existential pain is difficult to define, but is generally related to strong feelings of anguish and anxiety resulting of overstatement of physical pain (Strang *et al.*, 2004; Fishbain *et al.*, 2010). This existential or spiritual pain commonly promotes opioid addiction due to its initial response to opioids (Strang *et al.*, 2004; Fishbain *et al.*, 2010). Somatoform pain disorder also has a strong psychological role as the physical complaint is not associated with any medical condition or is in excess for what is expected from the physical findings (Yoshino *et al.*, 2013).

Taking time and causal factor into account, acute pain is mainly nociceptive, and chronic pain produced by nociceptive, neuropathic or existential stimuli. However, exceptions and mixed stimuli can exist (Fishbain *et al.*, 2010).

1.2 Pain neurophysiology

Pain involves dysfunction in several neural mechanisms. Although major progress has been made, several mechanisms are probably unknown and it is urgent to translate the pain research and mechanisms into clinical practice of pain management, to achieve an ideal relief with the best drug.

1.2.1 Peripheral pain mechanisms

Thermal, chemical or mechanical stimuli can trigger the pain process by activating the initial structures involved in nociceptive process, the primary afferent nociceptors. These nociceptors are peripheral with the cell body located in the dorsal root ganglion (DRG) and serve two major functions, transduction of the noxious stimuli in electrochemical impulses and subsequent transmission (Julius and Basbaum, 2001; Authors not listed, 2005; Woolf and Ma, 2007). Some nociceptors are lightly myelinated, the Aδ fibers, and are classified as fast-conducting nociceptive fibers, with rapid conduction of action potential (6-30 m/s). However, most are unmyelinated C fibers, with slower conduction (< 2m/s) and represent the majority of sensory neurons in the peripheral nervous system, being activated by thermal, mechanical and chemical stimuli (Woolf and Ma, 2007; Xu and Yaksh, 2011). “Fast pain” is usually a result of Aδ fibers activation and described as a short-lasting and pricking type of pain. Activation of C fibers leads to “slow pain”, a dull, not well localized, burning type of pain. The primary afferent nociceptors conduction leads to the activation of supraspinally projecting dorsal horn neurons and the more intense the stimulation, the higher the afferent input frequency and the frequency of dorsal horn neurons activation (Xu and Yaksh, 2011).

The stimulation of nociceptive primary afferents neurons results in the release of several neuropeptides from its terminals as substance P (SP), calcitonin gene-related peptide (CGRP) and neurokinin A (NKA) (Figure 3). Neuropeptides and excitatory transmitters (especially glutamate) activate numerous receptors such as kainate, α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionate (AMPA) and *N*-methyl-D-aspartate (NMDA), causing

rapid depolarization of the secondary afferent neurons in the dorsal horn (Authors not listed, 2005). Also, released neuropeptides activate tachykinin receptors leading to vasodilatation, edema and hyperalgesia and contributing to peripheral inflammatory process (neurogenic inflammation).

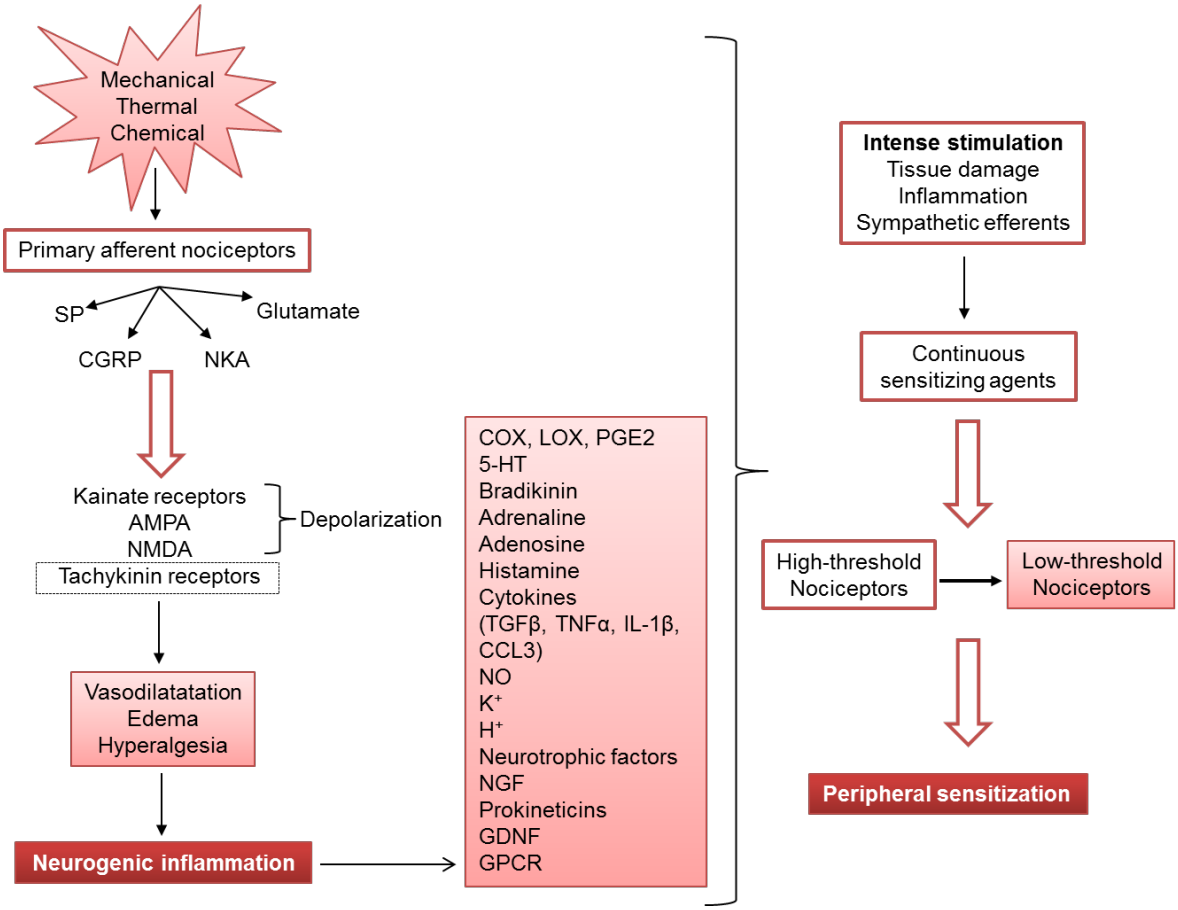


Figure 3. Peripheral pain mechanisms and sensitization. AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionate; CCL3, chemokine ligand 3; CGRP, calcitonin gene-related peptide; COX, cyclooxygenase; GDNF, glial cell-derived neurotrophic factor; GPCR, G-protein coupled receptor; 5-HT, 5-hydroxytriptamine; IL-1β, interleukin 1β; LOX, lipoxygenase; NGF, nerve growth factor; NKA, neurokinin A; NMDA, *N*-methyl-D-aspartate; NO, nitric oxide; PGE2, prostaglandin E2; SP, substance P; TGF-β, transforming growth factor β, TNF-α, tumor necrosis factor α.

All these stimuli activate high-threshold nociceptors, which signal transduction mechanisms include the transient receptor potential vanilloid (TRPV) family – activated by heat and capsaicin, and the acid-sensing receptors (ASIC) – activated by the low pH associated with ischemia and inflammation. Also, potassium and ligand-gated ion channels are activated, as TWIK-related potassium channel-1 (TREK-1, heat-sensitive potassium channels), TRP cation channel subfamily M member 8 (TRPM8, for cold

stimuli) or TRP cation channel subfamily A member 1 (TRPA1, intense cold that produces burning sensation) (Bandell *et al.*, 2004; Alloui *et al.*, 2006; Dhaka *et al.*, 2006; Bautista *et al.*, 2007). However, primary afferent nociceptors can adapt in response to inflammation or injury and repeated activation can modify the response to further stimuli, reducing the threshold response, which leads to hyperalgesia and allodynia (Woolf and Salter, 2000; Scholz and Woolf, 2002; Kumar and Saha, 2011). This neuroplasticity phenomenon is designated by peripheral sensitization and is extremely common in clinical pain, especially in inflammatory pain, some forms of neuropathic pain and in ongoing nociceptive stimulation (Woolf, 2004).

The release of SP, CGRP and NKA leads to neurogenic inflammation, as already mentioned. During this inflammatory process, several inflammatory mediators, neuropeptides and catecholamines are activated and released, as cyclooxygenase (COX), lipoxygenase (LOX), prostaglandin E2 (PGE2), serotonin (5-hydroxytriptamine, 5-HT), bradikinin, adrenaline, adenosine, histamine, cytokines, nitric oxide (NO), K^+, H^+, and neurotrophic factors (Woolf and Salter, 2000; Julius and Basbaum, 2001; Scholz and Woolf, 2002; Woolf and Ma, 2007; Kumar and Saha, 2011). Many other factors have been associated with this phenomenon in the last years, such as transforming growth factor β (TGFβ) member activin, tumour necrosis factor α (TNF-α), the chemokine ligand 3 (CCL3), prokineticins, proteases that activate G-protein coupled receptor (GPCR), glial cell-derived neurotrophic factor (GDNF) and nerve growth factor (NGF) (Zhang *et al.*, 2005; Jin and Gereau, 2006; Malin *et al.*, 2006; Vellani *et al.*, 2006; Xu and Hall, 2006; Dai *et al.*, 2007; Grant *et al.*, 2007; Watson *et al.*, 2008). These sensitizing agents either activate the neurons directly or sensitize them for other stimuli and activate second messenger cascades, producing intense stimuli and leading to peripheral sensitization (Bevan, 1996; Fornasari, 2012). This seems to occur through the phosphorylation of transducers and sodium voltage-gated channels (Nav) 1.7 and 1.8. PGE2 can reduce the nociceptors threshold by activating adenyl cyclase, leading to an increase in cyclic adenosine monophosphate (cAMP), which activates cAMP-dependent protein kinase (Woolf, 2004). On the other hand, bradikinin and leukotrienes can directly sensitize nociceptors and interleukin (IL)-1β and TNF-α can induce COX-2, that converts arachidonic acid to prostaglandin H (PGH) and finally to PGE2 (Woolf, 2004).

The large number of inflammatory molecules involved can, in part, explain the lack of an effective response to the treatment of inflammatory pain and the use of adjuvant medication for neuropathic pain besides nonsteroidal anti-inflammatory drugs (NSAIDs), as tricyclic antidepressants (TCA), anticonvulsants and antiarrythmics (2005).

Peripheral nerve injury can also occur, leading to altered afferent sensory input, inflammatory response with production of sensitizing agents, and to persistent pain, with hyperalgesia and allodynia (Xu and Yaksh, 2011). In healthy sensory nerve fibers, action potentials are a result of stimulation. However, impaired nerve fibers usually have ectopic discharges (Schaible and Richter, 2004). The increased spontaneous activity involve altered sodium (increased expression of tetrodotoxin-sensitive channels) and potassium (reduced) channel expression, increased expression of neuroma and DGR receptors and pathological activation by inflammatory mediators (TNF-α, NGF, catecholamines, bradikinin). Additional, the migration of non-neuronal inflammatory cells to DRG and dorsal horn, loss of inhibition mechanisms [as γ-aminobutyric acid (GABA)], pathological activation of injured nerve fibers by the sympathetic nervous system and altered neuropeptide expression may result in spontaneous activity of dorsal horn projection neurons (Schaible and Richter, 2004; Xu and Yaksh, 2011). Altogether, changes at the nerve injury location and DRG may originate the sharp, shooting and burning pain states in diabetic neuropathy, postherpetic neuralgia and peripheral nerve trauma.

1.2.2 Central perspective – dorsal horn mechanisms

Regardless of peripheral origin, nerve or tissue injury, the terminations of primary afferent nociceptors cause an input to the dorsal horns of the medulla and spinal cord, by transmitting the information to its neurons (secondary neurons). These synaptic transmissions encompass several excitatory (primary afferent nociceptors and neurons of spinal cord) and inhibitory (interneurons within the spinal cord and supraspinal sources) neurotransmitters and neuromodulators (Fornasari, 2012). Glutamate is the major excitatory neurotransmitter and mediates fast transmission by binding to AMPA receptors (Fornasari, 2012). Glutamate also interacts with NMDA receptors, but not during physiological nociceptive pain transmission, as these receptors remain physically blocked by a magnesium ion. However, intense or persistent peripheral stimuli lead to a massive release of glutamate and AMPA receptors activation results in the removal of the magnesium ion and subsequent NMDA activation (Figure 4) (Fornasari, 2012). These alterations play an important role in the central sensitization phenomenon, where low-level or subthreshold stimuli can lead to an aberrant response, allodynia, hyperalgesia and hypersensitivity (Woolf and Salter, 2000; Authors not listed, 2005; Fornasari, 2012). Together with glutamate, several other neuropeptides can be released, such as SP, NKA, CGRP and BDNF, acting on GPCR and receptor tyrosine kinases (Fornasari, 2012).

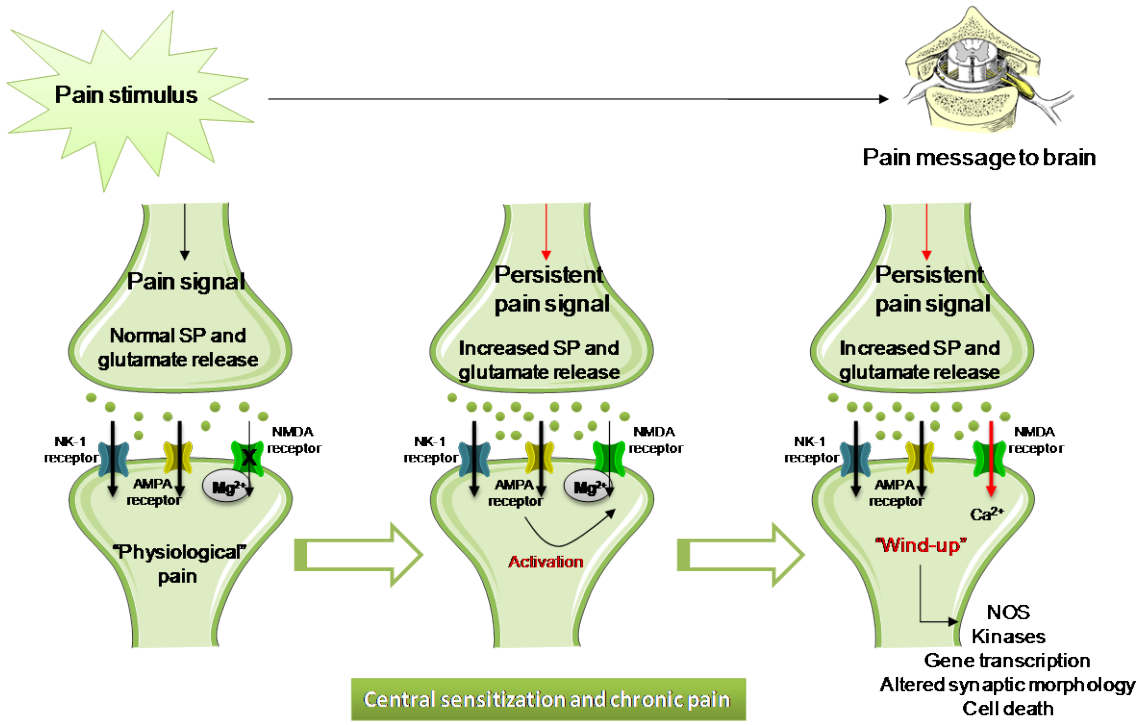


Figure 4. Pain transmission, central sensitization and chronic pain, resulting in hyperalgesia and allodynia. AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionate; NK-1, neurokinin 1; NMDA. *N*-methyl-D-aspartate; NOS, nitric oxide synthase; SP, substance P.

Central sensitization is an important phenomenon that especially occurs in neuropathic, functional and inflammatory pain, and in three stages: activation, modulation and modification (Woolf, 2004; Fornasari, 2012). During the activation stage, massive release of glutamate and neuropeptides and activation of AMPA and NMDA take place (Schaible and Richter, 2004; Fornasari, 2012). As already mentioned, NMDA is blocked by a magnesium ion, but successive synaptic depolarizations of this receptor lead to magnesium depletion and subsequent activation of NMDA regulated calcium channel, allowing an abnormal influx of calcium into the cell (Schaible and Richter, 2004; Authors not listed, 2005). This process is known as “wind-up” and calcium contributes to depolarize secondary neurons and act as a second messenger, activating protein kinases, which phosporylate receptors as NMDA (Woolf and Costigan, 1999; Costigan and Woolf, 2000; Schaible and Richter, 2004). This contributes to modify neural transmission and amplify the nociceptor response to stimuli, representing the second stage of central sensitization – modulation (Woolf and Costigan, 1999; Costigan and Woolf, 2000; Fornasari, 2012). The third stage – modification – encompasses the most dramatic changes. Within the second-order neurons, protein kinase activation may lead to gene

transcription, altered phenotype, changes in synaptic morphology and neural plasticity, and may lead to cell death (Woolf, 2004; Fornasari, 2012).

There is also evidence for interplay between NMDA and nociceptive and inflammatory components, as COX, NO synthase (NOS) and prostaglandins, and especially COX-2 have been shown to be induced in dorsal horn neurons, sustaining inflammatory hypersensitivity and neuropathic pain (Vane *et al.*, 1994; Salvemini, 1997; Wong *et al.*, 2000; Samad *et al.*, 2001; Ma and Eisenach, 2003). These are important evidences to support the use of NSAIDs in chronic neuropathic pain.

1.2.3 Interconnections in pain modulation

After nociceptors stimulation, the transmission of the information can be modulated at all levels and, when it reaches the dorsal horn, leads to inhibitory mechanisms, involving local inhibitory interneurons and descending pathways, in an attempt to limit the subsequent effect of stimulation and impulses. A model of this interaction was proposed by Melzack and Wall in 1965 (Melzack and Wall, 1965), designated by “gate theory” of pain (Figure 5).

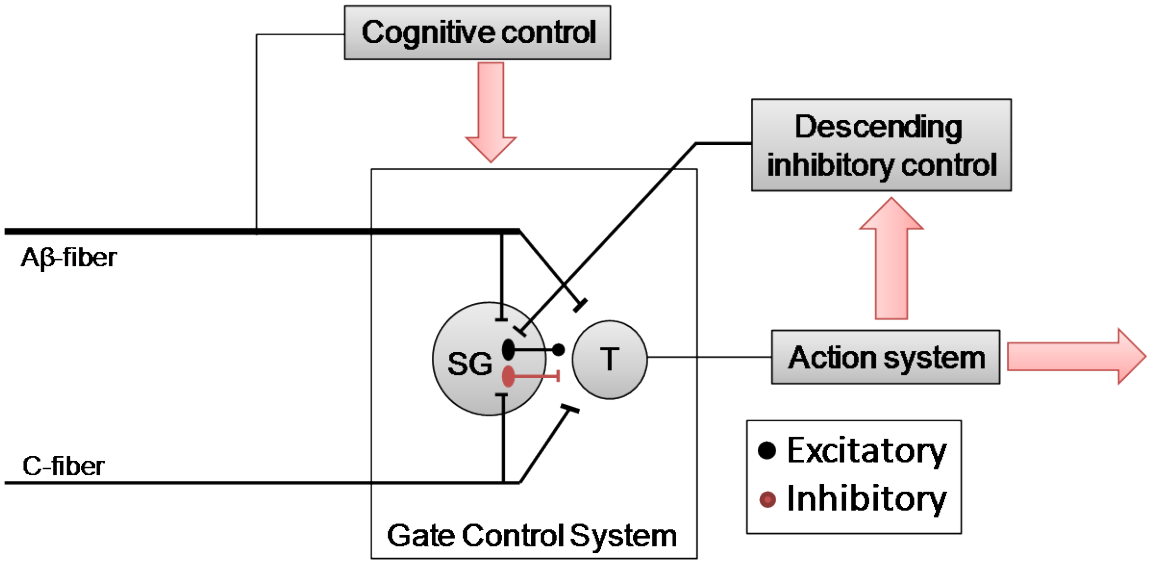


Figure 5. Gate theory of pain. SG, substantia gelatinosa; T, transmission cell [Adapted from (Melzack and Wall, 1965; Melzack, 1998)].

According to this, excitatory and inhibitory links and controls would affect the "gatekeepers", i.e., dorsal horn mechanisms that control the flow of nerve impulses from peripheral fibers. Then, pain can occur when the degree of sensory input exceeds the critical level (Authors not listed, 2005). Both GABA and glicine are involved in tonic inhibition and its down-regulation is implicated in neuropathic pain and allodynia. However, despite the significant impact of the gate theory in the understanding of pain concepts and treatments, it does not complete all the mechanisms and pathways. Some revisions were made, suggesting three interactive dimensions (Brown *et al.*, 2002; Authors not listed, 2005):

a) sensory-discriminative dimension (provides information on the location, magnitude, space and time of noxius stimuli);
b) motivational-affective dimension (activities in reticular and limbic structures);
c) cognitive dimension (neocortical and higher central nervous system process, using past experiences to predict outcomes of different responses).

Also, a new model has been thought, named Neuromatrix Theory, that complements previous knowledge with the premise that central brain processes can form the basis of pain, not focusing only in peripheral events (Authors not listed, 2005). Briefly, they defend the existence of a neurosignature, unique to each person, genetically determined but modified by intrauterine and life experience.

Modulation of spinal sensitization may also have implications in clinical practice. Reduction of excitatory amino acids as glutamate (anticonvulsants) may be a strategy, as the use of NMDA antagonists, to block initial stages of central sensitization, and NSAIDs.

1.2.3.1 Ascending pathways

Nociceptive inputs activate nociceptive dorsal horn neurons, especially ascending tract neurons, and can target three different supraspinal structures: the thalamus, the amygdala and the brain stem [mesencephalic dorsal reticular nucleus, midbrain periaqueductal gray (PAG), and rostral ventromedial medulla (RVM)], producing the conscious pain sensation (Schaible and Richter, 2004; Ossipov *et al.*, 2010; Quintero, 2013). These three structures intensely communicate with each other: thalamus sends projections to the cerebral cortex and amygdale and amygdale sends to the cerebral cortex and thalamus, besides

receiving from thalamus, spinal cord and also brain stem (Schaible and Richter, 2004). The pain sensation here produced has two components. One is the sensory discriminative component, with location, duration and intensity from the responsibility of the lateral thalamocortical system (neospinothalamic pathway). The second component is the affective aspect, as the unpleasant feeling and reactions, which is produced in the medial thalamocortical system (paleospinothalamic pathway with the relay nuclei in the central and medial thalamus and the anterior cingulated cortex (ACC), insula and prefrontal cortex) (Treede *et al.*, 1999; Carver and Foley, 2000; Schaible and Richter, 2004).

1.2.3.2 Descending pathways

The nociceptive processing can also be modulated by a descending tract, originated in the brainstem nuclei, which has the ability to suppress nociceptive information processing (Schaible and Richter, 2004). There are essentially three main paths (Millan, 2002):

a) a circuit cortex / hypothalamus / PAG / medulla / dorsal horn;
b) a second circuit of cortex / amygdala / PAG / medulla / dorsal horn;
c) a third path with cortex /PAG / medulla / dorsal horn.

After the cortical inputs reach PAG, projections are sent to the medulla and the spinal cord for inhibiting nociception (Ossipov *et al.*, 2010). Medulla includes a region named RVM, as already mentioned, whose projections to the dorsal horn can increase or decrease the nociceptive input (Schaible and Richter, 2004). Both antinociceptive effects of PAG and RVM on the spinal cord are especially mediated by 5-HT, noradrenaline (NA), glycine and GABA (Basbaum and Fields, 1978; Cui *et al.*, 1999; Carver and Foley, 2000; Authors not listed, 2005). Other compounds involved are enkephalin, β-endorphin and dynorphin, known as the most potent inhibitors of nociceptive activity and found in the specific nuclei in the brain stem, spinal cord, arcuate nucleus of the hypothalamus and in the pituitary (Carver and Foley, 2000). These compounds are endogenous opioid peptides that bind to specific receptors, opioid receptors μ (β-endorphin), δ (enkephalin) and κ (dynorphin), found in high concentration in cortical, brain stem and spinal cord (Carver and Foley, 2000).

Descending modulation is essential to pain discrimination and perception. As already mentioned, changes at NMDA receptors are essential for central sensitization. However,

loss of endogenous inhibitory mechanisms can also contribute (Scholz and Woolf, 2002). This reduced inhibition can result from down-regulation of neurotransmitters, peptides and receptors expressed in the dorsal horn (GABA, glycine, catecholamines and opioid receptors), but also from cell death of inhibitory interneurons after nerve injury and ectopic activity, leading to an increased dorsal horn excitability (Woolf and Decosterd, 1999; Authors not listed, 2005). Considering this, clinical pharmacotherapy to central pain may use agents for those targets, as TCAs, selective serotonin reuptake inhibitors (SSRIs), selective serotonin and noradrenaline reuptake inhibitor (SSNRI), anticonvulsants, opioids, α_2-agonists and GABA agonists (Authors not listed, 2005).

1.3 Cancer-related pain management

Cancer is a major world problem and every year millions of new cases are diagnosed. Unfortunately, is estimated that 70 to 90 % of patients with advanced cancer suffer significant pain (Andersen and Sjogren, 1998; Carver and Foley, 2000; Lötsch *et al.*, 2010) and around 5 million people are currently suffering from cancer pain with or without satisfactory treatment (Carver and Foley, 2000). Cancer-related pain is usually a result from tumor infiltration (bones, soft tissues, nerves, viscera, blood vessels), surgery, chemotherapy or radiation and is usual to classify it in somatic (the most common), visceral and neuropathic (the second most common) (Carver and Foley, 2000). However, cancer patients generally complain of mixed pain (Grond *et al.*, 1994; Portenoy *et al.*, 1994), and are often undertreated or may not respond optimally to the therapy (Mercadante, 2011), with an enormous impact on patient's quality of life. Due to the importance and prevalence of cancer-related pain and the lack of good analgesic treatment in a large number of patients, we will now focus on the available treatments and reasons for its variability.

Management of cancer-related pain can be made through the use of specific guidelines and algorithms (Portenoy *et al.*, 1987; Carver and Foley, 2000; Mercadante, 2011) and, in fact, patients treated according to these can experience a significant reduction in pain intensity (Du Pen *et al.*, 1999). Most of cancer pain patients are pharmacologically managed in accordance with WHO guidelines and its 3-step ladder model (Figure 6) (WHO, 1996), that has been extensively validated (Ventafridda *et al.*, 1987; Zech *et al.*, 1995; Mercadante, 1999).

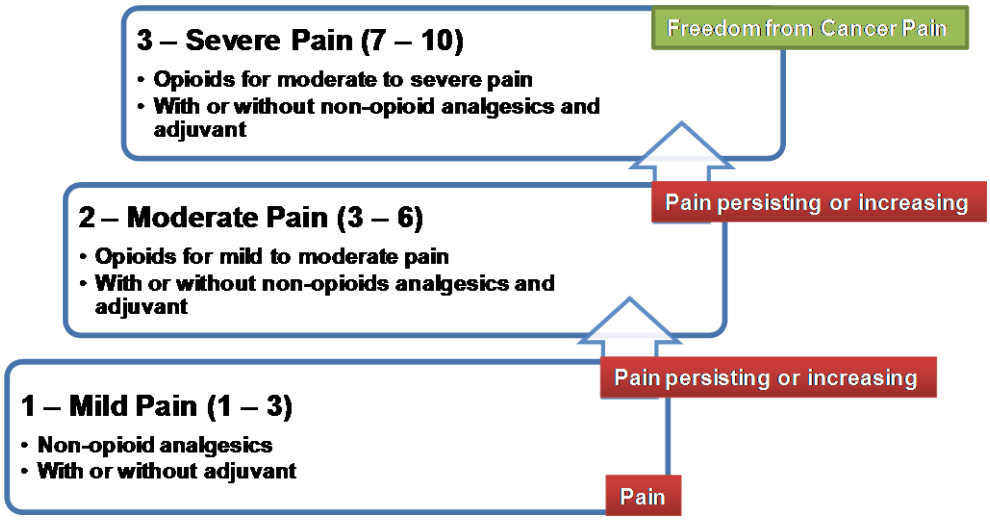


Figure 6. Three-step ladder model for pain management in cancer pain patients as suggested by WHO guidelines (WHO, 1996).

This “by the clock” medication approach also allows flexibility in the choice of analgesics and adjuvant treatment, and help cancer patients in a cost-effective manner, with its five rules (WHO, 1996; Vargas-Schaffer, 2010; Leung, 2012):

a) oral administration (when possible);
b) analgesics should be given at regular intervals, not on demand, and adjusted in accordance to patient’s level of pain;
c) the prescription should take into account the assessment of pain intensity;
d) dosing should be individualized;
e) patients, family and healthcare staff should be provided with all the necessary information about the drugs.

However, this ladder model has some limitations, especially in long-term survival, and hospitals-based palliative care approaches and new pain management models are welcomed (Kao *et al.*; Higginson *et al.*, 2002; Bakitas *et al.*, 2009; Ozcelik *et al.*, 2013), with continuous patient assessment and follow-up programs, mechanism-based and multimodal characteristics, combination therapies and interventions procedures.

Several modifications to the WHO 3-step ladder have been made, in order to obtain a better pain relief in cancer, but also in non-cancer chronic pain patients (Miguel, 2000;

Vadalouca *et al.*, 2008; Vargas-Schaffer, 2010). Some authors question the value of the ladder second step (Mercadante *et al.*, 1998; Grond *et al.*, 1999; Vielvoye-Kerkmeer *et al.*, 2000; Mystakidou *et al.*, 2003; Leung, 2012), but especially an additional fourth step based on interventional procedures seems to be required (Figure 7) (Krakowski *et al.*, 1996; Vargas-Schaffer, 2010). This adapted model has been proposed for adult chronic cancer and non-cancer pain, but also for pediatric pain, breakthrough and acute emergency pain, and allows a "step up, step down" bidirectional strategy (Krakowski *et al.*, 1996; Vargas-Schaffer, 2010).

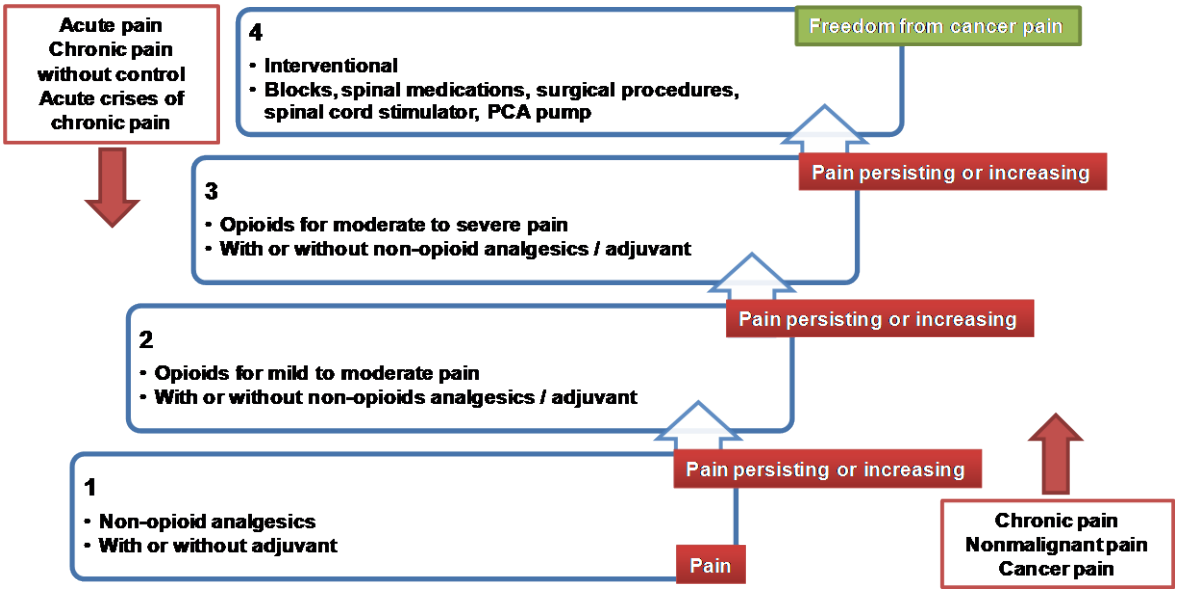


Figure 7. Proposed revision of the WHO model: a four-step ladder [adapted from (Vargas-Schaffer, 2010)]. PCA, Patient-controlled analgesia.

An interesting modified model based on the latest three-dimensional Neuromatrix pain theory was also suggested (Leung, 2012). As the cognitive and emotional dimensions were incorporated in pain processing, its management should also contain several other domains in a platform-based model (Figure 8) (Leung, 2012). This model incorporates opioids and non-opioids analgesics, adjuvant agents (anticonvulsants, muscle relaxants, antidepressants, cannabinoids), physiotherapy, physical therapy, surgical and neurosurgical procedures, cognitive behavioral therapy and psychological counseling, interpersonal reinforcement, mind-body integration, hypnosis and relaxation therapy, acupuncture, chiropractic and other complementary and alternative medicine (CAM) options (Leung, 2012). As in the revised model of bidirectional four-step ladder (Vargas-Schaffer, 2010), the clinician can move up or down the platforms, but it claims to be universally applicable to all pain scenarios (Leung, 2012).

Figure 8. Platform model for pain management [Adapted from (Leung, 2012)]. A, Physiotherapy and physical therapy; B, Mind–body integration (e.g. yoga, meditation and religious support); C, Hypnosis and relaxation therapy; D, Acupuncture; E, Chiropractic; F, External rub/lotions; G, Other CAM options (Tai chi, Tui Na); H, Muscle relaxants (e.g. cyclobenzaprine, baclofen and dantrolene); I, Injectable agents (steroids, local anesthetics); J, Interpersonal reinforcement (e.g. support group); K, Anticonvulsants (e.g. gabapentin, pregabalin and lamotrigine); L, Antidepressants (e.g. tricyclics antidepressants, SSRI, SSNRI); M, Compounds that act synergistically with opioids such as cannabinoids (nabilone); N, Cognitive behavior therapy and psychological counseling; O, Surgical and neurosurgical procedures (e.g. spinal cord stimulation, deep brain stimulation, spinal delivery of opioids, ganglion ablation by phenol or electrofrequency, sympathectomy).

Besides the new and revised models, the correct and more actual employ of the WHO method with the use of alternative administration routes and the correct pharmacological knowledge is still used. In fact, it can give an adequate pain control in most patients with advanced cancer and all healthcare workers should be informed and implement the WHO guidelines before introducing more recent models, still in validation (Mercadante, 2010). Also, a personalized and individual treatment still remains as the key for achieving the best pain relief, requiring a profound knowledge of drug characteristics, patient's responses and alternative treatments (Mercadante, 2010).

1.3.1 Pain assessment

One of the most important processes in Hospitals and Palliative Care Units for cancer pain management in order to achieve an effective individualized therapy is the regular assessment, preferably in all stages of disease. A correct pain assessment should be done accordingly to certain rules and guidelines (Ripamonti *et al.*, 2011):

a) pain must be assessed and re-assessed, identifying its cause, onset, type, site, duration, intensity, relief and temporal characteristics, as well as the presence of trigger factors and other symptoms or signs, helping to choose the best analgesic, which efficacy have also to be assessed;
b) the patient must also be assessed and re-assessed, with complete physical examination, identifying the interference of pain in the patient's quality of life, the impact of the disease and therapy, physical, psychological and functional status and the presence of symptoms and adverse effects associated with disease, therapy and cancer pain syndromes;
c) the ability to communicate with the patient and his family should be assessed and re-assessed, as they all need to understand the disease and therapy and the physician needs to understand the patient and family's requirements.

For an adequate and regular assessment, healthcare professionals are welcomed to use some validated assessment tools (Caraceni *et al.*, 2002). Considering the pain assessment limited to its intensity, a unidimensional structure can be used. However, taking into account that pain is a complex human experience, multidimensional tools have also been developed (Caraceni *et al.*, 2002). For a correct measurement and assessment of pain, the chosen tool must be valid and appropriate for the purpose.

The most frequently self-reporting standardized unidimensional scales are the visual analogue scale (VAS), the verbal rating scale (VRS) and the numerical rating scale (NRS) (Figure 9) (Caraceni *et al.*, 2002), which are well validated in cancer populations, with equivalent quality (Wallenstein *et al.*, 1980; Littman *et al.*, 1985; Jensen *et al.*, 1986; Caraceni *et al.*, 2002). Also, the Face Pain Scale Revised (FPS-R) can be extremely useful in the pediatric population (Hicks *et al.*, 2001). The number of words in the VRS or faces in FPS-R can vary (Caraceni *et al.*, 2002), but all the scales can be related to the numeric categorization of pain, helping to divide it in mild, moderate or severe and integrate the result in the WHO analgesic ladder (Figure 6). These scales can also be

used for measurement of pain relief, but its validity is limited to short-term intervention studies (maximum 24 hours) (Caraceni *et al.*, 2002).

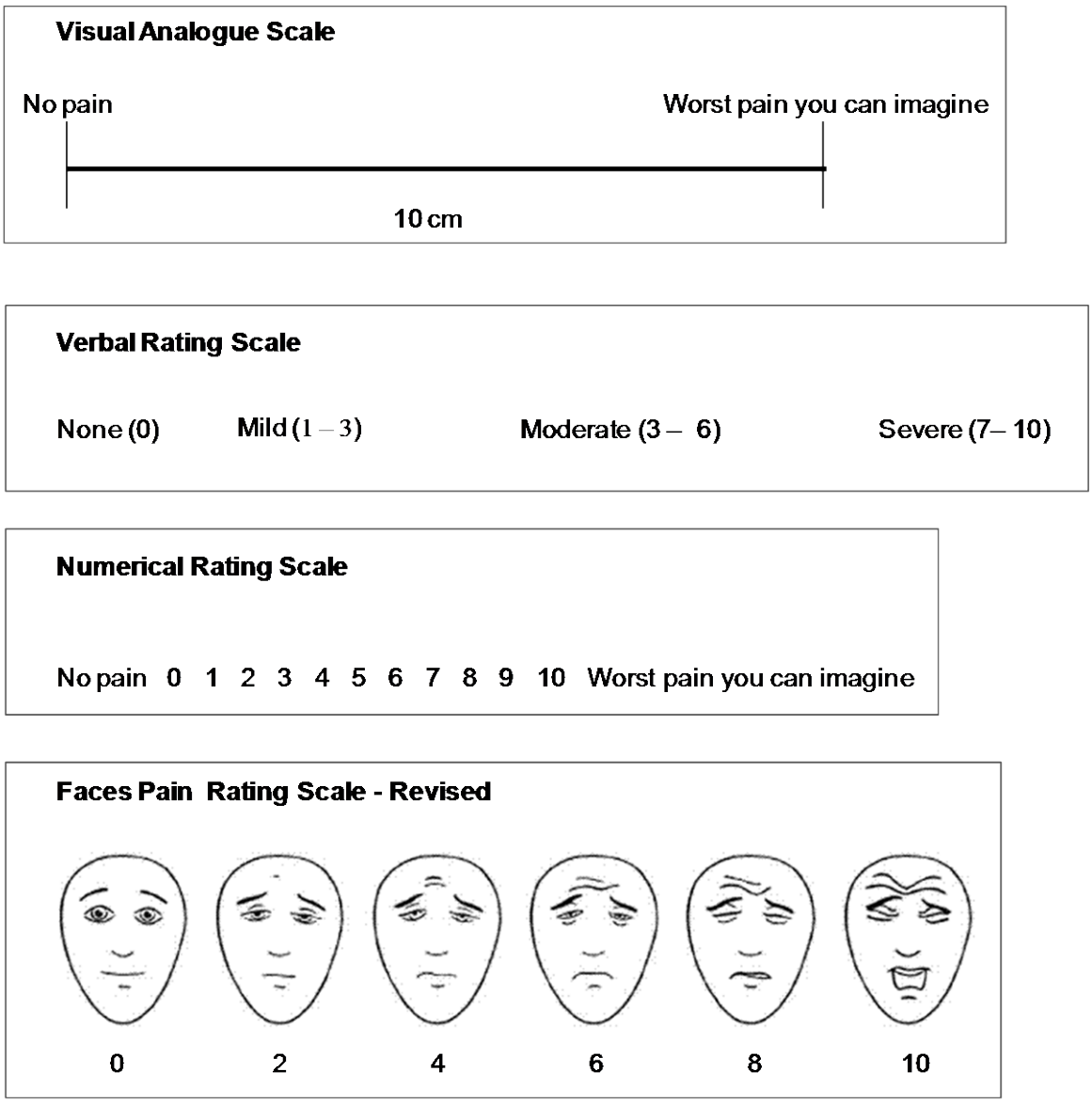


Figure 9. Pain intensity rating using four scales: visual analogue scale, verbal rating scale, numerical rating scale and faces pain rating scale revised.

Concerning the multidimensional tools, McGill Pain Questionnaire, Brief Pain Inventory and Memorial Pain Assessment Card are the most used, with the Brief Pain Inventory and the McGiil Pain Questionnaire being the most recommended (Caraceni *et al.*, 2002). These questionnaires are intended to collect information about the history, location, intensity, and quality of pain, interference of pain in patient's life and all pain dimensions

(Melzack, 1975; Serlin *et al.*, 1995). Additionally, several multidimensional measures and questionnaires of health-related quality of life have been developed and validated, including assessment of physical, psychological and social functions, along with several symptoms and life quality parameters (Hearn and Higginson, 1997; Jordhoy *et al.*, 2007). Despite a more comprehensive vision of pain and patient's status, these questionnaires are long and can be difficult to complete (Caraceni *et al.*, 2002).

As most of these scales and questionnaires depend on patient's status, older age and patients with limited cognitive skills or cognitive impairment may fail to be evaluated. In these situations, physicians and health care professionals may observe pain-related behaviors and discomfort (facial expressions, vocalization, movements, interactions, routine activity) to detect the presence of pain (Kaasalainen, 2007; van Herk *et al.*, 2007). In the last decade, improvements in this area are being made and some pain rating scales seem to be efficient in adults with cognitive impairment (Ware *et al.*, 2006) and several tools are now available for older, non-verbal or with cognitive impairment patients (Kovach *et al.*, 2002; Lane *et al.*, 2003; Herr *et al.*, 2006; Mahoney and Peters, 2008).

1.3.2 Pharmacological approaches

Pain relief can be achieved by several means, but pharmacological approach remains the mainstay of cancer pain management, as stated by WHO and its three-step model (WHO, 1996). Most importantly is the selection of the right analgesic, right dose and regular schedule to maximize analgesic effect and minimize adverse effects (Carver and Foley, 2000). Treating cancer pain with a sequential use of drugs starts with the non-opioid first step (e.g. paracetamol, NSAIDs and adjuvant drugs such as antidepressant or anticnvulsant drugs). Persisting pain leads to the introduction of an opioid for mild to moderate pain (e.g. codeine, hydrocodone, oxycodone, tramadol and dextropropoxyphene), with or without non-opioid or adjuvant drugs, and then a strong opioid to moderate to severe pain (e.g. morphine, hydromorphone, fentanyl, methadone, oxycodone, oxymorphone and levorphanol).

1.3.2.1 Non-opioid analgesics

Paracetamol and NSAIDs, including acetylsalicylic acid (ASA), are recommended as the first step of the WHO analgesic ladder, for mild cancer pain (WHO, 1996; Carver and

Foley, 2000; Mercadante, 2011; Ripamonti *et al.*, 2011). These compounds are usually administered *per os*, but their analgesia is limited by the designated “ceiling effect”, in which increasing the dose beyond a certain level will not produce an increase in the peak effect (Carver and Foley, 2000).

The mechanism of action of paracetamol has been a controversial subject for many years. However, recent studies pointed out that pharmacological action of paracetamol seems to result from the peripheral and especially central inhibition of the synthesis of prostaglandins from arachidonic acid, by inhibiting COX-1 and COX-2 (Graham *et al.*, 2013). Each enzyme possesses a cyclooxigenase and a peroxidase activity. Firstly, the cyclooxigenase activity occurs, with the oxidation of arachidonic acid to the hydroperoxide prostaglandin G2. Subsequently, this species is metabolized by the peroxidase activity to PGH2, and then to prostanoids (Graham *et al.*, 2013). Moreover, while the cyclooxigenase activity is dependent on the peroxidase function, the latter is independent (Smith *et al.*, 2000), with paracetamol as a substrate (Harvison *et al.*, 1988). Oxidation of paracetamol via peroxidase activity competes with the oxydation of a tyrosine residue to a tyrosine phenoxyl radical, considered essential for the cyclooxigenase activity of both COX-1 and COX-2 (Boutaud *et al.*, 2002). Due to paracetamol, the essential tyrosine radical becomes less available, resulting in the inhibition of cyclooxigenase activity.

However, there is an apparent COX-2 selectivity of paracetamol, indicated by its poor anti-platelet activity and good gastrointestinal tolerance, probably due to peroxide concentration (Graham *et al.*, 2013). In fact, in the presence of low concentrations of arachidonic acid, COX-2 pathway is preferentially activated, explaining the antinociceptive and antipyretic action of paracetamol, and the lack of its anti-inflammatory capacity in pathologies with high peroxide levels as rheumatoid arthritis and acute gout (Murakami *et al.*, 2000; Li *et al.*, 2008; Graham *et al.*, 2013). In addition, paracetamol inhibits other peroxidase enzymes, as myeloperoxidase, decreasing the formation of pro-inflammatory halogenating oxidants (Koelsch *et al.*, 2010; Graham *et al.*, 2013). Moreover, the antinociceptive action seems to be linked to other neuronal systems, as serotoninergic, opioid, endocannabinoid and cholinergic, where inhibitors of these systems can also block the analgesic effect of paracetamol (Pini *et al.*, 1997; Mallet *et al.*, 2008; Graham *et al.*, 2013). Paracetamol can also inhibit some nociceptive effects of excitatory neurotransmitters or factors, as glutamate, SP and noradrenaline (Choi *et al.*, 2001; Miranda and Pinardi, 2004). However, further studies are still required to conclude about the relation of therapeutic effect of paracetamol and neuroral systems.

Analgesic action of NSAIDs is very well-known and generally results from the peripheral inhibition of COX, decreasing prostaglandin synthesis. However, likewise paracetamol, analgesic effect of NSAIDs can also be mediated by a central COX inhibition (Malmberg and Yaksh, 1992; Graham *et al.*, 2013). In fact, several NSAIDs have demonstrated to inhibit SP and glutamate hyperalgesic effect by spinal COX inhibition (Malmberg and Yaksh, 1992), with ASA also inhibiting glutamate-induced nociceptive action, but not SP (Choi *et al.*, 2001). Moreover, other neuronal systems might also be related with NSAIDs antinociceptive effect, as serotoninergic system (Miranda *et al.*, 2003; Graham *et al.*, 2013).

The efficacy of these drugs in cancer pain has been reported and a number of non-opioid analgesics are available, the choice depending of the local availability and costs, as there is no evidence supporting the use of a drug over another (WHO, 1996; McNicol *et al.*, 2005; Mercadante, 2011; Ripamonti *et al.*, 2011). The combination of paracetamol with strong opioids has been reported as an improvement in pain relief and well-being and has become a routine in some hospitals (Stockler *et al.*, 2004; Axelsson *et al.*, 2008). However, this is used despite the small number of evidences of demonstrable additional analgesic effect and was not always confirmed by other studies (McNicol *et al.*, 2005; Israel *et al.*, 2010).

Non-opioid analgesics are especially helpful for pain caused by soft tissue and muscle infiltration and NSAIDs are very important for bone metastases-related pain, due to the high concentration of prostaglandins produced in the affected bone (WHO, 1996). Nevertheless, the long-term use of NSAIDs or COX-2 selective inhibitors needs to be monitored and reviewed due to its toxicity, namely gastrointestinal bleeding [aspirin, indomethacin, naproxen, sulindac, ketoprophen and piroxicam (Henry *et al.*, 1996)], platelet dysfunction, renal failure and risk of thrombotic cardiovascular adverse reactions (Ripamonti *et al.*, 2011). Also, risk of allergic phenomena should be taken into account, particularly for salicylates. Some adverse effects can be prevented by choosing analgesics with fewer or no antiplatelet effects (e.g., choline magnesium trisalicylate, paracetamol) or fewer gastrointestinal side effects (e.g., ibuprofen) (Carver and Foley, 2000).

1.3.2.2 Opioid analgesics

Opioid analgesics, with morphine as the prototype, remain as the mainstay treatment for cancer pain, despite their adverse effects and association with tolerance, dependence and addition (WHO, 1996). The widespread of opioids in chronic cancer pain is particularly related to the strong evidence of their efficacy, an increased knowledge of their clinical pharmacology and to the development of guidelines to guarantee a safe use (Geppetti and Benemei, 2009). Also, unlike NSAIDs, strong opioids do not appear to have a dose-related "ceiling", and generally a dose increase leads to a better analgesic effect, until the minimal effective dose is achieved.

Opioids exert their action by binding to G protein-coupled opioid receptor [classic μ, δ, κ receptors and "non-classic" nociceptin/orphanin FQ peptide (N/OFQ) receptor (NOP)] (McDonald and Lambert, 2013). Besides their known location in the nervous system (e.g. PAG, medial prefrontal cortex, amygdala, hippocampus, thalamus), opioid receptors are also distributed in peripheral organs, such as heart, lung, liver, gastrointestinal and reproductive tracts (Feng *et al.*, 2012; Bodnar, 2013). The activation of μ-opioid receptors seems to elicit the major behavioral responses, including analgesia, hyperlocomotion, respiratory depression, constipation and immunosuppression, as revealed by mice lacking μ-opioid receptor (Waldhoer *et al.*, 2004). Additionally, animal studies also revealed the important role of this opioid receptor sub-type in the neural circuit of reward (Hall *et al.*, 2001; Berrendero *et al.*, 2002; Waldhoer *et al.*, 2004).

Other opioid receptor subtypes also proved to be related to pain perception, stress response and affective reward states (Wang *et al.*, 2010; Bodnar, 2013; Zhou *et al.*, 2013). δ-opioid receptors have shown to exert some analgesic effects, with limited side effects (Waldhoer *et al.*, 2004), making it a promising target for new analgesics. These receptors have essentially an intracellular localization, rather than on the surface of most cells, which might explain the relatively high doses of δ-opioid agonists for analgesia (Cheng *et al.*, 1995). Along with analgesic effects, these receptors were also associated with the development of morphine dependence and tolerance (Abdelhamid *et al.*, 1991; Suzuki *et al.*, 1997) and beneficial effects in affective disorders (Gavériaux-Ruff and Kieffer, 2002). Concerning κ-opioid receptors, they have been especially related to dysphoria but also to stress-induced emotional responses (Waldhoer *et al.*, 2004) and the possible treatment of visceral pain (Gebhart *et al.*, 2000). On the other hand, NOP receptors were associated with anti-analgesic action and tolerance (McDonald and Lambert, 2005; McDonald and Lambert, 2011), but also to anxiety, feeding, learning and memory and urogenital activity (McDonald and Lambert, 2005).

In addition to the more well-known functions, opioid receptors have also been associated with ionic homeostasis, cell proliferation, neuroprotection, epileptic seizures, immune functions, feeding, obesity, cardiovascular regulation, learning and memory, gastrointestinal, renal and hepatic functions, general activity and neurodegenerative diseases (Feng *et al.*, 2012; Bodnar, 2013). Also, some studies suggest the existence of physical interaction between opioid receptors, which would contribute to their final effect, as the enhance of μ-agonists analgesic effect by δ-agonists or the reduction of the development of tolerance and dependence by μ-agonists by δ-antagonists (Miaskowski *et al.*, 1990; Ananthan, 2006).

Concerning opioid action in their receptors, especially in pain, the activation results in chain reactions that include several second messengers, as cAMP and ion channels such as the potassium or calcium (Figure 10) (McDonald and Lambert, 2013). The opioid receptors are part of a descending inhibitory system and their activation leads to a decrease of calcium entry into the cell and of neurotransmitter release, such as SP and CGRP, from primary afferents (Collin *et al.*, 1993; Kondo *et al.*, 2005; Geppetti and Benemei, 2009). Also, the potassium efflux in the post-synaptic neuron is enhanced leading to hyperpolarization and the nociceptive signal is interrupted (Geppetti and Benemei, 2009).

The main adverse effects patients develop are constipation, nausea, vomiting, urinary retention, pruritus and development of dependence, addition and tolerance. Dependence is related to the withdrawal symptoms if the opioid is abruptly discontinued or after the administration of an antagonist or mixed agonist-antagonist, and the symptoms intensity are related to the opioid, dose and duration of treatment. On the other hand, addition is related to a behavioral pattern of drug use characterized by continued craving for the drugs to obtain other effects than pain relief. Due to this possibility, the attempts of physicians and patients to not reach addition usually lead to lack of adequate cancer pain management. However, cancer pains chronically receiving opioids usually develop dependence but not addition (Porter and Jick, 1980; WHO, 1996; Carver and Foley, 2000).Tolerance represents the necessity of increasing the dose to provide the same effect. In cancer patients, dose escalation can happen due to pharmacologic tolerance but especially due to disease progression (Carver and Foley, 2000). One of the first signs of tolerance development is the patients' report of shorter duration of the analgesic effect that can be often mistaken as an early sign of addition (Carver and Foley, 2000). Switching to alternatives analgesics, adjuvant drugs, anesthetics and interventional procedures may be used to manage a tolerant patient (Carver and Foley, 2000).

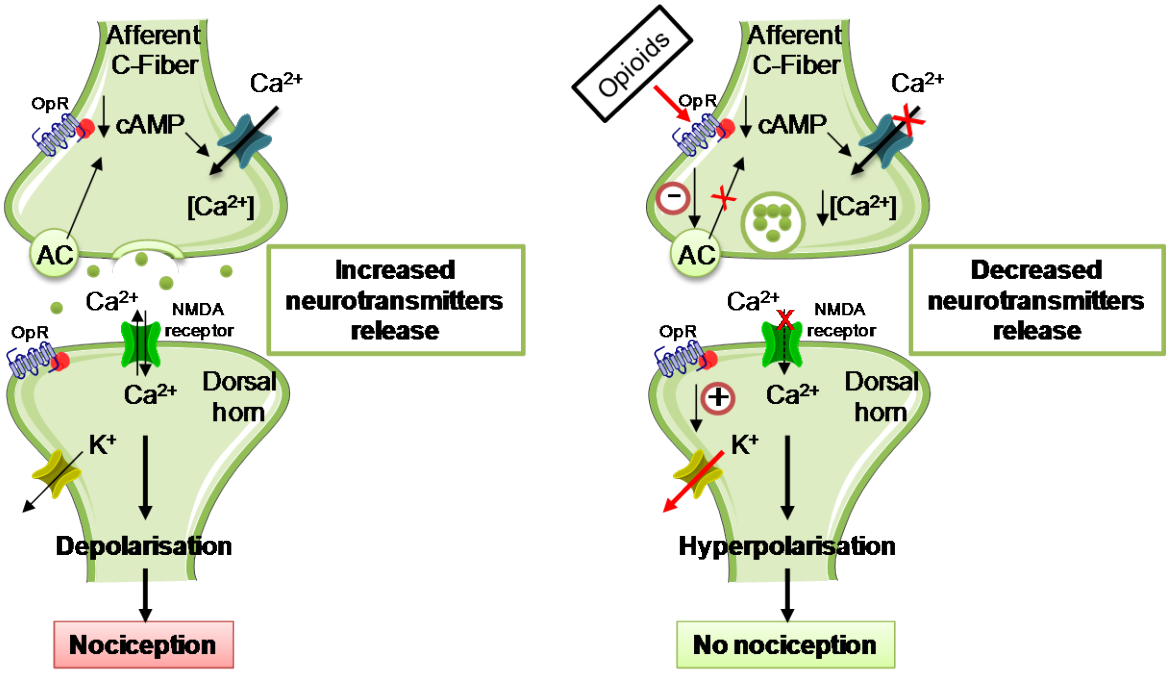


Figure 10. Opioids action in afferent C-fibers and post-synaptic neurons, leading to the analgesic effect. AC, adenylyl cyclase; cAMP, cyclic adenosine monophosphate; NMDA, *N*-methyl-D-aspartate; OpR, opioid receptor.

Additionally, signs of central toxicity can appear as drowsiness, cognitive impairment, confusion, hallucinations and myoclonic jerks, along with the development of hyperalgesia / allodynia (Carver and Foley, 2000; Ripamonti *et al.*, 2011). These effects can be managed by reducing opioid dose and co-administering another analgesic or switching to another opioid or route, which would be especially important in cases of opioid-induced hyperalgesia / allodynia (Cherny *et al.*, 2001). Moreover, certain drugs can relieve those symptoms as antiemetics, laxatives, benzodiazepines (for confusion). In case of (rare) severe opioid overdose, a short-acting antagonist, as naloxona, can be administered (Carver and Foley, 2000; Ripamonti *et al.*, 2011).

1.3.2.2.1 Mild to moderate pain

According with WHO step 2, mild to moderate pain should be treated with a weak immediate-release opioid (codeine, tramadol, dihydrocodeine, propoxyphene), which may have limited analgesic efficacy, plus paracetamol or NSAIDs. As already mentioned, this second step is surrounded by controversy and the efficacy and advantages of using this

step have been contested (Ventafridda *et al.*, 1987; Eisenberg *et al.*, 1994; Mercadante *et al.*, 1998; Grond *et al.*, 1999; Vielvoye-Kerkmeer *et al.*, 2000; Mystakidou *et al.*, 2003; Ripamonti *et al.*, 2011; Leung, 2012). Additionally, weak opioids have a dose-related "ceiling effect", as NSAIDs, leading some authors to defend the abolition of this second step and start an earlier use of low doses of morphine, but the studies are still inconclusive (Marinangeli *et al.*, 2004; Maltoni *et al.*, 2005; Mercadante *et al.*, 2006; Ripamonti *et al.*, 2011).

Codeine is a well-known opioid, however it can be poorly tolerated at higher doses and genetic variation of the major metabolic enzyme (cytochrome P450 2D6, CYP2D6) can lead to unexpected codeine and morphine concentrations, and therefore to unexpected adverse effects (Mikus *et al.*, 1991; Chary *et al.*, 1994). On the other hand, tramadol has been considered a safer opioid analgesic for mild to moderate pain, with lower probability of dependence and respiratory depression. However, the same genetic consideration of codeine has to be made for tramadol, as for dihydrocodeine and oxycodone, since they share the same metabolic pathway (O-demethylation) (Mikus *et al.*, 1991).

1.3.2.2.2 Moderate to severe pain

Strong opioids are definitely the recommended group of drugs for cancer-related pain. Morphine is the first-choice drug, the only opioid in WHO essential drug list for adults and children with pain and has been used for several years in Palliative Care Units and Hospitals, due to its efficacy, tolerance and low costs (Ripamonti *et al.*, 2011). After morphine administration, the drug undergoes a variety of metabolic pathways, but is extensively metabolized in the liver especially by Uridine 5'-diphospho-Glucuronosyltransferase 2B7 (UGT2B7) producing two important metabolites, morphine-6-glucuronide (M6G; 10-15 %) and morphine-3-glucuronide (M3G; 45-55 %), by glucuronidation of the 6-OH alcoholic group and the 3-OH phenolic group, respectively (Figure 11) (Carrupt *et al.*, 1991). M6G is a potent opioid receptor agonist with a higher analgesic activity than morphine, however M3G has no opioid action, thought it seems to have a role in the side-effects usually described, as well as hyperalgesia / allodynia, neurotoxicity and an antagonistic effect, decreasing morphine analgesia (Carrupt *et al.*, 1991; Christrup, 1997; Holthe *et al.*, 2002). As with codeine and tramadol, alterations in metabolism ratios might lead to different analgesic and adverse effects, especially in case of kidney disease, as both metabolites are especially eliminated by the kidney. Nevertheless, morphine has some properties that contribute to be considered a safe drug,

especially the linearity of morphine and metabolites pharmacokinetics after repeated administration, which probably indicates that its metabolic pathway is not subject to auto-induction.

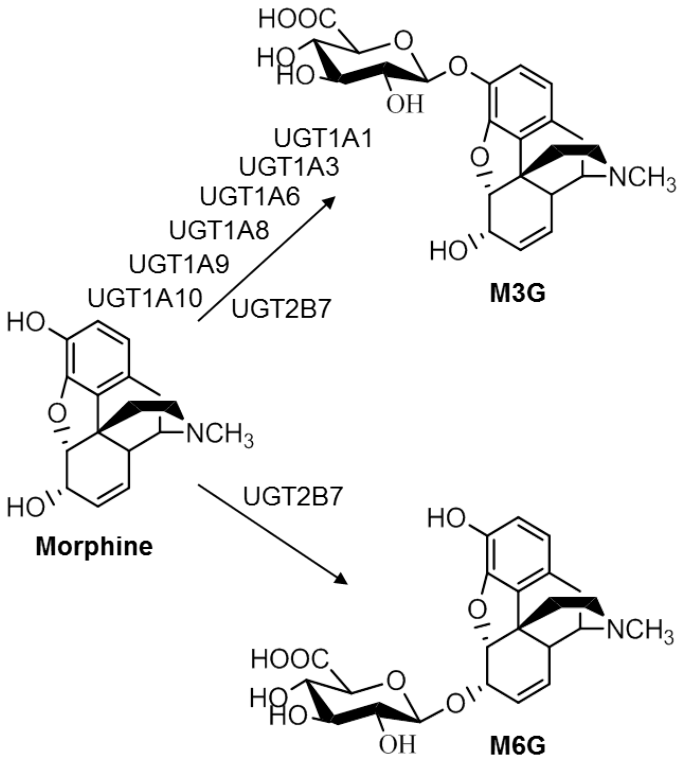


Figure 11. Morphine metabolism in M3G and M6G. M3G, morphine-3-glucuronide; M6G, morphine-6-glucuronide; UGT, UDP-Glucuronosyltransferase.

Nowadays, several other strong opioids are used across Europe, as methadone, oxycodone, hydromorphone, fentanil, alfentanil, buprenorphine, heroin, levorphanol and oxymorphone. A recent synthetic opioid is tapentadol, originally developed for moderate to severe chronic non-cancer pain (Hoy, 2012). Similarly to tramadol, this opioid has a double mechanism: μ-opioid receptor agonist (lower affinity than other strong opioids) and inhibition of NA reuptake, with an expected reduction of adverse effects profile and intensity (Kress, 2010; Hoy, 2012). Meanwhile, tapentadol efficacy in cancer pain patients was also described, but not a different intensity of adverse effects (Mercadante *et al.*, 2012). More studies are necessary to conclude about tapentadol advantages in cancer-related pain.

According to WHO guidelines, opioids should be preferably administered by oral route (WHO, 1996). However, in today's medicine, the chosen route of administration is

increasingly dependent on the patients' condition and pain assessment and patients requiring urgent relief should be treated and titrated with parenteral opioids (especially subcutaneous or intravenous), taking into account the equivalent dose and the relative potency ratio (Ripamonti *et al.*, 2011). Transdermal administration for fentanyl and buprenorphine has been increasingly used in patients unable to swallow and with poor compliance or tolerance to morphine (Ripamonti *et al.*, 2011). Also, buprenorphine has been shown to be a safe choice in patients with renal impairment and undergoing hemodialysis treatment (Boger, 2006). Other alternatives to oral morphine are the immediate and modified-release formulations of hydromorphone and oxycodone, and methadone, the latter to be used with greater caution (Ripamonti *et al.*, 2011).

In order to manage constipation, the most common and refractory side effect in cancer patients treated with opioids (Holzer *et al.*, 2009), opioid antagonists as naloxone, started to appear as an option, as they only affect gastrointestinal receptors, not diminishing central analgesic effects (Gaertner and Schiessl, 2013). Naloxone is a peripherally operating opioid antagonist, with low bioavailability due to a substantial first-pass hepatic metabolism, and often used with oxycodone (Reid *et al.*, 2006). Later on, the efficacy of an oxycodone / naloxone prolonged-release combination was reported for chronic non cancer pain patients (Simpson *et al.*, 2008; Lowenstein *et al.*, 2010). Studies for cancer-related pain also took place and the fixed combination seemed a promising approach (Ahmedzai *et al.*, 2012; Mercadante and Giarratano, 2013). However, further studies are necessary and precaution should be taken in dose escalation, that might increase the bioavailability of naloxone, and also in patients with hepatic malfunction, as naloxone will not undergo complete hepatic metabolism and might reverse opioid analgesia at the central opioid receptors (Gaertner and Schiessl, 2013).

1.3.2.2.3 Breakthrough pain

Breakthrough pain, as already mentioned, is defined as a transitory increase in pain intensity in patients on analgesic treatment regularly administered, with an opioid-controlled baseline pain. To treat this type of pain it is necessary to establish rescue doses of opioids (Mercadante, 2010). The physician can use a rapid onset and short half-life opioid, as immediate-release morphine, in about 10 – 15 % of the total daily dose, every 2 – 3 hours (Mercadante, 2010; Ripamonti *et al.*, 2011). However, more than four rescue doses indicate that the baseline opioid treatment has to be adapted (Ripamonti *et al.*, 2011). Oral transmucosal administrations, as the new rapid onset formulations of

fentanil, can lead to pain relief in a similar way of intravenous morphine (10 – 15 minutes), but only in active and collaborating patients (Ripamonti *et al.*, 2011). New effervescent buccal tablets, intranasal or sublingual fentanil formulation have emerged, became more accepted and the pain relief is achieved similarly (Grape *et al.*, 2010; Davis, 2011).

1.3.2.3 Adjuvant drugs for analgesia

According to WHO analgesic ladder, besides opioid and non-opioid analgesics, physicians can also employ some adjuvant drugs to enhance the analgesic effect and diminish opioid doses (Figure 12). This situation occurs especially for the treatment of cancer-related neuropathic pain, generally a result of regional nerve damage from tumor infiltration into nerves and plexuses, radiation, fibrosis, chemotoxicity or surgical injury (Portenoy, 1989). Another usual situation for the use of adjuvant drugs is bone pain. In both cases, the ideal is to choose an individualized, simple but potent combination of drugs.

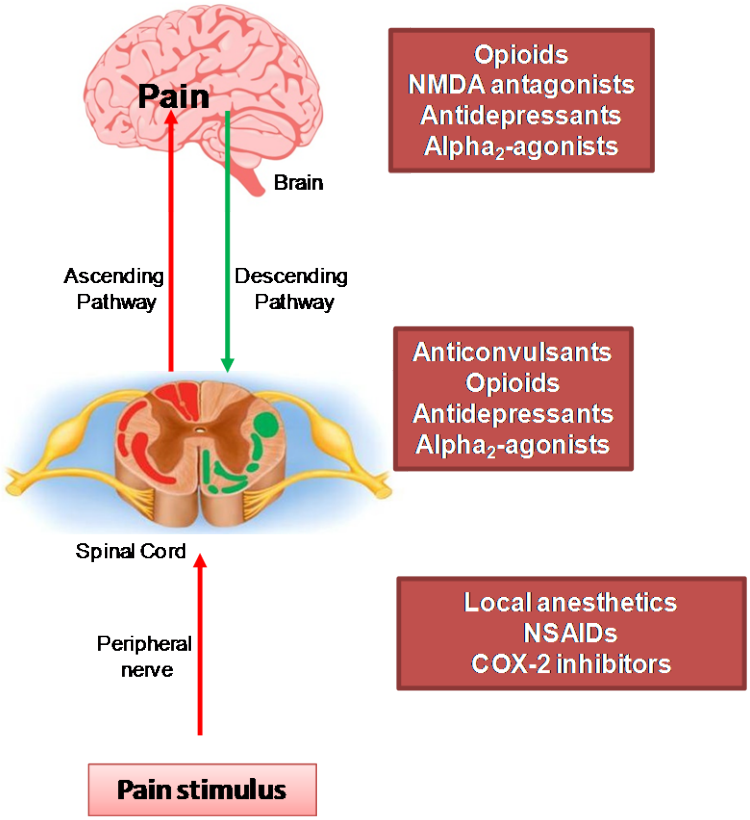


Figure 12. Pharmacological modulation with opioids and adjuvant drugs. COX, cyclooxygenase; NMDA, *N*-methyl-D-aspartate; NSAIDs, nonsteroid anti-inflammatory drugs.

1.3.2.2.1 Antidepressant drugs

Antidepressants drugs have been probably the most helpful class of drugs for neuropathic pain (Sindrup *et al.*, 2005), despite the few number of studies referring to the use of antidepressant agents for treatment of cancer pain (McGeeney, 2008). As already mentioned, noradrenergic and serotoninergic systems are involved in pain mechanisms and the influence of antidepressants in these two systems, promoting the endogenous descending antinociceptive system, can explain their analgesic effect (Carver and Foley, 2000). Besides their strong adverse effects, TCA seem to be the most effective group, particularly amitriptyline, but reports of the efficacy of imipramine and desipramine exist (Kishore-Kumar *et al.*, 1990; Max *et al.*, 1992; Zin *et al.*, 2008). Another advantage of TCA is related to its sedatives properties, particularly helpful in patients with insomnia (McGeeney, 2008). Among SSRI, paroxetine has also demonstrated efficacy, with fewer side effects than TCA (Sindrup *et al.*, 1990) and more recent antidepressants as venlafaxine and duloxetine seem very promising for cancer-related pain, also with fewer adverse effects (McGeeney, 2008; Zin *et al.*, 2008; Mercadante, 2011).

1.3.2.2.2 Anticonvulsant drugs

Anticonvulsant drugs are the second most well-studied class for neuropathic pain, after antidepressants (McGeeney, 2008). Among them, carbamazepine, gabapentin and pregabalin represent drugs of choice for trigeminal neuralgia and other neuropathic pain, with Food and Drug Administration (FDA) approval (Carver and Foley, 2000; McGeeney, 2008; Mercadante, 2011). Topiramate, oxcarbazepine and lamotrigine are also used off-label for different pain syndromes, while phenytoin, phenobarbital, levetiracetam and zonisamide are nowadays rarely prescribed (McGeeney, 2008).

Anticonvulsants are effective adjuvant drugs for cancer-related pain due to their mechanism of action, especially by modulating voltage-gated ion channels (sodium and calcium) and enhancing GABA mechanism. Gabapentin and pregabalin are structural analogues of GABA, however their pharmacological action is accomplished by modulating specific voltage-gated calcium channels and calcium influx is reduced (Luo *et al.*, 2001; Lesser *et al.*, 2004; Shimizu *et al.*, 2004). There are more evidences supporting their efficacy in chronic non-cancer pain (McGeeney, 2008) but major advantages of these compounds are the very few drug-drug interactions and the low percentage that binds to plasma proteins (McGeeney, 2008).

1.3.2.2.3 Oral and local anesthetic agents

Oral anesthetics have been reported for the management of neuropathic pain, with mexiletine being considered the safest drug (Carver and Foley, 2000). Non-systemic means, namely topical local anesthetics or capsaicin, can also be used for cancer-related neuropathic pain (Carver and Foley, 2000; NCCN, 2006; McGeeney, 2008). Among these, lidocaine patch 5 % has been approved by FDA (Galer *et al.*, 2002). The lidocaine patch has beneficial effects for the patient by two mechanisms: reduces the ectopic activity in Na_V channels of damaged nerves, and the patch itself provides a mechanical barrier that decreases allodynia (Fields *et al.*, 1998; Sawynok, 2005). The efficacy has already been reported, including in cancer pain patients (Rowbotham *et al.*, 1996; Galer *et al.*, 2002; Meier *et al.*, 2003; Fleming and O'Connor, 2009; Lopez Ramirez, 2013).

1.3.2.2.4 Bone pain and bisphosphonates

Bone metastatic disease often implies several skeletal complications, such fracture, spinal compression and/or skeletal related events, i.e., bone surgery, inducing serious pain and morbidity (Gaertner and Schiessl, 2013). Bisphosphonate drugs (clodronate, pamidronate, ibandronate, zoledronic acid) have been reported to reduce skeletal complications, particularly severe bone pain associated with bone metastatic disease (Coleman, 2004, 2005). These compounds are used in patients with bone lesions from solid tumors but also in multiple myeloma, with ibandronate and zoledronic acid showing the highest potency (Carver and Foley, 2000; Gaertner and Schiessl, 2013; Kmetec and Hajdinjak, 2013). Some studies also claim that bisphosphonates may be useful for pain and skeletal complications but also for improved survival, due to their capacity of inhibit bony attachment of cancer cells, decrease cytokine production and induce apoptosis of tumor cells (Mercadante, 1997; Pereira *et al.*, 1998).

Despite the use of bisphosphonates, these drugs cannot avoid skeletal related events in about 50 % of patients (Van Poznak *et al.*, 2011). However, the identification of osteoprotegerin (OPG) and its ligands [receptor activator of nuclear factor kappa-B ligand (RANKL), also known as osteoprotegerin ligands (OPGL)] as critical for bone remodeling has opened new pathways for bone pain and skeletal related events (Gaertner and Schiessl, 2013) and RANKL inhibition may be helpful. Denosumab, a RANKL inhibitor, seems to prevent skeletal related events and cancer pain due to bone metastases better

than bisphosphonates (Fizazi *et al.*, 2011; Lipton *et al.*, 2012), but further studies and economical costs have to be considered (Gaertner and Schiessl, 2013)

1.3.2.2.5 Corticosteroids

Corticosteroids are widely used as adjuvant analgesics for pain syndromes associated with raised intracranial pressure, acute spinal cord compression, superior vena cava syndrome, metastatic bone pain, neuropathic pain due to infiltration or compression by tumor, and hepatic capsular distension (Carver and Foley, 2000; Jost, 2005; McGeeney, 2008). Pain patients with advanced cancer may benefit from steroids administration in pain management, with reduced opioid doses and improved quality of life, but also in appetite, nausea and mood (Della Cuna *et al.*, 1989; Carver and Foley, 2000; Lauretti *et al.*, 2013).

1.3.2.2.6 Cannabinoids

In the last years, cannabinoids have emerged as a possible new class of adjuvant drugs for chronic cancer and non-cancer pain (Pertwee, 2006). The theory behind their use is related to the fact that cannabinoids seem to mimic endogenous cannabinoids (anandaminde, 2-arachidonoyl glycerol) and bind to cannabinoid receptors (CB), CB1 and CB2 (Pertwee, 2006). Pain relief has been described for dronabinol and annabidiol, as well as a joint effect of opioids and cannabinoids (Welch and Stevens, 1992; Pertwee, 2006; Portenoy *et al.*, 2012). Some authors tried to explain this effect by the location of receptors of both classes in the descending pain pathway and the fact that cannabinoids seem to elicit the release of endogenous opioid precursors (Gaertner and Schiessl, 2013). Recently, nabiximols, a novel cannabinoid formulation with extract of *Cannabis sativa* that has shown an analgesic effect in peripheral neuropathic pain (Nurmikko *et al.*, 2007), was studied in cancer pain patients. The results were disappointing, though pointing to some advantages in pain intensity at lower doses, showing that the merits of cannabinoids in cancer-related pain are yet limited and further studies are necessary (Portenoy *et al.*, 2012; Gaertner and Schiessl, 2013).

1.3.2.2.7 Ketamine and dextromethorphan

Ketamine has been administered off-label at sub-anesthetic doses for cancer pain, in combination with opioids (Kerr *et al.*, 2011). Ketamine is a non-competitive antagonist of NMDA receptors that are involved in pain transmission and processing as already mentioned. Also, ketamine interrupts cholinergic transmission and inhibits reuptake of NA and 5-HT (Gaertner and Schiessl, 2013). The administration of ketamine has not been based in clinical and controlled studies, but a multisite, double-blind, randomized, placebo-controlled trial made recently by Hardy and collaborators (Hardy *et al.*, 2012) has shown disappointing results, with no differences comparing with the placebo group and an intense incidence of adverse effects. Dextromethorphan, another non-competitive antagonist of NMDA receptors has also been used in combination with morphine, but again no clinical benefit was found in cancer pain patients (Dudgeon *et al.*, 2007; Mercadante, 2011). Still, NMDA receptor antagonists are studied as an analgesic-target.

1.3.2.4 Other analgesic / adjuvant agents and future perspectives

Other adjuvant agents are used and several new perspectives are being investigated. Ziconotide is a N-type voltage-sensitive calcium channel antagonist that blocks the entry of calcium. It was approved by FDA for severe chronic pain by intrathecal administration in patients intolerant or refractory to other treatment (Wermeling, 2005). Ziconotide was already studied in cancer pain patients, improving pain intensity, but has several possible adverse effects, as neurologic impairment and psychiatric symptoms (Staats *et al.*, 2004). Given the potential serious risks, evidence of efficacy and advantages of ziconotide in cancer pain with unsuccessful treatment history is yet too weak (Mercadante, 2011).

Intensive efforts are still being made for new drug development, for many potential targets. Leconotide, a new calcium channel blocker promises powerful anti-hyperalgesia by intravenous administration without the dangerous side effects of its predecessor ziconotide (Mercadante, 2011).

Ralfinamide, a α-aminoamide derivative, is a novel promise for neuropathic pain and seems to have a combined mechanism, including inhibition of sodium and calcium currents, inhibition of SP release and NMDA antagonism (Yamane *et al.*, 2007). Ralfinamide has demonstrated analgesic effects in animal models but further studies are required.

New targets are also been explored for chronic pain, which can be tested in chronic cancer pain later. NGF has shown to contribute to persistent pain and anti-NGF therapies are also under study, as this factor seems to be integrally involved in up-regulation, sensitization and disinhibition of multiple neurotransmitters, ion channels and receptors in the primary afferent nerve and dorsal root ganglia fibers (Hefti *et al.*, 2006). Like-wise, TRPA1 receptors and its agonists revealed to be pronociceptives and the block of these receptors could be useful. In fact, antagonists of TRPA1 have shown to reduce hyperalgesia in animal models and seem promising for neuropathic and inflammatory pain (Petrus *et al.*, 2007; Eid *et al.*, 2008). Another approach is the development of selective ligands to $GABA_A$ receptors, which are involved in pain transmission and have shown an antinociceptive activity in experimental models of pain (Hwang and Yaksh, 1997; Kaneko and Hammond, 1997). Imidazoline (I_2) receptors agonists are also under investigation. Despite a little theoretical basis, comparing to the previous targets, ligands of I_2 receptors have shown to alleviate acute visceral, neuropathic and inflammatory pain and increase the antinociceptive effect of opioids (Ferrari *et al.*, 2011).

Several pharmacological approaches are now in use and under investigation. However, as conventional drug treatment has shown several limitations, several other therapies are also combined, like psychosocial interventions (Gaertner and Schiessl, 2013), radiotherapy (Ripamonti *et al.*, 2011), surgery and interventional approaches (Bhaskar, 2012). Genetic approaches are also under investigation, as the development of viral vectors for gene therapy (Huang *et al.*, 2011), microRNAs (Chen *et al.*, 2013; Kress *et al.*, 2013) and pharmacogenetic / pharmacogenomic studies.

1.4 Genetic polymorphisms, pain perception and morphine requirements

Under-treatment of cancer-related pain remains a significant problem, despite the several guidelines, opioids, non-opioids and adjuvant drugs. As already mentioned, opioids are the mainstay treatment for cancer-related pain, with morphine as first-line drug (WHO, 1996). However, interindividual variability is becoming a major concern and a possible reason for the lack of good analgesic effect. Perception of pain varies greatly among people, which implies wide variations in morphine dosage, pharmacological efficacy and tolerability (Aubrun *et al.*, 2003; Ross *et al.*, 2005; Shi *et al.*, 2010). Moreover, it is estimated that about 30 % of cancer pain patients are non-responders to morphine (Riley *et al.*, 2006; Kasai *et al.*, 2008). Although age, gender, race/ethnicity, mood states and stress can be pointed as influencing factors (Zhou *et al.*, 1993; Cepeda *et al.*, 2001; Pleym

et al., 2003; Klepstad *et al.*, 2005; Chakrabarti *et al.*, 2010; Sibille *et al.*, 2011), an important cause is thought to be of pharmacogenetic nature. In fact, studies on inbred strains of laboratory mice have shown that genetic factors explain up to 30 to 76 % of pain variance (Mogil *et al.*, 1999; Lariviere *et al.*, 2002). Additionally, twin studies have also suggested that heritability estimates up to 70 % for clinical pain conditions and up to 60 % for sensitivity for certain stimuli (LaCroix-Fralish and Mogil, 2009; Nielsen *et al.*, 2012). Hence, in the past decade, efforts have been made to identify genetic factors, especially single nucleotide polymorphisms (SNP) that can explain the interindividual variability in pain sensitivity and morphine dose requirements, especially in polymorphisms of opioid receptors, transporters and metabolizing enzymes (Belfer *et al.*, 2004; Lötsch and Geisslinger, 2006; Kadiev *et al.*, 2008; Kasai *et al.*, 2008; Jannetto and Bratanow, 2010; Kleine-Brueggeney *et al.*, 2010; Muralidharan and Smith, 2011), and in modulators/suppressors and neurotransmitters involved in perception and processing of pain information (Lötsch and Geisslinger, 2006; Shi *et al.*, 2010). We will now focus on the major genetic variants that were already associated with pain status. However, rare genetic conditions, such as congenital insensitivity to pain or congenital indifference to pain, were not considered. Likewise, SNP / molecules related to pain circuits but never studied in pain populations were also not subject of study in the present thesis.

1.4.1 Pain transmission and perception

1.4.1.1 Catecholaminergic and serotoninergic systems

1.4.1.1.1 Metabolism: catechol-O-methyl transferase and monoamine oxidases

The catecholaminergic and serotoninergic systems seem to be involved in pain transmission and processing, with several polymorphic candidate genes in the biosynthesis, transport and metabolism (Figure 13).

Catechol-O-methyltransferase (COMT) regulates catecholamines inactivation and the influence of the SNP Val(108/158)Met (G1947A) in pain has been subject of investigation (Zubieta *et al.*, 2003; Diatchenko *et al.*, 2006; Nackley *et al.*, 2007; Jensen *et al.*, 2009; Mobascher *et al.*, 2010; Belfer and Segall, 2011; Hickey *et al.*, 2011; Kolesnikov *et al.*, 2011). The Val(108/158)Met polymorphism leads to an amino acid substitution, valine (Val) by methionine (Met) (Zubieta *et al.*, 2003), which leads to a reduction in its activity (Zubieta *et al.*, 2003; Zhang *et al.*, 2009; Shi *et al.*, 2010). Met/Met genotype is associated

with the lowest activity of COMT, Met/Val with intermediate and Val/Val with the highest (Zubieta *et al.*, 2003). Individuals homozygous for Met allele have been reported to exhibit increased pain sensitivity and lower μ-opioid system activation during sustained pain (Zubieta *et al.*, 2003; Jensen *et al.*, 2009; Mobascher *et al.*, 2010; Vossen *et al.*, 2010), as well as higher sensory and affective ratings and a more negative internal affective state (Zubieta *et al.*, 2003). These differences are most felt in patients with chronic pain, and could be related with opioid-induced hyperalgesia and tolerance (Jensen *et al.*, 2009). Also, the associated increase in pain sensitivity appears to be blocked by $\beta_{2/3}$ antagonists, revealing the important role of catecholamines in pain sensitivity (Nackley *et al.*, 2007). Val(108/158)Met SNP have also been associated with morphine requirements. Carriers of Met/Met genotype were unexpectedly associated with lower morphine requirements than patients homozygous for the Val allele (Rakvåg *et al.*, 2005; Reyes-Gibby *et al.*, 2007; Rakvag *et al.*, 2008), explained by a compensatory increased of μ-opioid receptor density and binding potential (Chen *et al.*, 1993; Zubieta *et al.*, 2003). Nevertheless, contradictory information has been reported in recent years (Klepstad *et al.*, 2011; Kolesnikov *et al.*, 2011).

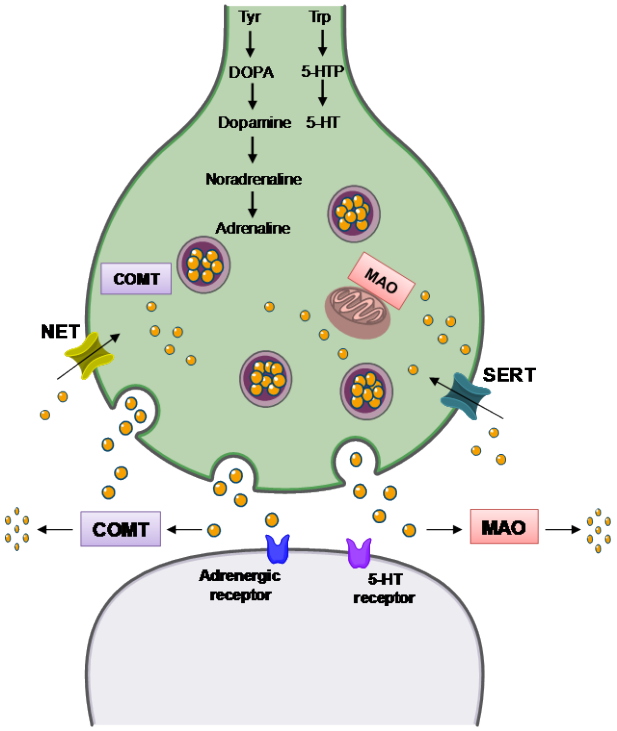


Figure 13. Schematic representation of the several phases that can be altered by genetic variation: biosynthesis, transport, metabolism and receptor activation. COMT, catechol-O-methyltransferase; 5-HT, 5-hydroxytryptamine; 5-HTP, 5-hydroxytryptophan; MAO, Monoamine oxidase; NAT, noradrenaline transporter; SERT, serotonin (5-HT) transporter; Trp, tryptophan; Tyr, tyrosine.

Despite Val(108/158)Met being the most studied *COMT* SNP, several new functional polymorphisms were identified and seems that other SNP, especially rs6269 (A/G), rs4633 (C/T) and rs4818 (C/G), can influence enzyme activity and pain sensitivity, along with Val(108/158)Met (G/A). In fact, three common haplotypes defined can determine COMT enzymatic activity and account for approximately 11 % of the variability in pain response (Diatchenko *et al.*, 2005; Diatchenko *et al.*, 2006), with the ACCG haplotype exhibiting the lowest enzymatic activity and protein expression (Nackley *et al.*, 2006). Moreover, being heterozygous for ATCA and ACCG haplotypes, it was strongly associated with high sensitivity to experimental pain (Diatchenko *et al.*, 2005). In another study, *COMT* haplotypes were constructed, based on 11 SNPs, in a sample of cancer pain patients receiving morphine and the most common haplotype was related to lower morphine requirements (Rakvag *et al.*, 2008).

Monoamine oxidases (MAO) isoforms MAO-A (*MAOA* gene) and MAO-B (*MAOB* gene) are capable of metabolizing 5-HT and NA. SNPs in *MAOA* were weakly associated with female postoperative pain intensity (Kim *et al.*, 2006), but not *MAOB*. However, a polymorphism in intron 13 of *MAOB* was significantly correlated with male postoperative pain intensity (Sery *et al.*, 2006). The correlation of genetic variation of MAO and pain is still inconsistent.

1.4.1.1.2 Reuptake transporters

Reuptake transporters can influence catecholamines and 5-HT concentration, and its importance is highlighted by the role of TCAs, SSRI and SSNRIs as analgesic adjuvant drugs, that block the NA transporter (NAT) and serotonin transporter (SERT). Polymorphisms in the *NAT* gene, also known as *solute carrier family 6 member 2* (*SLC6A2*), were only weakly associated with analgesic onset time in patients with postoperative pain (Kim *et al.*, 2006) and their real role has to be further studied in clinical trials that assess their influence in pain relief produced with TCAs and SNRIs.

Concerning the *SERT* gene (also known as *5HTT* or *SLC6A4*), two main functional variants are especially known: 5-HTT Linked Polymorphic Region (5-HTTLPR) and second intron (STin2) variable number tandem repeat (VNTR) (Gentile *et al.*, 2011). The 5-HTTLPR variant is a 44-base pair (bp) insertion/deletion that generate a long or short allele and was suggested as a risk factor for some painful conditions (fibromyalgia and tension-headache), but not migraine (Buskila *et al.*, 2007; Park and Moon, 2010; Schurks

et al., 2010). Additionally, the short allele, which results in reduced SERT expression, was related to lower heat, cold and pressure pain sensitivity (Lindstedt *et al.*, 2011). The VNTR polymorphism represents a 17-bp VNTR in intron 2, producing alleles with 9, 10 or 12 repeats and seems to be associated with protective phenotypes against migraine (Schurks *et al.*, 2010). However, all these preliminary results need further confirmation.

The dopamine transporter (DAT, also known as SLC6A3) is responsible for the reuptake of dopamine and its influence on pain is also being studied. A VNTR polymorphism in the 3'-untranslated region of *DAT1* gene was found to be associated with chronic headache (Cevoli *et al.*, 2006) and cold pain tolerance, suggesting that low dopaminergic activity can be associated with high pain sensitivity (Treister *et al.*, 2009).

1.4.1.1.3 Receptors

The effects of catecholamines and 5-HT are a result of their binding to specific receptors, and genetic variation in the receptors may affect the response. 5-HT binds to a family of receptors and 5-HT_1, 5-HT_2, 5-HT_3 and their subtypes have been implicated in nociception (Hoyer *et al.*, 1994). There are three common SNP in *5-HT1B* gene, which encodes the subtype 5-HT_{1B}: T(-261)G, A161T and G861C. However, clinical studies didn't yet demonstrate an influence of these polymorphisms in pain sensitivity. Concerning dopamine, a 48-bp VNTR in exon 3 of the dopamine receptor D_4 gene (*DRD4*), has been associated with clinical pain in fibromyalgia and migraine patients (Dan *et al.*, 2004; Cevoli *et al.*, 2006).

1.4.1.1.4 Biosynthesis

Genetic variation in genes involved in catecholamines and 5-HT biosynthesis can also influence these neurotransmitters concentration. The enzyme guanosine triphosphate cyclohydrolase (GCH1) catalyzes the rate-limiting step in the synthesis of tetrahydrobiopterin (BH4) (Figure 14), an essential co-factor for 5-HT and NA biosynthesis, and was already reported as upregulated in neuropathic pain (Costigan *et al.*, 2002).

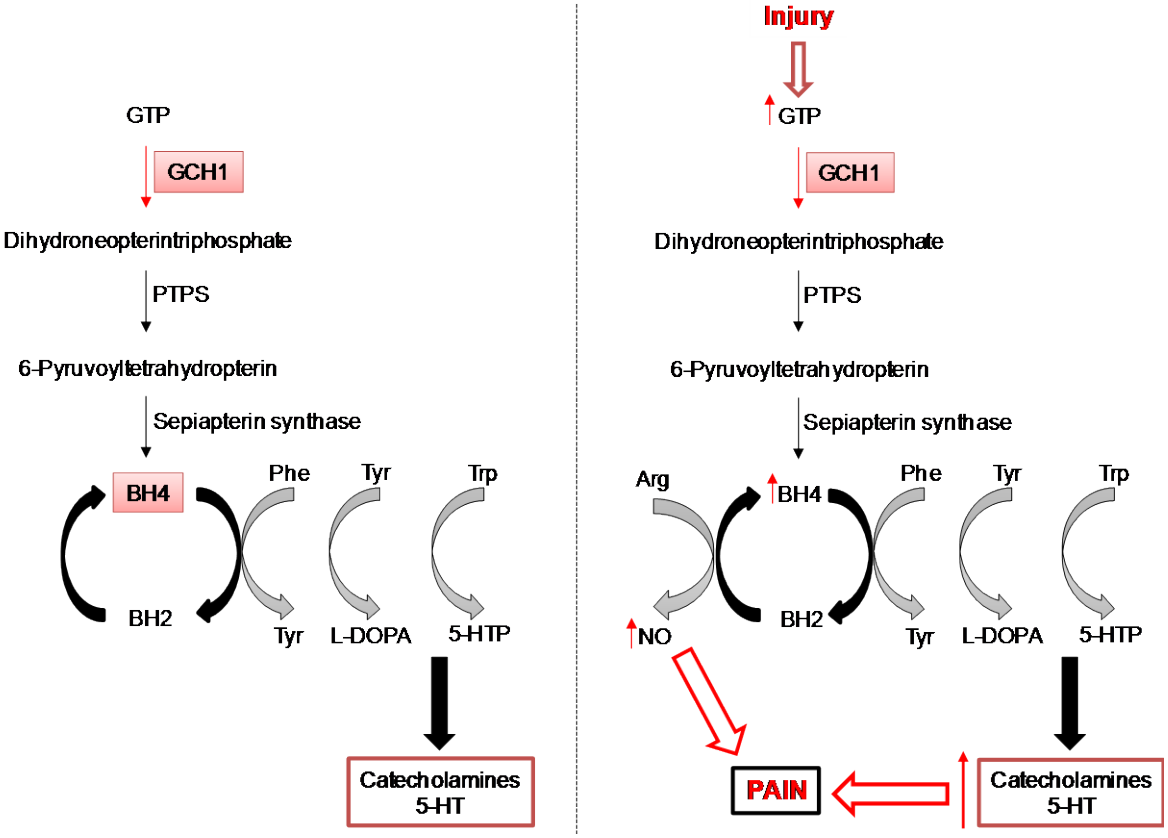


Figure 14. Tetrahydrobiopterin synthesis and its influence in pain [adapted from (Pasternak and Inturrisi, 2006; Clot *et al.*, 2009)]. Arg, arginine; BH2, dihydrobiopterin; BH4, tetrahydrobiopterin; GCH1, guanosine triphosphate cyclohydrolase; 5-HT, 5-hydroxytryptamine; 5-HTP, 5-hydroxytryptophan NO, nitric oxide; Phe, Phenylalanine; PTPS, 6-pyruvoyl tetrahydropterin synthase; Trp, tryptophan; Tyr, tyrosine.

An haplotype of 15 SNP in *GCH1* gene was already associated with reduced pain sensitivity in patients with neuropathic pain (Tegeder *et al.*, 2006) and several SNP were associated with reduced upregulation of GCH1 (Tegeder *et al.*, 2006; Antoniades *et al.*, 2008; Tegeder *et al.*, 2008). Three variants of this haplotype, rs8007267 (G/A), rs3783641 (A/T) and rs10483639 (C/G) were found to have reliability, specificity and sensitivity for the genetic diagnosis of pain sensitivity, replacing the need for testing the 15 variants (Lotsch *et al.*, 2007). Later, the influence of the reduced-function haplotype in cancer pain therapy was reported, with a longer interval between cancer diagnosis and opioid therapy initiation in homozygous carriers of the genetic variants (Lötsch *et al.*, 2010). In fact, the reduced upregulation haplotype of *GCH1* probably led to a reduced expression of BH4, delaying the need for opioid therapy and suggesting partial GCH1 blockade or BH4 inhibition as targets for the management of cancer pain (Lötsch *et al.*, 2010).

Major polymorphisms for catecholaminergic and serotoninergic systems are resumed in Table 1:

Table 1. Major polymorphisms in catecholaminergic and serotoninergic systems that can affect opioids requirements, pain transmission and perception.

Gene	Polymorphism or Haplotype	Pain Phenotype	Reference
Metabolism			
COMT	Val(108/158)Met (rs4680)	Pain sensitivity Morphine requirements Alteration of μ-opioid system in sustained pain Influences in sensory and affective ratings	(Zubieta *et al.*, 2003; Rakvåg *et al.*, 2005; Reyes-Gibby *et al.*, 2007; Rakvag *et al.*, 2008; Jensen *et al.*, 2009; Mobascher *et al.*, 2010; Vossen *et al.*, 2010; Kolesnikov *et al.*, 2011)
	Haplotype: rs6269, rs4633 and rs4818, rs4680	Pain sensitivity Morphine requirements	(Diatchenko *et al.*, 2005; Rakvag *et al.*, 2008)
MAOA	rs3788862, rs2283724, rs1800659, rs979605, rs2064070	Pain intensity	(Kim *et al.*, 2006)
MAOB	rs1799836 (A/G polymorphism in intron 13)	Pain intensity	(Sery *et al.*, 2006)
Transporters			
NAT	rs40434	Analgesic onset time	(Kim *et al.*, 2006)
SERT	rs2066713	Analgesic onset time	(Kim *et al.*, 2006)
	5-HTTLPR	Pain syndromes Thermal and pressure pain sensitivity	(Gunne, 1963; Buskila *et al.*, 2007; Park and Moon, 2010; Schurks *et al.*, 2010; Lindstedt *et al.*, 2011)
	rs57098334 (STin2 VNTR)	Protective phenotype in migraine patients	(Schurks *et al.*, 2010)
DAT1	VNTR polymorphism	Headache Thermal pain sensitivity	(Cevoli *et al.*, 2006; Treister *et al.*, 2011)

Table 1. Major polymorphisms in catecholaminergic and serotoninergic systems that can affect opioids requirements, pain transmission and perception (cont.).

Gene	Polymorphism or Haplotype	Pain Phenotype	Reference
Receptors			
DRD_4	48-bp VNTR	Clinical pain in fibromyalgia and migraine patients	(Dan *et al.*, 2004; Cevoli *et al.*, 2006)
Biosynthesis			
GCH1	Hapolotype: rs8007267, rs3783641, rs10483639	Neuropathic pain Pain sensitivity Interval between cancer diagnosis and opioid therapy	(Tegeder *et al.*, 2006; Lotsch *et al.*, 2007; Lötsch *et al.*, 2010)

bp, base pair; COMT, catechol-O-methyltransferase; DAT, dopamine transporter; DRD_4,dopamine receptor 4; GCH1, guanosine triphosphate cyclohydrolase; 5-HTTLPR, 5-hydroxytriptamine linked polymorphic region; MAO, monoamine oxidase; SERT, serotonin transporter; VNTR, variable–number tandem repeat.

1.4.1.2 Other genes affecting pain transmission and perception

1.4.1.2.1 Transient receptor potential channels

TRP channels are involved in the nociception system, as already mentioned. TRPA1 is activated by noxius cold temperature and the SNP rs1198795 (G/T) was associated with different cold-withdrawn time (Kim *et al.*, 2006). TRPV, another subfamily, is associated with warm and noxius heat sensations and genetic variation in TRPV1 may also influence the response to noxius temperature stimuli. The SNP rs8065080 (Ile585Val) have an amino acid alteration and were related to longer pain-response time to cold stimuli in healthy female (Kim *et al.*, 2004).

1.4.1.2.2 Ion channels

Voltage-gated ion channels as Na_V and potassium (K_V) are key regulators of membrane potential in excitable tissues as sensory neurons, with opposite actions (Catterall *et al.*, 2005). Among the Na_V subtypes already identified, $Na_V1.7$ has an essential role in nociception transmission (Nassar *et al.*, 2004) and the R1150W SNP, a G/A substitution,

was correlated with altered pain perception (Reimann *et al.*, 2010). Concerning K_V channels, potassium voltage-gated channel subfamily S member 1 (*KCNS1*) gene encodes the α-subunit of $K_V9.1$ subtype and was identified as a putative pain gene (Costigan *et al.*, 2010). The SNP I489V in *KCNS1* has been studied in humans, with the valine allele being associated with higher pain intensity, and the SNP was proposed as a prognostic indicator for chronic pain risk (Costigan *et al.*, 2010), but additional studies are required.

P2X7 receptor, encoded by the highly polymorphic *P2RX7* gene, belongs to the ionotropic ATP-gated receptor family and seems to be associated to chronic pain (Chessell *et al.*, 2005; Sorge *et al.*, 2012). Some SNP were already studied in mice and humans, influencing pain behavior and suggesting new targets of pain treatment individualization (Sorge *et al.*, 2012).

1.4.1.2.3 Fatty acid amide hydrolase

Fatty acid amide hydrolase (FAAH) degrades the fatty acid amide family of endogenous signaling lipids including the endogenous cannabinoid anandamide, which has been implicated in the suppression of pain. Animal studies revealed that mice without the *FAAH* gene had prolonged pain-response latencies to temperature stimuli (Lichtman *et al.*, 2004). The SNP rs324420 (C385A; Pro129Thr) leads to an amino acid alteration, reducing cellular expression of the enzyme in human lymphocytes, which could result in different pain sensitivity (Chiang *et al.*, 2004). However, it was not associated with thermal-pain response (Kim *et al.*, 2006). Men carrying the variant alleles rs932816 A, rs4141964 C and rs2295633 A had increased cold pain intensity and carriers of the rs4141964 C allele had shorter cold withdrawal time than non-carriers (Kim *et al.*, 2006). These results could be due to an increased enzyme activity and subsequent accelerated endocannabinoid degradation (Lotsch and Geisslinger, 2011).

1.4.1.2.4 Melanocortin-1 receptor

Melanocortin-1 receptor (MCR1) is encoded by the gene *MCR1* and is especially known for its role in hair and skin pigmentation, with *MCR1* variants associated with red hair and fair skin. However, in the last decade, some studies claimed a possible association with pain, but opposite studies associated inactivating variants to higher tolerance to electrical

stimulus (Mogil *et al.*, 2005) and lower tolerance to thermal pain stimulus (Liem *et al.*, 2005). Opioid analgesia has also been associated with *MC1R* variants, with women with two non-functional alleles related with stronger analgesic effect from pentazocine (Mogil *et al.*, 2003). However, analgesic effects mediated by M6G did not produce sex-specific analgesia and all individuals with non-functional alleles variants (R151C, R160W, and D294H) displayed reduced sensitivity to noxious stimuli and increased analgesic response to M6G (Mogil *et al.*, 2005).

Table 2 resumes polymorphisms that can influence pain transmission, besides catecholaminergic and serotoninergic systems.

Table 2. Other polymorphisms that can influence pain transmission and perception.

Gene	Polymorphism or Haplotype	Pain Phenotype	Reference
TRPA1	rs1198795 (G/T)	Thermal pain sensitivity	(Kim *et al.*, 2006)
TRPV1	rs8065080 (Ile585Val)	Thermal pain sensitivity	(Kim *et al.*, 2004)
$Na_V1.7$	rs6746030 (R1150W)	Pain perception	(Reimann *et al.*, 2010)
KCNS1	rs734784 (I489V)	Pain intensity	(Costigan *et al.*, 2010)
P2RX7	rs7958311 (G853A)	Pain intensity	(Sorge *et al.*, 2012)
FAAH	rs932816, rs4141964, rs2295633	Thermal pain sensitivity	(Kim *et al.*, 2006)
MCR1	rs1805007 (R151C), rs1805008 (R160W), rs1805009 (D294H)	Thermal and noxius pain sensitivity Response to M6G Opioids analgesic effect	(Mogil *et al.*, 2005) (Liem *et al.*, 2005) (Mogil *et al.*, 2003) (Mogil *et al.*, 2005).

FAAH, Fatty acid amide hydrolase; KCNS1, K^+ voltage-gated channel subfamily S member 1; M6G, morphine-6-glucuronide; MCR1, Melanocortin-1 receptor; $Na_V1.7$, Voltage-gated sodium channel; TRP, Transient receptor potential channels.

1.4.2 Inflammation

In the last years, proinflammatory cytokines as IL 1, 2, 6, 8, 15, 18, interferon γ (IFN-γ) and TNF-α appear to have a central role in pain and hyperalgesia and have already demonstrated to interfere in the nociceptive transmission, neuropathic pain and analgesics efficacy (Hutchinson *et al.*, 2008; Kawasaki *et al.*, 2008; Shi *et al.*, 2010; Albulescu *et al.*, 2013). Cancer and its treatments also induce a release of proinflammatory cytokines that might contribute to the feeling of pain (Oh *et al.*, 2001) and polymorphisms in genes encoding cytokines might interfere in pain perception and morphine response. Main polymorphisms in cytokine genes are resumed in Table 3:

Table 3. Major polymorphisms in cytokines genes related to pain phenotypes.

Gene	Polymorphism or Haplotype	Pain Phenotype	Reference
IL1A	rs 1800587 [C(-889)T]	Pain intensity	(Solovieva *et al.*, 2004)
IL1B	rs1143634 (C3954T)	Pain intensity and duration	(Solovieva *et al.*, 2004)
IL1RN	G1812A	Pain occurrence, intensity, duration and limitations of daily activities	(Solovieva *et al.*, 2004)
	86-bp VNTR	Postoperative morphine requirements	(Bessler *et al.*, 2006)
IL6	rs1800795 [G(-174)C]	Opioid requirements in lung cancer patients	(Reyes-Gibby *et al.*, 2008)
	rs1800797 [A(-596)G]; rs1800796 [G(-572)C]; rs1800795; rs13306435 (T15A) (GGGA)	Pain duration in sciatica patients	(Karppinen *et al.*, 2008)
IL8	rs4073 [T(-251)A]	Pain intensity in lung and adenocarcinoma of the pancreas patients	(Reyes-Gibby *et al.*, 2007; Reyes-Gibby *et al.*, 2009)
TNFA	rs1800629 G(-308)A	Pain intensity in lung cancer patients	(Reyes-Gibby *et al.*, 2008)

bp, base pair; IL, interleukin; TNF-α, tumor necrosis factor α; VNTR, variable number repeat.

IL-1 has been implicated in pain sensitivity (Watkins and Maier, 2002; Gabay *et al.*, 2011) and its activity is determined by IL-1α (*IL1A* gene), IL-1β (*IL1B* gene), and an endogenous competitive inhibitor, IL-1 receptor antagonist (IL-1Ra, *IL1RN* gene). IL-1β is capable of inducing hyperalgesia and allodynia (Falchi *et al.*, 2001), as well as decreasing the effect of morphine (Shavit *et al.*, 2005; Mika *et al.*, 2008). *IL1A*, *IL1B* and *IL1RN* are mapped to a closely linked area and polymorphisms C(-889)T in *IL1A,* C3954T and C(-511)T in *IL1B* and an 86-bp VNTR in *IL1RN* seem to influence IL-1 production (di Giovine *et al.*, 1992; Tountas *et al.*, 1999; Hulkkonen *et al.*, 2000; Lacruz-Guzman *et al.*, 2013). Concerning pain, the simultaneous carriage of *IL1A* -889T and *IL1RN* 1812A alleles was associated with pain intensity and *IL1B* C3954T and *IL1RN* G1812A with multiple pain phenotypes, in patients with low back pain (Solovieva *et al.*, 2004). The 86-bp VNTR was related with higher morphine requirements in postoperative female patients (Bessler *et al.*, 2006).

IL-6 is also implicated in the pathophysiology of pain, with knockout mice demonstrating a reduced response and higher tolerance to the analgesic effect of morphine (Bianchi *et al.*, 1999). The G(-174)C polymorphism is one of the most extensively studied and has been related with lower levels of plasma IL-6 in healthy subjects (Fishman *et al.*, 1998) and higher opioids requirements in lung cancer patients (Reyes-Gibby *et al.*, 2008). An haplotype based in four SNP [A(-596)G, G(-572)C, G(-174)C, T15A) was constructed and carriers of GGGA were related with the number of days with pain in sciatica patients (Karppinen *et al.*, 2008).

Another proinflammatory cytokine involved in pain is IL-8, whose up-regulation after tissue injury was associated with post-surgery pain intensity (Wang *et al.*, 2009). Concerning *IL8* SNP, T(-251)A, a common polymorphism in the promoter region, was correlated with cytokine levels (Hull *et al.*, 2000) and severe pain in patients with lung cancer (Reyes-Gibby *et al.*, 2007) and adenocarcinoma of the pancreas (Reyes-Gibby *et al.*, 2009).

TNF-α is one of the first cytokines formed in inflammatory processes, simultaneously with IL-1β, and has been related with hyperalgesia and allodynia in neuropathic pain models (Reeve *et al.*, 2000). Also, administration of etanercept or infliximab that neutralize TNF-α, resulted in decreased mechanical hyperalgesia (Segond von Banchet *et al.*, 2009). A widely studied SNP is the G(-308)A, which was already associated with increased TNF-α expression (Wilson *et al.*, 1997) and also to pain intensity in lung cancer patients (Reyes-Gibby *et al.*, 2008).

1.4.3 Genetic variants in morphine pharmacodynamics

1.4.3.1 Opioid receptors

Along with SNP in important molecules in pain transmission, there are some important candidate genes that can be considered to influence morphine response and the analgesic effect by affecting its pharmacokinetics or pharmacodynamics (Figure 15).

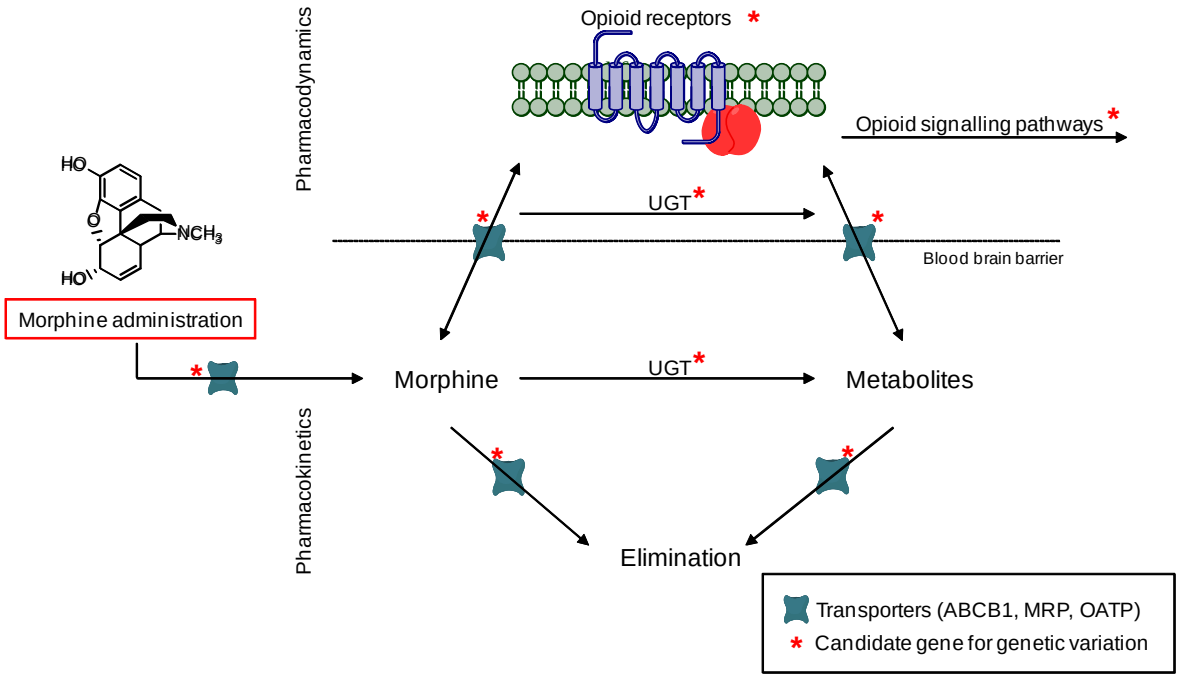


Figure 15. Possible candidate genes for genetic variation in morphine pharmacokinetics and pharmacodynamics. ABCB1, ATP-binding cassette B1; MRP, multidrug resistance-associated proteins; OATP, organic anion-transporting polypeptides; UGT, UDP-Glucuronosyltransferase.

The most studied SNP is the μ-opioid receptor gene (*OPRM1*). As already mentioned, morphine exert its analgesic effect by binding to opioid receptors, and the connection to μ-opioid receptor seems to be especially important and responsible for the major analgesic and adverse effects. A widely studied and frequent polymorphism in Caucasians (10 – 30 %) is the SNP A118G, with the substitution of an adenosine by a guanine at position 118, leading to the loss of the N-glycosylation site (Klepstad *et al.*, 2005; Vuilleumier *et al.*, 2012). Despite the still existence doubts about the real consequences and mechanisms, this SNP became of major interest due to the pharmacological and physiological alterations that seems to promote. It was already suggested that the SNP affects the binding characteristics (Bond *et al.*, 1998; Kroslak *et al.*, 2007) or mRNA expression levels

(Zhang *et al.*, 2005), but the results were not always consistent (Beyer *et al.*, 2004; Oertel *et al.*, 2009). Recently, a study with humanized mouse model has shown that in *118GG* sensory neurons morphine presented a lower efficacy and potency (Mahmoud *et al.*, 2011). Accordingly, human clinical studies suggest that individuals homozygous for the wild-type A allele seem to require less morphine to achieve pain control, including cancer pain patients (Klepstad *et al.*, 2004; Reyes-Gibby *et al.*, 2007; Sia *et al.*, 2008; Tan *et al.*, 2009). However, controversy results have also been described and the real importance of this isolated SNP is still an issue (Klepstad *et al.*, 2011).

Besides A118G, several other SNP of OPRM1 are described and a limited number [G(-172)T, IVS2+31G>A, IVS2+691G>C, C5433T, C32459T, A50665G, G51325C and T80547C) was already studied in cancer patients on morphine (Klepstad *et al.*, 2004; Ross *et al.*, 2005), but no significant associations were found. Additionally, the SNP S268P in *OPRM1* leads to an amino acid change, resulting in altered receptor desensitization and signaling, and in vitro decreased morphine potency and efficacy (Koch *et al.*, 2000).

Polymorphisms in δ- and κ-opioid receptor genes have also been described, but were especially studied and related to addition behaviors (Zhang *et al.*, 2008).

1.4.3.2 Molecules interfering in opioid signaling pathways

1.4.3.2.1 G-protein-activated inwardly rectifying potassium

G-protein-activated inwardly rectifying potassium (GIRK) channels are activated by the release of β/γ subunits of $G_{i/o}$ protein, playing a critical role in opioid signaling after their binding to the receptors. Four subtypes were already identified in mammals (Wickman *et al.*, 1997) and *Girk2* (*KCNJ6*) and *Girk3* (*KCNJ9*) genes appear to be associated with pain and morphine effect, as knockout mice revealed hyperalgesia and reduced analgesic efficacy of morphine (Marker *et al.*, 2004). Later, the SNPs G(-1250)A and A1032G in *KCNJ6* gene were analyzed in patients who underwent major open abdominal surgery and genotype AA of A1032G SNP and haplotype -1250G/1032A were correlated with increased postoperative analgesic requirements. Additionally it was suggested that the result for the AA carriers of the A1032G SNP was due to a lower *KCNJ6* gene expression levels and consequent insufficient analgesic effects (Nishizawa *et al.*, 2009). In another study, besides higher opioids requirements for analgesic effect, homozygous individuals

for allele A of SNP A1032G were also related with increased opioid requirements in opiate substitution therapy (Lotsch *et al.*, 2010).

1.4.3.2.2 β-arrestin

β-arrestin2, coded by the gene *ARRB2*, is an intracellular protein that inhibits active receptors and is a negative regulator of opioid receptor signaling (Raehal and Bohn, 2005). Studies in β-arrestin2 knockout mice have shown an enhanced morphine analgesia (Bohn *et al.*, 1999) and SNPs (T8622C, A1082G, A8864G, A11143G) in the *ARRB2* gene seem to be associated with differences between morphine responders and morphine non-responders, especially T8622C (Ross *et al.*, 2005).

1.4.3.2.3 Signal transducer and activator of transcription 6

Signal transducer and activator of transcription 6 (Stat6) is a transcription factor that has the ability to alter μ-opioid receptor gene expression. The gene encoding Stat6 is highly polymorphic and seems that the SNPs C(-1714)T and C9065T might affect the response to morphine (Ross *et al.*, 2005).

1.4.4 Genetic variants in morphine pharmacokinetics

1.4.4.1 Morphine metabolism

Morphine is essentially metabolized by UGT2B7 to the toxic and hyperalgesic M3G and the analgesic M6G, as already mentioned (Figure 11) (Christrup, 1997; Holthe *et al.*, 2002). Due to the different pharmacological activities, variability in metabolites formation may influence morphine efficacy and pain relief. The variability of metabolites formation has been described, but the correlation with genetic factors was not yet established (Klepstad *et al.*, 2005; Innocenti *et al.*, 2008).

One of the most studied SNP in *UGT2B7* gene is the C802T, also known as His268Tyr, which is linked with T801A and can cause an enzyme with either histidine (His) or tyrosine (Tyr) in the amino acid 268. A homozygous individual for T801C802 produces an enzyme

with His268 (*UGT2B7*1*) and an individual A801T802 produces a Tyr268 (*UGT2B7*2*) (Bhasker *et al.*, 2000; Holthe *et al.*, 2002). Several studies have focused in the SNP C802T of UGT2B7 and its influence in morphine and other compounds glucuronidation and contradictory results have been described (Holthe *et al.*, 2002; Hirota *et al.*, 2003; Sawyer *et al.*, 2003; Saeki *et al.*, 2004; Ross *et al.*, 2005; Levesque *et al.*, 2007; Parmar *et al.*, 2011). In addition, a recent study associated *UGT2B7*2* genotype to the frequency of nausea (Fujita *et al.*, 2010).

Another well described SNP in *UGT2B7* is G(-840)A, located in the promoter region, which is linked to five other variants: -1248G, -1241C, -1054C, -268G, and -102C (Duguay *et al.*, 2004). The carriers of allele G in the SNP G(-840)A was recently associated with reduced glucuronidation of morphine in patients with sickle cell disease, leading to variability in morphine hepatic clearance (Darbari *et al.*, 2008). Additionally, heterozygous for a genetic variation in the regulatory part of the UGT2B7 gene, the SNP G(-79)A, has been related with lower levels of M6G. Several other polymorphisms are present in UGT2B7 gene but their role in morphine metabolism is still unknown (Holthe *et al.*, 2003; Nagar and Remmel, 2006).

Despite UGT2B7, other UGT isoforms seem to be involved in M3G formation, like UGT1A1, 1A3, 1A6, 1A8, 1A9, and 1A10 (Stone *et al.*, 2003; Ohno *et al.*, 2008). Genetic variability in *UGT1A1* and *UGT1A8* genes appear to influence morphine metabolism and metabolic ratios in cancer pain patients, together with clinical factors, but further studies are necessary (Fladvad *et al.*, 2013).

1.4.4.2 Transporters

Opioids absorption, distribution and excretion can be affected by several factors and genetic variability in drug transporters can also affect the metabolites concentration and consequently morphine analgesic effect. *ATP-binding cassette B1* (*ABCB1*) codes for P-glycoprotein (Pgp), which regulates the efflux of morphine from the brain (Cordon-Cardo *et al.*, 1989; Xie *et al.*, 1999) and reduced Pgp activity/levels may result in enhanced analgesia after systemic administration of morphine (King *et al.*, 2001). Polymorphisms in the *ABCB1* gene frequently alter Pgp transport characteristics or Pgp expression (Gerloff, 2004). Three of the most frequent and most studied SNPs in ABCB1 are C3435T, C1236T and G2677T/A. The C3435T SNP is associated with altered Pgp expression and transport function, with homozygous individuals for T allele exhibiting lower mRNA expression

(Wang *et al.*, 2005). Additionally, this SNP was related with variability in morphine analgesic effect in cancer patients (Campa *et al.*, 2007). C1236T was found to be in linkage disequilibrium with C3435T and was also related to different opioid doses requirements, higher in T allele homozygous (Kleine-Brueggeney *et al.*, 2010). Moreover, cancer pain patients homozygous for 1236T or with TT/TT diplotype at 2677 and 3435 SNPs were correlated with reduced fatigue (Fujita *et al.*, 2010).

Also, multidrug resistance-associated proteins (MRP, *ABCC*) and organic anion-transporting polypeptides (OATP) are involved in transmembrane movements of a variety of substrates, including opioids, especially MRP2, OATP1A2 and OATP1B3 (van de Wetering *et al.*, 2007; Kadiev *et al.*, 2008). Genetic variation in genes encoding these transporters is described and a study by Lee and collaborators related SNP in *OATP1A2* gene and a reduced uptake capacity of opioids (Lee *et al.*, 2005). However, the role of polymorphisms in these transporters in pain is not yet clarified.

The most important polymorphisms related to of morphine are resumed in Table 4:

Table 4. Major polymorphisms affecting morphine pharmacodynamics and pharmacokinetics.

Gene	Polymorphism or Haplotype	Pain Phenotype	Reference
Receptor			
OPRM1	rs1799971 (A118G)	Morphine efficacy, potency and requirements	(Klepstad *et al.*, 2004; Reyes-Gibby *et al.*, 2007; Sia *et al.*, 2008; Tan *et al.*, 2009; Mahmoud *et al.*, 2011)
	S268P	Morphine efficacy and potency (*in vitro*)	(Koch *et al.*, 2000)
Signaling			
Girk2	rs2836016 [G(-1250)A], rs2070995 (A1032G)	Opioids requirements	(Nishizawa *et al.*, 2009; Lotsch *et al.*, 2010)
ARRB2	rs1045280 (T8622C), rs3786047 (A1082G), rs2271167 (A8864G), rs2036657 (A11143G)	Morphine responders *vs*. morphine non-responders	(Ross *et al.*, 2005)
Stat6	C(-1714)T and C9065T	Response to morphine	(Ross *et al.*, 2005)
Metabolism			
UGT2B7	hCV32449742 [C802T (His268Tyr) + T801A]	Controversy results in morphine metabolism Morphine-related symptoms	(Holthe *et al.*, 2002; Hirota *et al.*, 2003; Sawyer *et al.*, 2003; Fujita *et al.*, 2010)
	G(-840)A	Morphine metabolism	(Darbari *et al.*, 2008)
	rs 7668282 [G(-79)A]	M6G concentration	(Holthe *et al.*, 2003; Nagar and Remmel, 2006)
UGT1A1, *UGT1A8*	Haplotypes UGT1A1/UGT1A8	Morphine metabolism and metabolic ratios	(Fladvad *et al.*, 2013)
Transporters			
ABCB1	rs1045642 (C3435T), rs1128503 (C1236T), rs2032582 (G2677T/A)	Morphine-related symptoms (analgesic and adverse effects)	(Campa *et al.*, 2007; Fujita *et al.*, 2010; Kleine-Brueggeney *et al.*, 2010)

ABCB1, ATP-binding cassette B1; *ARRB2*, β-arrestin2 gene; Girk2, G-protein-activated inwardly rectifying K^+ 2; *OPRM1*, μ-opioid receptor gene; Stat6, Signal Transducer and Activator of Transcription 6; UGT, UDP Glucuronosyltransferase.

Numerous genes were already analyzed in several target-molecules, as mentioned, but many other polymorphic candidate genes involved in pain mechanisms are waiting to be tested. However, human genetic studies are often inconsistent, even with usual and widely tested SNPs. Large clinical studies with multiple haplotypes, correctly designed and executed are necessary but remain a challenge until today. Meanwhile, additional information can be also obtained by genome-wide association studies and epigenetics, and hopefully we will be able to pave the way towards an individualized pain therapy.

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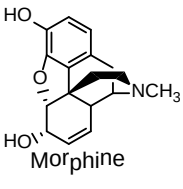
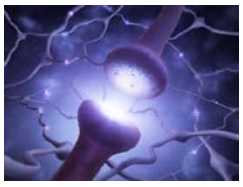
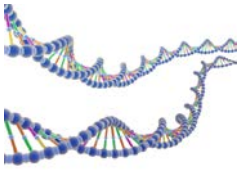
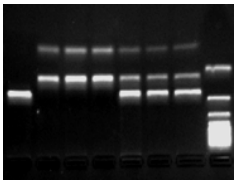
CHAPTER II

OBJECTIVES




The overall aim of the present thesis was to search for predictive biomarkers in morphine-treated patients that may help to introduce a tailored treatment for cancer-related pain.

Clinical practice of pharmacologic pain therapy faces daily a large inter-individual variability of the desired and unwanted effects of administered analgesics. Thus, in most cases it is unpredictable to know which patients are likely to develop an appropriate response. Genetic factors might affect variations of morphine sensitivity, pharmacokinetics and pharmacodynamics. Adequate studies on the relationship between gene polymorphisms and response to morphine will contribute to a better understanding of the inter-variability in response to morphine treatment and enable personalized pain treatment by predicting morphine sensitivity and requirement for each patient, which can be useful for clinical application.

The strategy pursued to achieve the main objective proposed comprised the following steps:

a) To define a pharmacogenomic profile of morphine-treated cancer patients in a clinical setting of Oncological Palliative Care, and relate it with pain response and morphine sensitivity.

b) To develop and validate a sensitive and specific high-performance liquid chromatography (HPLC) assay for the quantification of morphine and glucuronides in several *antemortem* and *postmortem* matrices, namely brain, kidneys, liver, urine, plasma and whole blood.

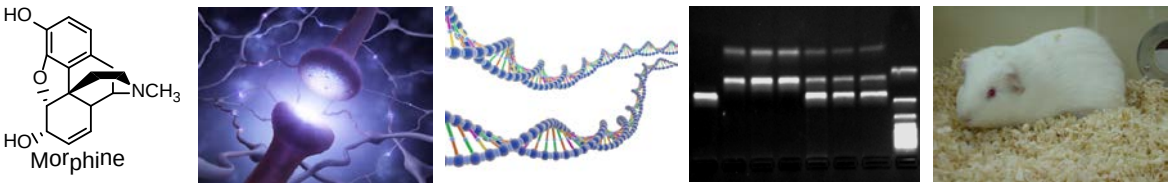
c) To define the pharmacogenomic profile using the detection of genomic variations in genes associated with morphine metabolism, drug transporters, opioid receptors and perception and processing of pain and correlate with clinical assessment and analytical morphine and metabolite concentrations, to understand its functional relevance.

d) To develop an animal model for the study of pharmacokinetics of morphine and pain assessment, in guinea pigs.

e) To understand the relevance of mechanisms involved in morphine pharmacokinetics in analgesia, through the study of the influence of morphine metabolism induction and inhibition and pain assessment in an animal model.

CHAPTER III

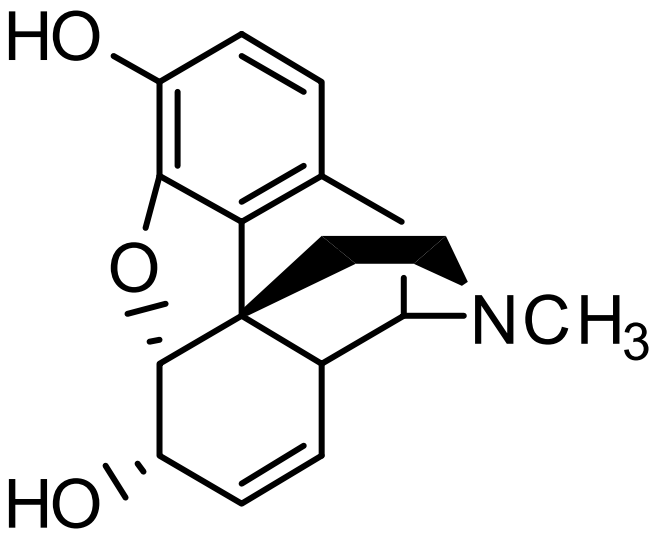
ORIGINAL RESEARCH



Study I

Quantification of morphine and its major metabolites M3G and M6G in *antemortem* and *postmortem* samples

(Submitted for publication)



Morphine

Quantification of morphine and its major metabolites M3G and M6G in *antemortem* and *postmortem* samples

Running title: Morphine quantification in *antemortem* and *postmortem* samples

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Abstract

Morphine is one of the most effective agents for the control of significant pain, primarily metabolized to morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G). While M6G is a potent opioid agonist, M3G has no opioid action and seems to have a role in the side-effects usually described. In this study, a reversed-phase high-performance liquid chromatographic method with diode-array and electrochemical detection was developed for the simultaneous determination of morphine, M3G and M6G in *antemortem* and *postmortem* samples (plasma, whole blood, urine, liver, kidney and brain). Morphine, glucuronides and internal standard were extracted by double solid-phase extraction and the separation was carried out with a Waters Spherisorb® ODS2 reversed-phase column and potassium phosphate buffer:acetonitrile containing sodium dodecyl sulfate as the mobile phase. The method proved to be specific with good linearity for all analytes in a calibration range from 1-600 ng/mL. Limits of detection in the studied matrices ranged from 0.4-4.5 ng/mL for morphine, 2.7-6.1 ng/mL for M3G and 0.8-4.4 ng/mL for M6G. Also, the method proved to be accurate with adequate precision and recovery. The proposed method can be successfully applied to quantify morphine and its metabolites in several biological samples, covering the major routes of distribution, metabolism and elimination of morphine.

Keywords: Morphine, morphine-3-glucuronide, morphine-6-glucuronide, metabolism, HPLC-DAD-electrochemical

Introduction

Morphine, an alkaloid present in the poppy plant, is one of the most effective agents for the short- and long-term control of significant pain. Accordingly to World Health Organization guidelines, morphine is the mainstay of pharmacological treatment for moderate-to-severe acute and chronic cancer-related pain (WHO, 1996; Ross *et al.*, 2005). However, despite its widespread clinical use, this opioid displays wide variations in its pharmacological efficacy and tolerability, presenting some side-effects that can compromise the patient safety / compliance and its analgesic effectiveness.

Morphine is extensively metabolized in the human liver especially by UDP-Glucuronosyltransferase 2B7 (UGT2B7) producing two important metabolites, M6G (10-15 %) and M3G (45-55 %), by glucuronidation of the 6-OH alcoholic group and the 3-OH phenolic group, respectively (Figure 1) (Carrupt *et al.*, 1991).

Other UGT isoforms seem to be involved in M3G formation, like UGT1A3, 1A6, 1A8, 1A9, and 1A10 (Stone *et al.*, 2003). M6G is a potent opioid receptor agonist with higher analgesic activity as compared to morphine (Carrupt *et al.*, 1991; Osborne *et al.*, 1992). M3G has no opioid action and it seems to have a role in the side-effects usually described, namely hyperalgesia / allodynia, neurotoxicity and an antagonistic effect, decreasing morphine analgesia (Carrupt *et al.*, 1991; Christrup, 1997; Holthe *et al.*, 2002).

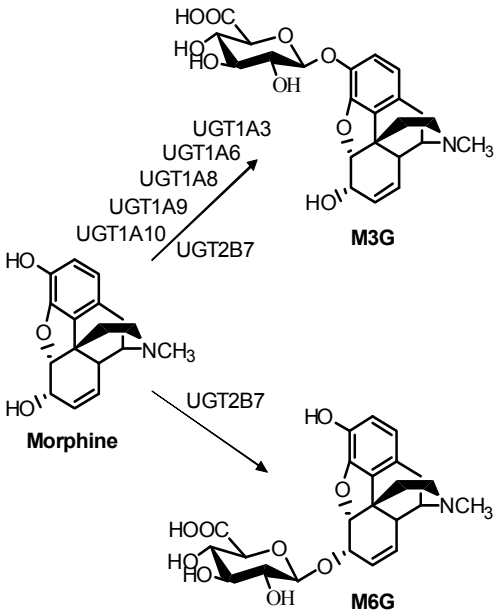

Figure 1. Morphine metabolism in M3G and M6G. M3G, morphine-3-glucuronide; M6G, morphine-6-glucuronide; UGT, UDP-Glucuronosyltransferase.

A variability of metabolites formation has been described in humans (Holthe *et al.*, 2002; Sawyer *et al.*, 2003; Klepstad *et al.*, 2005) and the different roles played by each compound may also account for different pain intensities and morphine requirements (Klepstad *et al.*, 2000). Therefore, the quantification of morphine and its glucuronide metabolites and calculation of metabolic ratios have become of increasingly interest for a better understanding of morphine efficacy and side-effects and also for the interpretation of toxic deaths involving heroin or morphine (Staub *et al.*, 1990; Bosch *et al.*, 2007).

Several analytical methodologies have been described for the quantification of morphine alone or in combination with its metabolites, in a variety of biological matrices (Samuelsson *et al.*, 1993; Smith *et al.*, 1999; Edwards and Smith, 2005; Kudo *et al.*, 2006; Musshoff *et al.*, 2006; Bosch *et al.*, 2007; Santos *et al.*, 2008). Since the direct quantitation of M3G and M6G has proved to be unsuccessful by gas chromatography accopled with mass spectrometry (GC-MS) (Bosch *et al.*, 2007), analysing only free and total morphine after hydrolysis (Kudo *et al.*, 2006), the majority of the quantification methods are based on liquid chromatography (LC) accopled with ultraviolet (UV)/diode array (DAD) detection (Bourquin *et al.*, 1997), electrochemical (Meng *et al.*, 2000; Ary and Rona, 2001), fluorescence (Huwyler *et al.*, 1995; Beike *et al.*, 1999; Meng *et al.*, 2000) or mass spectrometry (MS) (Edwards and Smith, 2005; Musshoff *et al.*, 2006). As MS is still more sensitive and specific than UV, DAD, electrochemical or fluorescence, LC-MS methods have emerged as the most suitable for quantification of morphine metabolites, despite their high costs, which decreases its availability and utilization. Thus, robust methods are required for the quantification of morphine, M3G and M6G, with lower costs than LC-MS but with similar sensitivity and specificity. The coupling of detectors can be a strategy for achieving this objective. In this study, we use both DAD and electrochemical detectors, accordingly with other reports (Ary and Rona, 2001; Fujita *et al.*, 2010). Electrochemical detection has been known as a highly sensitive technique, capable of detecting in the fentomol range, with a good linear response for several analytes (Takata and Muto, 1973; Acworth, 2011). This sensitivity is a major advantage, especially for morphine quantification, usually in lower concentration in chronic pain patients. Furthermore, it is also of major importance the development of methods that can quantify simultaneously the three compounds in several *ante* and *postmortem* matrices making the analysis faster and more efficient in both circumstances. Besides its interest, few

methodologies were described for simultaneous quantification of morphine and its glucuronides metabolites in *postmortem* fluids and organs. In this work we develop and validate an analytical method to quantify morphine, M3G and M6G by HPLC-DAD-electrochemical detection, in six different biological matrices, namely plasma, urine, whole blood, liver, brain and kidney, covering *ante* and *postmortem* analysis.

Methods

Reagents and Standards

Morphine hydrochloride, M3G hydrochloride and M6G hydrochloride were purchased from Lipomed (Arlesheim, Switzerland). Phenacetin (internal standard, IS), triethylamine, sodium dodecyl sulfate and hydrochloric acid were obtained from Sigma-Aldrich (St. Louis, MO). Methanol, acetonitrile, sodium dihydrogen phosphate and phosphoric acid were acquired from Merck (Darmstad, Germany). OASIS® weak cation exchange (WCX) cartridges, 60 mg, 3 mL were obtained from WATERS (Milford, MA). Bond Elut® C18 cartridges, 100 mg, 1mL were purchased from Agilent. All chemicals and reagents were of analytical grade or from the highest available grade.

Biological specimens

Antemortem and *postmortem* (autopsies performed 6h after death) negative morphine samples (whole blood, plasma, urine, liver, brain and kidney) were collected from rodents (*Cavia porcellus)*, according to previously proposed procedures (Dinis-Oliveira *et al.*, 2010). This species is considered the ideal model for studies involving morphine and its metabolites, since the pattern of metabolism is the most similar to humans, with an average M6G:M3G *ratio* of 1:4 in *Cavia porcellus* and 1:7 in humans (Kuo *et al.*, 1991).

Organ samples were homogenized in ice-cold deionized (1:4 w/v, Ultra-Turrax®). The homogenate was kept on ice and centrifuged at 13000*g*, 4ºC, 10 min. Aliquots of the resulting supernatants were stored (− 80°C) for posterior quantification.

Whole blood (1.5 mL) was diluted with phosphate buffer 0.01 M (1:2 v/v), submitted to two freeze-thawing cycles and centrifuged at 3000 rpm, 4ºC, 10 min. Plasma and urine samples were directly subjected to extraction by solid phase extraction (SPE).

Preparation of standard stock and fortified solutions

Stock solutions of morphine, M3G and M6G were prepared in deionized water at the concentration of 1 mg/mL. Quality control samples were subsequently prepared by serial dilutions of the stock solution in each matrix to yield the working solutions (1, 10, 20, 50, 100, 250, 600 ng/mL). A stock solution of the IS phenacetin was prepared in methanol (10 mg/mL). All the solutions were prepared daily and stored at -80°C.

Solid phase extraction

Morphine, M6G, and M3G were extracted by two-step solid-phase extraction (SPE) (Figure 2) according with Meng and collaborators (Meng *et al.*, 2000), with slight modifications. Briefly, for the extraction, 30 µL of the internal standard phenacetin at 10 mg/mL were added to 1.5 mL of plasma and 2 mL of urine/organ

homogenate or whole blood supernatant. The sample was then transferred to C18 cartridges, which have been previously conditioned with 2 mL of methanol and 2 mL of phosphate buffer (10 mM, pH = 9.5). The cartridge was then washed with 2 mL of phosphate buffer (10 mM, pH = 9.5) and eluted with methanol with 0.5 % of triethylamine. The eluate was dried with a nitrogen stream and posteriorly reconstituted with 1 mL of 80 % acetonitrile and transferred into a weak cation exchange (WCX, Oasis®) cartridge, previously conditioned with 4 mL of acetonitrile. After washing the cartridge with 4 mL of acetonitrile, the compounds were eluted with 1.5 mL of 80 % methanol containing 0.05 M HCl. The eluate was dried in a Labconco® evaporator. Samples were reconstituted with 50 µL of mobile phase and 40 µL were injected in the HPLC system.

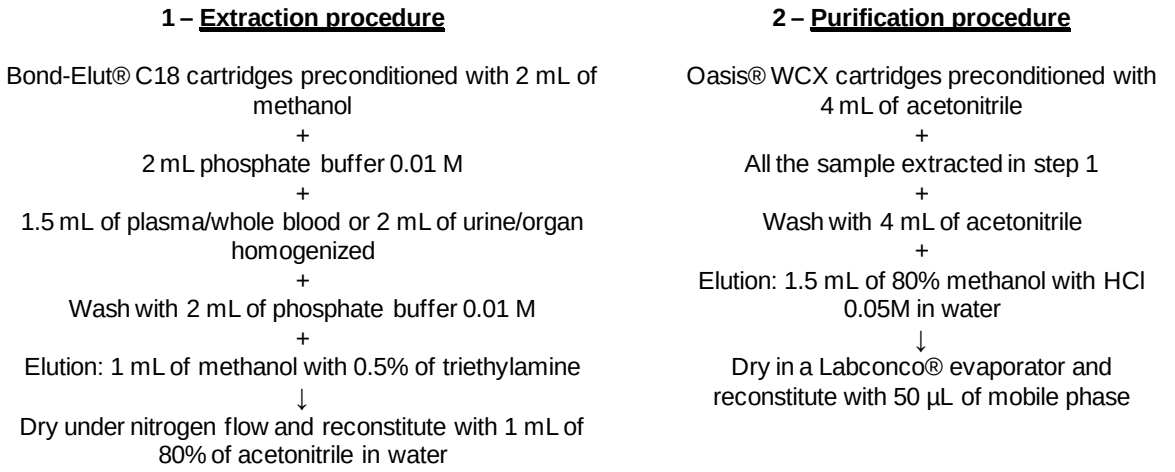

Figure 2. Sample preparation procedure. (1) Extraction of morphine, its metabolites and the internal standard (phenacetin) with SPE. (2) Purification of the sample extracted with a second SPE.

Chromatographic conditions

The HPLC system consisted in a HPLC Waters® 2690 system and analytes were separated at ambient temperature in a Waters Spherisorb® ODS2 reversed-phase column (250 mm x 4.6 mm x 5 µm). The mobile phase consisted of 0.01 M potassium phosphate buffer:acetonitrile (85:15 v/v) containing 0.04 mM sodium dodecyl sulfate and the flow rate was 1 mL/min. The eluent was filtered through 0.45 µm membrane and degassed with nitrogen stream. Quantification of M3G was performed in a DAD Waters® 996, at 210 nm. Quantification of M6G and morphine were performed at Coulochem® II 5200A, with 0.200 V for cell 1, 0.350 V for cell 2 and 0.400 V for guard cell. The analysis of the chromatogram was performed using a Waters Millennium[32] software.

Method validation

The validation of the method was performed according to the European Medicines Agency (EMA) (EMA, 2011), and other studies (Gouveia *et al.*, 2012; Costa *et al.*, 2013; Pinho *et al.*, 2013).

Selectivity

In order to detect any possible interferences, six blank samples (no analyte or IS added) of each matrix were extracted as previously described and analyzed by HPLC-DAD-electrochemical to detect possible interferences with morphine, M3G or M6G. Chromatographic selectivity was evaluated by the presence or absence of co-eluting peaks at the retention times of the analytes at the lower limit of quantification (LLOQ). The absence of interfering components is accepted when the response is less than 20% of the LLOQ for the analyte and 5% for the IS.

Carry-over

Carry-over was assessed by injecting blank samples after a high-concentration standard at the upper limit of quantification. Carry-over should not be greater than 20 % of the lower limit of quantification and 5 % for the IS.

Linearity

The method linearity was evaluated by the regression curves (*ratio* of analyte peak area and IS peak area *vs* analyte concentration) and expressed by the squared correlation coefficient (r^2). Three independent calibration curves (y = mx + b) were obtained using different concentrations of morphine and metabolites (1, 10, 20, 50, 100, 250, 600 ng/mL) and the mean slopes were obtained in order to calculate the concentration of unknown concentrations. In addition, a blank sample (processed matrix sample without analyte and without IS) and a zero sample (processed matrix with IS) were also analyzed but not used in the calculation of the calibration curve parameters. Linearity was accepted if $r^2 \geq 0.98$.

Limits of detection and lower limit of quantification

Limit of detection (LOD) and LLOQ were determined from the calibration curves data, as follows: LOD = $3\sigma/m$ and LLOQ = $10\sigma/m$, where σ is the standard deviation of the response and m is the slope of the calibration curve. For LOD, a retention time within ± 0.2 minutes of the average retention time of standards was also considered. For LLOQ, imprecision ≤ 20 % was accepted.

Precision and accuracy

Intra-day precision was determined by preparing and analyzing on the same day 3 replicates of 3 different concentrations (low, medium and high: 20, 250, 600 ng/mL) of the 3 analytes. The inter-day precision was evaluated by repeating the intra-day precision study in 3 different days for all the compounds. Precision was determined by calculating the mean, standard deviation and coefficient of variation (CV%) of the replicated analysis. A CV% value of ≤ 15 % was considered satisfactory.

Accuracy was assessed by spiking blank matrix with the same 3 different concentrations and through the calculation of the percent deviation between the calculated value and the nominal value [Accuracy (%) = (experimental concentration ⁄ theoretical concentration) × 100]. A deviation percentage of ≤15 % was considered satisfactory.

Recovery

The recovery was evaluated by analyzing two sample groups of the same concentrations (20, 250 and 600 ng/mL) in triplicate, but differently processed. In the first group, morphine, its metabolites and IS were

analyzed following the extraction procedure mentioned above. In the second group, all the four compounds were added to the elution solvent before drying. The recovery was evaluated by the comparison of the mean response of the two groups. The response of the unextracted group represents 100 % recovery. Analytical recovery between 80 and 120 % was considered acceptable.

Results and Discussion

Method Validation

Solid-phase extraction, chromatographic separation and detection

The applied double SPE procedure allowed the pre-concentration of the analytes but also the achievement of a cleaner extract, allowing us to develop a more sensitive and specific methodology.

To obtain the best peak resolution and separation of all the compounds, several parameters were tested, such as different mobile phase percentages, flow rate of the mobile phase and injection volume. An injection volume of 40 µL and the total time of analysis was 40 minutes were considered optimal. The retention times for M3G, M6G, morphine and IS, were respectively 9.8, 15.1, 25.3 and 35.2 minutes (Figure 3).

Selectivity

Several blank samples of plasma, whole blood, urine, liver, kidney and brain were analyzed to evaluate chromatographic interferences. No interference peaks were detected, either in the retention times of morphine and metabolites or in the IS retention time, confirming the selectivity of the method. Therefore, all standard solutions were prepared in the different matrix to mimic real conditions.

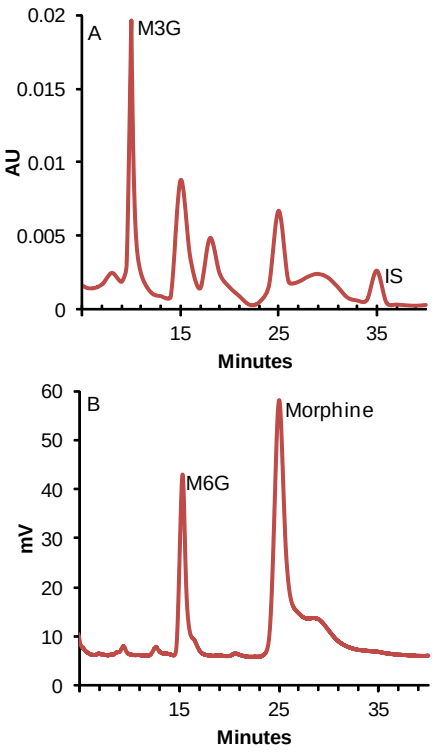

Figure 3. Chromatogram of morphine and metabolites in plasma, at 600 ng/mL. (A) DAD detector. (B) Coulometric detector. IS, Internal Standard; M3G, morphine-3-glucuronide; M6G, morphine-6-glucuronide.

Carry-over

Each injection of high-concentration calibration standard was followed by a blank sample injection (mobile phase). The obtained carry-over results were <20 % of the LLOQ and <5 % for the IS, which are within the proposed acceptance limits (EMA, 2011).

Linearity

The weighted least squares linear regression equations and coefficients of correlation were calculated using three independent curves. Results are presented as mean ± standard deviation and y and x represent the

relationship between the peak area ratio and the corresponding calibration concentrations, respectively. The method was linear at the concentration range of 1-600 ng/mL, with coefficients higher than 0.99 over the concentration range, confirming the linearity of the method for each compound (Table 1-3).

Table 1 - Linear regression analysis of morphine standard solutions in the different biological matrices (1-600 ng/mL) performed on three different days.

Sample	n =3	y = mx + b	r^2	LOD (ng/mL)	LLOQ (ng/mL)
Plasma	1	y = 0.0559x + 0.0075	0.9969	0.41	1.24
	2	y = 0.0567x + 0.0295	0.9977		
	3	y = 0.0563x + 0.008	0.9976		
Whole blood	1	y = 0.0112x + 0.0076	0.9958	2.0	6.2
	2	y = 0.0111x + 0.0158	0.9969		
	3	y = 0.0114x + 0.0019	0.9966		
Urine	1	y = 0.0654x + 0.1127	0.9978	0.5	1.5
	2	y = 0.0664x + 0.0068	0.9962		
	3	y = 0.0663x + 0.0158	0.9970		
Kidney	1	y = 0.0347x + 0.4287	0.9950	0.7	2
	2	y = 0.0345x + 0.3247	0.9980		
	3	y = 0.0337x + 0.469	0.9908		
Liver	1	y = 0.0676x + 0.0853	0.9999	4.5	4.4
	2	y = 0.0675x + 0.0022	0.9986		
	3	y = 0.0673x + 0.0045	0.9986		
Brain	1	y = 0.0714x - 0.2003	0.9960	0.4	1.4
	2	y = 0.0722x - 0.1202	0.9928		
	3	y = 0.0758x - 0.2322	0.9952		

LLOQ, lower limit of quantification; LOD, limit of detection

Table 2 - Linear regression analysis of M3G standard solutions in the different biological matrices (1-600 ng/mL) performed on three different days.

Sample	n =3	y = mx + b	r^2	LOD (ng/mL)	LLOQ (ng/mL)
Plasma	1	y = 0.0101x + 0.0442	0.9984	2.8	8.5
	2	y = 0.0107x – 0.0213	0.9900		
	3	y = 0.0103x + 0.0206	0.9915		
Whole blood	1	y = 0.005x + 0.0048	0.9974	5.3	16.1
	2	y = 0.0049x + 0.01	0.9979		
	3	y = 0.005x + 0.0061	0.9975		
Urine	1	y = 0.0022x + 0.0073	0.9985	6.0	18.2
	2	y = 0.0021x + 0.0006	0.9994		
	3	y = 0.0021x + 0.0043	0.9993		
Kidney	1	y = 0.0038x + 0.0485	0.9998	6.1	18.4
	2	y = 0.0036x + 0.0873	0.9935		
	3	y = 0.004x + 0.0217	0.9968		
Liver	1	y = 0.0123x + 0.0393	0.9980	2.7	8.0
	2	y = 0.0124x + 0.0363	0.9983		
	3	y = 0.0125x + 0.0835	0.9995		
Brain	1	y = 0.0036x + 0.0151	0.9991	4.7	14.1
	2	y = 0.0035x + 0.0119	0.9975		
	3	y = 0.0035x + 0.0191	0.9994		

LLOQ, lower limit of quantification; LOD, limit of detection; M3G, morphine-3-glucuronide

Table 3 - Linear regression analysis of M6G standard solutions in the different biological matrices (1-600 ng/mL) performed on three different days.

Sample	n =3	y = mx + b	r^2	LOD (ng/mL)	LLOQ (ng/mL)
Plasma	1	y = 0.0214x + 0,034	0.9980	1.0	3.2
	2	y = 0.0211x + 0,0925	0.9989		
	3	y = 0.0219x + 0,0487	0.9986		
Whole blood	1	y = 0.0053x + 0.0103	0.9973	4.4	13.2
	2	y = 0.0053x + 0.0211	0.9970		
	3	y = 0.0053x + 0.0066	0.9980		
Urine	1	y = 0.0088x + 0.0148	0.9977	2.4	7.4
	2	y = 0.0088x + 0.0199	0.9971		
	3	y = 0.0088x + 0.0183	0.9968		
Kidney	1	y = 0.019x + 0.1247	0.9991	1.0	3.2
	2	y = 0.0189x + 0.1204	0.9987		
	3	y = 0.0184x + 0.1105	0.9958		
Liver	1	y = 0.0138x + 0.0427	0.9994	1.6	5.0
	2	y = 0.0136x + 0.0416	0.9996		
	3	y = 0.0138x + 0.0479	0.9991		
Brain	1	y = 0.0132x - 0.1961	0.9982	0.8	2.3
	2	y = 0.0127x - 0.1741	0.9984		
	3	y = 0.0133x - 0.1962	0.9980		

LLOQ, lower limit of quantification; LOD, limit of detection; M6G, morphine-6-glucuronide

Limit of detection and lower limit of quantification

LOD and LLOQ results are shown in Table 1-3. The LOD and LLOQ obtained for the three compounds in the several matrices are in agreement with the ones described for these compounds in the literature in real samples.

Precision and accuracy

Precision and accuracy results are presented in Table 4. All the CV% values calculated for intra and inter-day precision studies of all three compounds did not exceed 15 %, so the method was considered precise for morphine, M3G and M6G. Regarding accuracy, values in the range of 91.7-114.3 % for plasma, 88.9–111.2 % for whole blood, 89.8–114.8 % for urine, 97.3–113.2 % for kidney, 94.7–117.7 % for liver and 96.1–114.4 % for brain were determined, which are within the proposed acceptance limits for this parameter (100 ± 15 %). Associated with lower CV% (0.2-11.0 %), these results suggest that the extraction was equally efficient for the three different concentrations evaluated (Table 4).

Recovery

Values for the recovery of all the three compounds in the different matrix were in the range of 79.9-94.9 % for the three chosen concentrations.

Table 4 – Precision and accuracy (%) for morphine, M3G and M6G quantification

		Morphine				M3G				M6G			
Sample	Concentration (ng/mL)	Intra-day precision (%, n=3)	Inter-day precision (%, n=3)	Accuracy (%, n=3)	Recovery (%, n=3)	Intra-day precision (%, n=3)	Inter-day precision (%, n=3)	Accuracy (%, n=3)	Recovery (%, n=3)	Intra-day precision (%, n=3)	Inter-day precision (%, n=3)	Accuracy (%, n=3)	Recovery (%, n=3)
Plasma	20	2.1	2.8	114.3	92.2	8.4	5.0	101.3	89.0	4.3	3.4	111.4	90.2
	250	1.1	2.0	91.7	89.2	2.1	6.8	101.1	83.2	2.3	1.4	98.0	85.1
	600	0.3	0.7	102.4	79.9	1.2	4.2	102.1	86.2	0.8	0.9	102.2	90.6
Whole	20	4.9	1.3	104.0	89.6	4.1	8.2	111.2	81.3	7.5	11.0	111.0	85.7
Blood	250	5.6	1.7	88.8	92.1	4.9	0.8	92.5	88.5	7.2	3.8	99.6	81.3
	600	1.2	1.3	101.6	80.7	2.8	2.6	101.2	89.1	3.1	1.1	103.5	89.9
Urine	20	6.8	9.6	89.8	93.1	6.0	2.1	105.1	93.2	3.6	9.6	114.8	91.6
	250	2.1	1.5	90.4	91.1	5.0	1.9	95.4	88.4	5.4	0.6	90.7	93.8
	600	2.4	0.9	101.1	90.3	5.0	5.3	100.6	93.1	1.9	0.5	101.4	93.4
Kidney	20	8.6	6.2	109.6	85.4	5.0	10.2	99.9	88.3	7.3	3.2	113.2	87.9
	250	4.7	6.5	111.4	90.2	10.4	8.4	101.2	88.7	5.8	5.5	98.3	91.4
	600	5.0	2.9	97.4	91.1	5.6	5.0	100.1	91.6	7.6	2.6	97.3	88.5
Liver	20	4.8	2.7	112.7	91.3	3.1	2.5	110.4	92.2	2.3	1.9	112.3	85.3
	250	2.0	4.1	94.7	94.3	3.5	7.5	96.3	90.2	1.6	1.8	103.6	93.4
	600	1.6	0.2	100.7	89.8	1.2	0.6	100.6	89.6	0.6	0.5	99.3	90.1
Brain	20	6.4	2.8	97.1	89.6	0.8	2.9	101.7	85.1	10.6	8.9	114.4	81.6
	250	4.8	5.5	114.9	94.9	1.3	0.2	95.0	89.9	3.1	3.9	97.2	87.3
	600	3.0	2.8	98.0	90.3	6.9	3.2	99.8	88.5	4.6	2.2	100.5	84.1

M3G, morphine-3-glucuronide; M6G, morphine-6-glucuronide.

Conclusions

A selective, precise, accurate and reproducible analytical method to quantify morphine and metabolites in *ante mortem* and *post mortem* samples was developed. The described method has good sensitivity with LOD comparable to LC/MS methodologies (in the ng/mL and ng/g range) (Bosch *et al.*, 2007), but with a much less expensive equipment. Morevover, it was possible to validate the assay for different *ante mortem* and *post mortem* matrices, namely plasma, urine, whole blood, liver, brain and kidney. The proposed method can be successfully applied in the quantification of morphine and metabolites, covering the routes of distribution, main metabolism and elimination of morphine.

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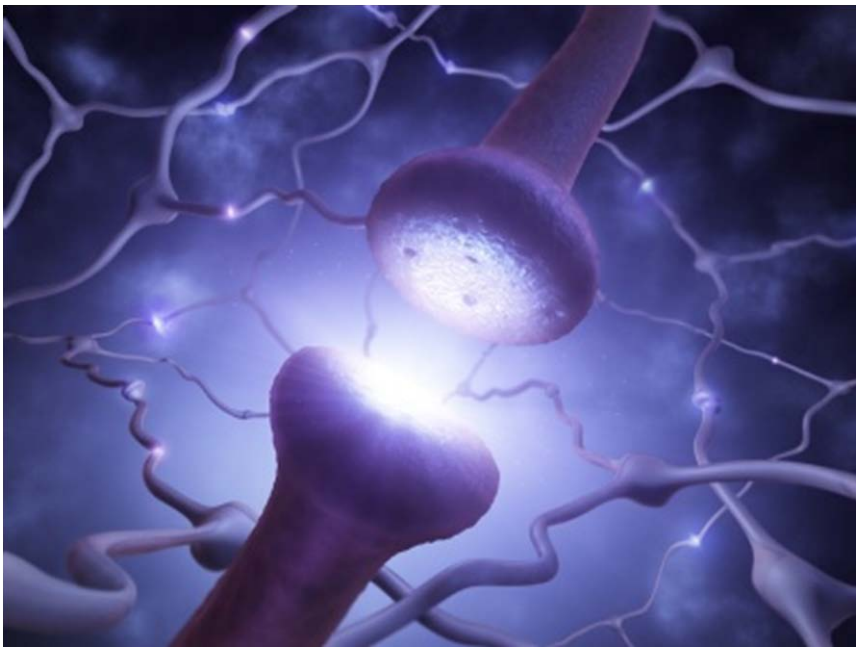
Study II

COMT Genetic Polymorphisms are associated with opioid dose requirements in cancer patients

(Submitted for publication)



COMT Genetic Polymorphisms are associated with opioid dose requirements in cancer patients

Running title: COMT and opioids requirements

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Abstract

Genetic variability may result in significant differences in the response to opioids. Polymorphisms in genes encoding μ-opioid receptor (*OPRM1*), ATP-binding-cassette-sub-family-B-member-1 transporter (*ABCB1*) and catechol-O-methyltransferase enzyme (*COMT*) may influence pharmacokinetics and pharmacodynamics of opioids, as well as the nociception mechanism. Our purpose was to investigate the repercussions of the mentioned polymorphisms on pain-related parameters in cancer patients. DNA samples from cancer patients were genotyped for the polymorphisms in *OPRM1* (rs1799971), *COMT* (rs4680), and *ABCB1* (rs1128503,rs1045642) with real-time PCR. Doses were re-expressed as oral morphine equivalents. We examined the relation between these polymorphisms and opioid dose, pain intensity, performance status, adverse effects, age, sex, metastases and breakthrough pain. Total opioid consumption was related to the polymorphism Val(108/158)Met in *COMT* gene. Carriers of Met allele were significantly associated with a requirement of higher opioids doses (p = 0.008, Fischer's exact test), and the same result was obtained with logistic regression analysis, adjusted to age and sex (p = 0.013; p = 0.003 using Bootstrap analysis). Our results suggest that genetic variation at COMT enzyme may be correlated with the dose requirement and/or response to opioids in cancer patients.

Keywords: Catechol-O-Methyl Transferase (COMT), Val(108/158)Met polymorphism, cancer-related pain, pain management, opioid analgesics.

Introduction

The World Health Organization treatment guidelines include opioid analgesics as mainstay for moderate to severe acute and chronic cancer-related pain (WHO, 1996; Ross *et al.*, 2005). However, the perception of pain varies greatly among people, which implies wide variations in opioids dosage, pharmacological efficacy and tolerability (Aubrun *et al.*, 2003; Ross *et al.*, 2005; Shi *et al.*, 2010). An important cause of this interindividual variability may be of pharmacogenetic nature, due to polymorphisms in opioid receptors, transporters and metabolic enzymes (Lötsch and Geisslinger, 2006; Kasai *et al.*, 2008; Kleine-Brueggeney *et al.*, 2010; Muralidharan and Smith, 2011). Also, perception and processing of pain information involves a significant number of modulators/suppressors that are also plausible candidates to interfere with opioids action (Lötsch and Geisslinger, 2006; Shi *et al.*, 2010).

Among the various genes involved in pain, the μ-opioid receptor (*OPRM1*) gene, encoded by the genetic locus *OPRM1*, has been subject of investigation for some single nucleotide polymorphisms (SNP) that seemed to influence opioids binding and activity. The SNP A118G (rs1799971) is relatively frequent in Caucasians (10-14 %) (Klepstad *et al.*, 2005) and causes an amino acid alteration from asparagine to aspartatic acid in exon 1, (Klepstad *et al.*, 2005) which seems to influence opioids action. In spite of an

increased affinity and potency shown *in vitro* for homozygous G (Bond *et al.*, 1998), clinical studies suggest that individuals homozygous for the wild-type A allele seem to require a lower morphine dose to achieve pain control (Klepstad *et al.*, 2004; Reyes-Gibby *et al.*, 2007; Sia *et al.*, 2008; Tan *et al.*, 2009). However, controversial results have also been described (Beyer *et al.*, 2004; Klepstad *et al.*, 2011).

Opioids absorption, distribution and excretion can be affected by several factors, including their transport across biological membranes. Among several transport systems, efflux-carriers of the ATP-binding cassette (ABC) family represent a major factor in the disposition of drugs and xenobiotics (Gerloff, 2004). P-glycoprotein (Pgp), the gene product of multidrug resistance protein 1 (MDR1, *ABCB1*), is probably the most studied one (Gerloff, 2004). Since opioids are Pgp substrates (Xie *et al.*, 1999), polymorphisms in the *ABCB1* gene might influence the pharmacological and toxicological effects of these drugs by altering Pgp transport characteristics expression (Gerloff, 2004). Two of the most frequent SNP in *ABCB1* are synonymous polymorphisms, C3435T (rs1128503) and C1236T (rs1045642). The C3435T SNP is associated with altered Pgp expression and transport function and homozygous individuals for T allele exhibit a lower mRNA expression, due to an alteration in its stability (Wang *et al.*, 2005). It has been reported that C1236T is in *linkage disequilibrium* with C3435T and that is also probably related to different opioid doses requirements, with higher opioid doses needed in T allele homozygous individuals (Kleine-Brueggeney *et al.*, 2010).

The influence of the polymorphic catechol-O-methyl-transferase (*COMT*) gene in pain has also been subject of investigation (Zubieta *et al.*, 2003; Diatchenko *et al.*, 2006; Nackley *et al.*, 2007; Mobascher *et al.*, 2010; Ahlers *et al.*, 2012; Martínez-Jauand *et al.*, 2013). This enzyme is a key modulator of dopaminergic and noradrenergic neurotransmission and it is postulated to have a role in pain. The Val(108/158)Met polymorphism is a nonsynonymous SNP, resulting in an amino acid substitution, valine (Val) by methionine (Met) (Zubieta *et al.*, 2003). This amino acid interchange is associated with altered thermostability of the enzyme that leads to a three-to-four fold reduction in its activity (Zubieta *et al.*, 2003; Zhang *et al.*, 2009; Shi *et al.*, 2010). Individuals with the Met/Met genotype have the lowest activity of COMT, heterozygous are intermediate and those with Val/Val genotype have the highest activity of the enzyme (Zubieta *et al.*, 2003). The different COMT activities resulting from this SNP may have a serious impact in several physiological functions, including pain perception (Emin Erdal *et al.*, 2001; Diatchenko *et al.*, 2006; DeYoung *et al.*, 2010). In the last decade, several studies have shown an association between the Val(108/158)Met SNP and pain sensitivity (Zubieta *et al.*, 2003; Jensen *et al.*, 2009; Mobascher *et al.*, 2010), relating individuals homozygous for Met allele with increased pain sensitivity and lower μ-opioid system activation during sustained pain (Zubieta *et al.*, 2003; Jensen *et al.*, 2009; Mobascher *et al.*, 2010; Vossen *et al.*, 2010; Ahlers *et al.*, 2012; Martínez-Jauand *et al.*, 2013). All the effects were opposite in the Val/Val individuals. Regarding a possible association

of the SNP with opioid dose, carriers of Met/Met genotype were unexpectedly associated with lower morphine requirements than patients homozygous for the Val allele (Rakvåg *et al.*, 2005; Reyes-Gibby *et al.*, 2007; Rakvag *et al.*, 2008). Nevertheless, contradictory information has been reported in recent years (Klepstad *et al.*, 2011; Kolesnikov *et al.*, 2011) and the association of the Met allele with lower consumption of morphine has not always been verified. An association between the Val/Val genotype and lower opioids requirements or pain intensity would be more consistent with the results previously described of a lower μ-opioid system activation and increased sensitivity to pain in patients with Met allele. These controversy results prompted us to an investigation in this field.

Therefore, the aim of our exploratory study was to evaluate the role of *OPRM1*, *ABCB1* and *COMT* genotypes on several pain-related parameters on pain-treated patients, namely the opioid dose requirements, pain intensity, performance status, adverse effects, age, sex, bone or CNS metastases and breakthrough pain.

Methods

Ethics

All data were obtained with the informed consent of the participants prior to their inclusion in the study, according to Helsinki Declaration principles. The study was also approved by the Hospital (Portuguese Institute of Oncology - Porto) Ethical Internal Commission.

Subjects

We conducted a hospital-based study analyzing 30 Caucasian individuals admitted in the Portuguese Institute of Oncology, Porto, Portugal between 2010 and 2011. All the patients were in-patients from the Palliative Care Unit-Network or followed for pain consultation and were recruited according to the criteria: expected survival above 1 month, with at least 1 week of oral or subcutaneous opioid treatment for cancer-related pain, must read and write, not in confusional state and without cardiovascular, renal or hepatic dysfunction. Data concerning time to adverse effects associated with opioid therapy (fatigue, pruritus, anorexia, perspiration, nausea and vomiting, diarrhea, xerostomia, cough, dyspnea, insomnia, drowsiness, nervousness, sadness and confusion), time to switch for another pain-relief regimen due to inadequate analgesia or intolerable side effects, overall survival time, cancer diagnosis, age, sex and ethnicity were obtained from clinical files. Daily opioid doses were collected from the patients' ward charts and were re-expressed as oral morphine equivalents (OMEQ) (Cepeda *et al.*, 2010).

Assessments

Pain was measured daily, through evaluation of average and maximal pain during the last 24 h using a numeric 11-point scale, where 0 represents "no pain" and 10 means "worst pain possible" (Klepstad *et al.*, 2002). Patient's internal state and side effects associated with opioid therapy were assessed daily through a 5-point scale: "no", "mild", "moderate", "intense", and "maximum" (Aaronson *et al.*, 1993; Laugsand *et al.*, 2011). Patients' functional status was

evaluated by the Eastern Cooperative Oncology Group (ECOG) performance status scale (Oken *et al.*, 1982).

Blood samples and pharmacogenetic analysis

Blood samples were collected by venipuncture after achieving a stable painkilling opioid dose. Genomic DNA was extracted from peripheral blood samples by using QIAMP DNA Blood Mini kit (QIAGEN®), according to the manufacturer's protocol.

All genotypes were determined by direct allelic discrimination in the ABI Prism Real Time PCR System 7300 and Taqman^{TM} Allelic Discrimination. Genotyping of *OPRM1* (rs1799971) (Sia *et al.*, 2008), *COMT* (rs4680) (Mobascher *et al.*, 2010) and *ABCB1* (rs1045642, rs1128503) (Levran *et al.*, 2008) were performed as previously described. Probe sequences for VIC/FAM are described in the Supplementary Table 1. Allelic discrimination PCR reactions were carried out in 6 µL volumes using 2.5 µL of TaqMan® Universal PCR Master Mix (2×), 0.125 µL of 40× assay mix, 2.375 µL of sterile H_2O and 1 µL of genomic DNA. Amplification of DNA was carried out on an ABI 7300 using the following conditions: 95ºC for 10 min, followed by 45 cycles of 95ºC for 15s and 60ºC for 1 min. Data capture and analysis were performed through the ABI 7300 Real Time PCR System (Applied Biosystems) and the Sequence Detection Systems software (Applied Biosystems version 1.2.3). Quality control included the use of non-template controls in all runs and blind replicate genotype assessment on 10 % of the samples. We observed concordance among duplicates.

Statistical analysis

Data analysis was performed using the computer software Statistical Package for Social Sciences (SPSS) for Windows (version 18.0) and GraphPad Prism® for Windows (version 5.0). For the analysis, daily OMEQ was divided in four groups, according to Edmonton classification (Bruera *et al.*, 1995; Bercovitch and Adunsky, 2004): Low (< 60 mg/24 h), Moderate (60-299 mg/24 h), High (300-599 mg/24h) and Very High (≥ 600 mg/24 h). In a second step, analysis was performed comparing two groups accordingly to the lower limit of OMEQ: < 60 mg/24 h and ≥ 60 mg/24 h. Differences in proportions were evaluated by univariate comparisons of genotype frequencies using the X^2 test, Fisher's exact test and bootstrapping analysis, and a $p<0.05$ was considered statistically significant. The results of the second analysis were also analyzed by logistic regression, adjusted to age, gender, and stress and mood state. We evaluated the statistical power of the sample using EPI6 software.

Results

Patients

Thirty patients receiving chronic opioids for cancer-related pain were admitted in this study (Table 1). No statistically significant association ($p > 0.05$) was found between the patient's characteristics, pain assessment, adverse effects and other symptoms and the genotype groups of *OPRM1*, *ABCB1* and *COMT* SNP (data not shown).

Table 1: Patients' data.

Variable	Patients (n = 30)	Variable	Patients (n = 30)
Sex		Pain category	
Male	15	Visceral pain	3
Female	15	Nociceptive pain	7
Age	56.97 ± 12.77	Neuropathic pain	6
Tumour		Nociceptive + Neuropathic pain	3
Lung	4	Mixed pain	11
Urologic	3	Pain Intensity	3.43 ± 2.73
Breast	6	Maximum Pain	5.04 ± 3.65
Prostate	3	OMEQ (mg/24 h)	181.41 ± 37.93
Gastrointestinal	1	Breakthrough pain	
Female reproductive organs	3	Yes	19
Others	10	No	11
Metastasis		Rescue opioid (breakthrough pain)	
No	11	No	13
Liver	6	Morphine	15
Bone	14	Tramadol	1
CNS	3	Methadone + Morphine	1
Lung	7	OMEQ (mg/24 h) for breakthrough pain	48.60 ± 27.48
Other	5		
ECOG	2.28 ± 1.34		

All numbers are absolute numbers or mean ± SD. No statistically significant differences were observed between groups. Categorical data were analyzed using the chi-square test. CNS, central nervous system; ECOG, Eastern Cooperative Oncology Group; OMEQ, Oral Morphine Equivalents.

Genotype distribution

Regarding *OPRM1* A118G SNP, genotype frequencies were: 70 % AA, 23.3 % A/G and only 6.7 % GG. For *ABCB1* C3435T genotype frequencies were: 23.3 % CC, 63.3 % C/T and 13.3% TT. *MDR1* C1236T SNP evidenced a distribution of: 26.7 % CC, 56.7 % C/T and 16.7% TT. Concerning *COMT* Val(108/158)Met SNP genotype frequencies were: 30.3 % Val/Val, 56.7 % Val/Met and only 10 % Met/Met. In a second examination, the Val/Met group was analyzed together with the Met/Met group resulting that the Met allele was present in 20 patients (66.7 %). Allele frequencies and the results of the X^2 test showed that there was no significant departure from Hardy-Weinberg equilibrium.

Daily oral morphine equivalents requirements and genotypes

Considering daily OMEQ requirements, there were no significant differences ($p > 0.05$) when comparing the different genotypes of *OPRM1* and *ABCB1* SNP. However, there differences were found when comparing the different *COMT* genotypes with opioid requirements. It was possible to observe that patients with de Val/Val genotype required the lower dose (95.08 ± 27.76 mg/24 h) and that the presence of the Met allele was related

with an increase in morphine dose requirements (195.68 ± 45.94 mg/24 h for Val/Met genotype and 388.33 ± 258.78 mg/24 h for Met/Met genotype). Due to the low frequency of Met/Met genotype, all the analyses were performed with the Val/Val group (n = 10) *vs.* presence of Met allele (n = 20). Significant differences (p = 0.008, Fisher's exact test for two OMEQ groups) were found between the two groups of genotypes and morphine dose requirements (Table 2 and Figure 1), 95 % patients with Met allele in *COMT* Val(108/158)Met polymorphism requiring significantly higher daily doses of opioids when compared with the Val/Val genotype.

The same result was obtained for the two OMEQ classes by logistic regression, adjusted to age and gender (p = 0.013, Fig. 1; p = 0.003 using Bootstrap analysis). Furthermore, when the adjustment for logistic regression was according to stress and mood state, results were also significant (p = 0.016; p = 0.019 using Bootstrap analysis). The evaluation of the power of the sample indicated that for an 80 % power/95 % confidence will be required 36 cases and for 80 % power/90 % confidence, at least 30 cases are required.

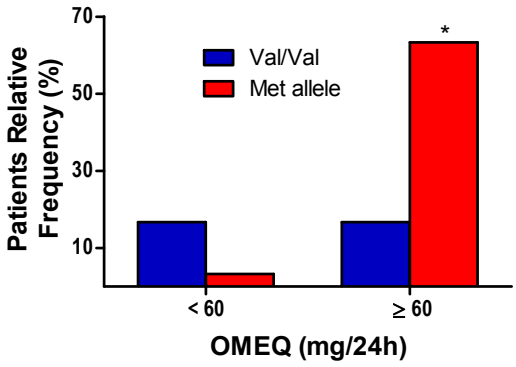

Figure 1. Two OMEQ classes *vs COMT* Val(108/158)Met SNP. Fisher's exact test (p < 0.05). Significant differences (p = 0.008) were found between the two groups of genotypes and morphine dose requirements, which was also confirmed by logistic regression, adjusted to age and gender (p = 0.013; p = 0.003 using Bootstrap analysis) and to stress and mood state (p = 0.016; p = 0.019 using Bootstrap analysis). OMEQ, Oral Morphine Equivalents.

Table 2: Patients' classification through 4 OMEQ classes for Val(108/158)Met genotype groups.

OMEQ (mg/24h)	**Patients (n = 30)**	**Val/Val (n = 10)**	**Met Allele (n = 20)**	***p* value** (Fisher's exact test)
Low: < 60 mg/24h	6	5	1	0.008*
Moderate: 60-299 mg/24h	18	4	14	
High: 300-599 mg/24h	4	1	3	
Very High: ≥ 600mg/24h	2	0	2	

All numbers are absolute numbers. Fisher's exact test (p < 0.05): *Model 1 – Low vs. Moderate/High/Very High, p = 0.008; Model 2 – Low/Moderate vs. High/Very High, p = 0.326; Model 3 – Low/Moderate/High vs. Very High, p = 0.436.

Discussion

In the present study we analyzed the association of four frequent SNP involved in different phases of pharmacokinetics and pharmacodynamics of opioids on several pain-related parameters of pain-treated patients. While the SNP related to *OPRM1* and *ABCB1* evidenced no statistically significant association with patient's characteristics, opioids requirements, adverse effects or pain assessment, the present study suggests an association of *COMT* Val(108/158)Met polymorphism with OMEQ requirements of patients suffering from cancer-related pain. Individuals with Met allele were related first with four groups of OMEQ, revealing a significant association. Due to the low number of cases in some of the groups, the variable OMEQ was re-grouped and the statistical analysis performed through the Fisher's exact test, enlightening a statistically significant result (p = 0.008). Formal corrections for multiple comparisons were not performed, since this exploratory study focuses on only few scientifically sensible comparisons. Fisher's exact test and Bootstrap re-sampling strategy were used to analyze the results and statistical significance of major findings was obtained, suggesting that the presence of the Met allele implies higher doses of opioids to eliminate pain in a small population of patients with cancer-related pain.

COMT is a key enzyme for norepinephrine, epinephrine and dopamine metabolism. Several studies have shown that Val(108/158)Met polymorphism affects the thermostability of the enzyme (Lotta *et al.*, 1995; Chen *et al.*, 2004; Zhang *et al.*, 2009) and that different levels of COMT activity may influence the functions regulated by these monoamines, including pain and μ-opioid system. Zubieta and collaborators (Zubieta *et al.*, 2003) observed, through positron emission tomography studies, that homozygous Met allele individuals are characterized by diminished regional μ-opioid system responses to pain, a decreased release of endogenous opioids and increased sensitivity to pain. These results were corroborated by recent studies (Jensen *et al.*, 2009; Mobascher *et al.*, 2010). No correlation was found between the initial response to the pain stimulus and *COMT* Val(108/158)Met polymorphism (Kim *et al.*, 2004; Diatchenko *et al.*, 2006; Jensen *et al.*, 2009). Nevertheless, during sustained pain, the inhibitory pain system is continuously challenged and the differences become relevant (Jensen *et al.*, 2009; Loggia *et al.*, 2011). Hence, this polymorphism may have an enormous importance in chronic pain patients, including cancer-related pain.

The influence of *COMT* Val(108/158)Met polymorphism in pain processing may be explained by the higher levels of extraneuronal catecholamines in brain. Higher synaptic cleft levels of dopamine and chronic overactivation of dopamine 2 (D_2) receptors may result in a potential inhibition of morphine analgesia, as it was observed in animal studies (Kolesnikov *et al.*, 2011). Additionally, animal experiments have shown that hyperalgesia can be induced by β_2-adrenergic stimulation (Khasar *et al.*, 1999) and $\beta_{2/3}$-adrenergic antagonists can block pain sensitivity induced by COMT inhibition (Nackley *et al.*, 2007). Therefore, accumulation of norepinephrine and

epinephrine may result in overactivation of the nociceptive $\beta_{2/3}$-adrenergic pathways. In accordance, the effect of propranolol on pain reduction (Tchivileva *et al.*, 2010) and opioid-induced hyperalgesia (Chu *et al.*, 2012) was already described in humans.

Controversial studies describe a possible relation between *COMT* Val(108/158)Met polymorphism and morphine requirements. A potential association between Met/Met genotype and lower doses of morphine requirements was suggested (Rakvåg *et al.*, 2005; Reyes-Gibby *et al.*, 2007; Rakvag *et al.*, 2008). Although not expected, the results were explained by the compensatory increased of μ-opioid receptor density and binding potential in different brain regions, in Met/Met carriers (Chen *et al.*, 1993; Zubieta *et al.*, 2003). Nevertheless, we observed the opposite effect, since carriers of the Met allele required higher doses of opioids. Indeed, Met/Met individuals have an increased expression of μ-opioid receptor at baseline, but during sustained pain they have a decreased activation of the μ-opioid system (Zubieta *et al.*, 2003; Ross *et al.*, 2008). Furthermore, in the study of Jensen and colleagues (Jensen *et al.*, 2009) no differences in the analgesic effect were found, after the injection of the opioid.

The current study suggests a possible association between *COMT* Val(108/158)Met polymorphism and the need of higher doses of opioids in cancer patients. However, the influence of this polymorphism in the efficacy of pain modulation or/and the susceptibility to opioid-induced hyperalgesia and tolerance is still a matter of debate (Jensen *et al.*, 2009). Both situations may lead to the increased pain sensitivity reported in Met carriers, although the mechanisms involved are different. Further studies are necessary to answer this question.

Some limitations may be considered in our study. The number of individuals involved is small, especially for the Met/Met COMT genotype and we had to combine heterozygous and homozygous Met carriers. Also, it would be important to analyze other SNP in *COMT* gene that may influence the activity of the enzyme. On the other hand, the *COMT* Val(108/158)Met polymorphism is a functional polymorphism with a well-documented impact on enzyme activity and animal and human physiology. In addition, alleles have a similar frequency in a Caucasian population (Palmatier *et al.*, 1999), helping to overcome the small number of patients included (Jensen *et al.*, 2009). Furthermore, we think it must be considered the importance of exploratory studies in different populations and, to the best of our knowledge, our study is the first to be reported in the Iberian population.

The potential interactive effect of other polymorphisms in genes encoding opioid receptors and transporters was also examined and seemed to not influence total opioid consumption in this population. However, the analysis of these four SNP in a larger number of individuals may provide more information about this association.

Conclusions

Pain is a complex trait and the influence of genetics in pain sensitivity and efficacy of analgesics is an ongoing challenge. Our preliminary results suggest that COMT

Val(108/158)Met polymorphism may affect chronic opioids dose requirements in cancer pain patients. It also highlights the importance of non-opioids systems in the nociception processes.

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Author Disclosure Statement

No competing financial interests exist.

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Zubieta J-K, Heitzeg MM, Smith YR, Bueller JA, Xu K, Xu Y*, et al.* COMT val158met genotype affects μ-opioid neurotransmitter responses to a pain stressor. Science 2003; 299 (5610): 1240-43.

Supplementary Tables

Supplementary Table 1: Probe sequences for VIC/FAM

SNP	VIC/FAM sequences
OPRM1 (rs1799971)	GGTCAACTTGTCCCACTTAGATGGC[A/G]ACCTGTCCGACCCATGCG GTCCGAA
COMT (rs4680)	CCAGCGGATGGTGGATTTCGCTGGC[A/G]TGAAGGACAAGGTGTGC ATGCCTGA
ABCB1 (rs1045642)	TGTTGGCCTCCTTTGCTGCCCTCAC[A/G]ATCTCTTCCTGTGACACCA CCCGGC
ABCB1 (rs1128503)	GCCCACTCTGCACCTTCAGGTTCAG[A/G]CCCTTCAAGATCTACCAG GACGAGT

SNP, Single Nucleotide Polymorphism

Study III

Genetic profile and cancer-related pain: a tale from two outlier cases with bone metastatic disease

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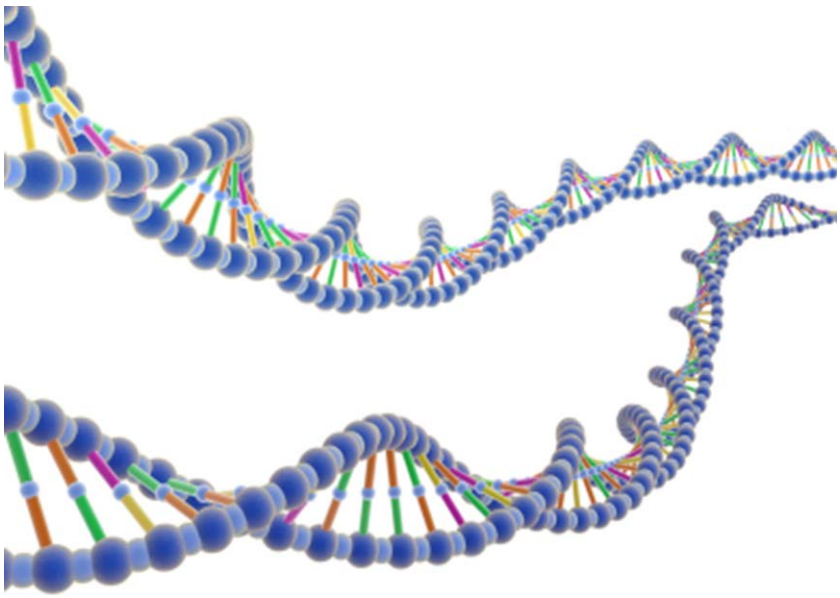

Pain Medicine

Pain Medicine 2013; *: **–**
Wiley Periodicals, Inc.

Genetic Profile and Cancer-Related Pain: A Tale from Two Outlier Cases with Bone Metastatic Disease

Dear Editor,

Morphine is the mainstay of pharmacological treatment for moderate-to-severe cancer-related pain. However, different analgesic response is an important problem in palliative care (Muralidharan and Smith, 2011). Genetic variations seems to represent an important cause of this interindividual variability in polymorphisms of opioid receptors, transporters and metabolizing enzymes, as well as in modulators/suppressors involved in perception and processing of pain information (Muralidharan and Smith, 2011). Therefore, genetic study of outlier cases might be an excellent opportunity to analyze the influence of some single nucleotide polymorphisms (SNP) in nociception and morphine requirements.

Therefore, genetic study of outlier cases might be an excellent opportunity to analyze the influence of some single nucleotide polymorphisms (SNP) in nociception and morphine requirements.

Here we present the study of a genetic profile of two cases: one patient considered a low responder (Patient 1) and one considered sensitive to morphine (Patient 2), requiring about 40-fold less morphine. The difference in morphine requirements prompted us to study SNP that include different phases of analgesic response: μ-opioid receptor (OPRM1; rs1799971), catechol-O-methyltransferase (COMT; rs4680), multidrug resistance protein 1 (ABCB1; rs1128503, rs1045642), organic anion-transporting polypeptides 1A2 (OATP1A2; rs11568563) and UDP-Glucuronosyltransferase-2B7 (UGT2B7; hCV32449742: rs7439366, rs7438284). Plasma concentrations of morphine and major metabolites (morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G)) were also determined (Meng *et al.*, 2000) and metabolic *ratios* were calculated.

The first patient, a 23-year-old female presenting an osteosarcoma, bone metastasis and complains of mixed pain (nociceptive and neuropathic pain), was receiving 800 mg/day of morphine. Co-administered drugs were gabapentin (1700 mg/day) and prednisolone (20 mg/day). Despite medication, the pain relief was not adequate and the patient complained of high pain intensity (average: 6; maximum: 9) and breakthrough pain, requiring an extra dose of morphine (100 mg/day). No remarkable adverse effects were observed and there was no presence of co-morbidity or renal and hepatic malfunction. The functional status was scored 3 by the Eastern Cooperative Oncology Group (ECOG) performance status scale.

The second patient, a 63-year-old male presenting a prostate cancer, bone metastasis and complains of mixed pain, was receiving 20 mg/day of morphine. Co-administered

drugs were diazepam (18 mg/day), omeprazole (20 mg/day) and prednisolone (20 mg/day). Despite higher levels of sadness and anxiety ("maximum" *vs*. "no" and "intense" *vs*. "no", respectively), the pain relief was adequate, with low pain intensity (average: 3; maximum: 5) and no breakthrough pain. No remarkable adverse effects were observed and there was no presence of co-morbidity or renal and hepatic malfunction. The functional status was scored 3.

The results are presented in Table 1 and Figure 1.

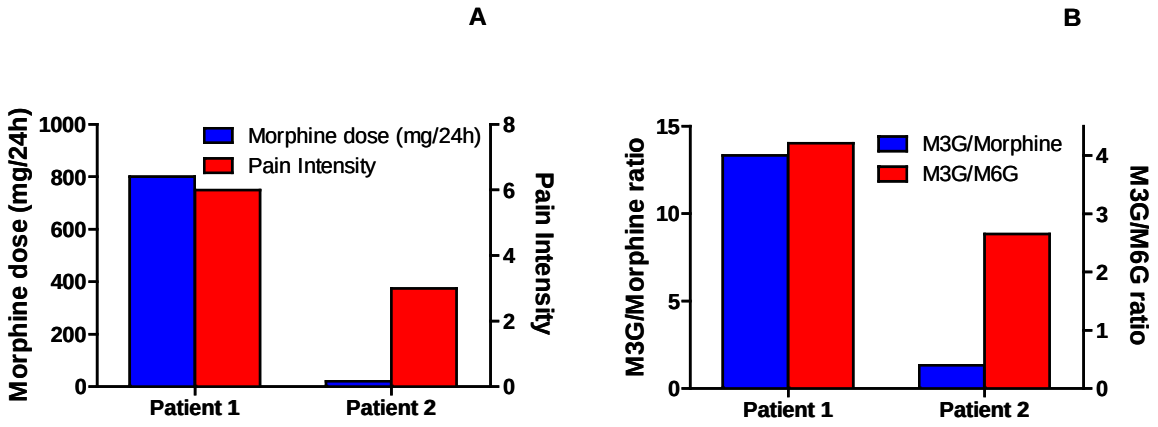

Figure 1. **A**. Pain intensity and morphine requirements for the two cases of patients. In spite of a morphine dose 40 times higher, Patient 1 presented higher levels of pain intensity. **B**. Differences in M3G/Morphine and M3G/M6G *ratios* for the two cases. Patient 1 is a homozygous T801C802 (His268; *UGT2B7*1*), presenting M3G/Morphine and M3G/M6G *ratios* 10 and 2-fold higher, respectively, than Patient 2.

Table 1: Genotyping metabolic *ratios* of morphine and metabolites.

	Patient 1	**Patient 2**
Genotyping		
OPRM1 A118G	GA	AA
COMT Val(108/158)Met	Val/Met	Val/Val
ABCB1 C3435T	CT	CT
ABCB1 C1236T	CT	CT
UGT2B7 T801A	TT	AA
UGT2B7 C802T	CC	TT
OATP1A2 A516C	AA	AA
Metabolic *ratios*		
M3G/Morphine	13.33	1.33
M6G/Morphine	3.17	0.5
M3G/M6G	4.21	2.65

All numbers are absolute numbers. ABCB1 ATP-binding cassette, sub-family B; COMT, catechol-O-methyl transferase; M3G, morphine-3-glucuronide; M6G, morphine-6-glucuronide; Met, Methionine; OATP1A2, organic anion-transporting polypeptides 1A2; OPRM1, μ-opioid receptor; UGT2B7, UDP-Glucuronosyltransferase-2B7; Val, Valine.

The current report describes two cases of cancer patients in palliative care: one low responder and one sensitive to morphine. Both patients were diagnosed with mixed pain, similar metastasis and all received similar treatment. Besides that, there were still major differences in daily morphine requirements, breakthrough pain and pain intensity (Table 1 and Figure 1A).

SNP in *OATP1A2* and *ABCB1* evidenced no association with morphine requirements, adverse effects or pain assessment. However, this study provides insights regarding a possible influence of SNP in *OPRM1*, *UGT2B7* and *COMT* (Table 1).

Concerning *OPRM1* A118G SNP, AA individuals were already related to lower requirements of morphine (Sia *et al.*, 2008). We observed that Patient 1 was a heterozygous, thus likely to require higher dose of opioids, compared to AA individuals (Patient 2).

In relation to *COMT* Val(108/158)Met SNP, the presence of Met allele leads to a reduction in the activity of the enzyme (Zubieta *et al.*, 2003), diminished regional activation of μ-opioid, decreased release of endogenous opioids and increased pain sensitivity over time, even after administration of opioids, and especially during sustained pain (Jensen *et al.*, 2009; Loggia *et al.*, 2011). We observed that the patient with higher morphine requirements and pain intensity (Patient 1) was a carrier of the Met allele, while Patient 2 was a homozygous for Val allele, thus showing lower pain intensity and consequently needing lower morphine doses.

Morphine is essentially metabolized by UGT2B7 to form M3G and M6G, which have different pharmacological activities. Differences were found for *UGT2B7* C802T and T801A, with Patient 1 being a homozygous T801C802 (His268; *UGT2B7*1*) and Patient 2 a homozygous A801T802 (Tyr268; *UGT2B7*2*). This genetic variation has been subject of several studies, with contradictory results (Holthe *et al.*, 2002; Parmar *et al.*, 2011), but recent studies indicate a lower glucuronidation capacity of the UGT2B7 Tyr268 isoform (Parmar *et al.*, 2011). There are also some significant differences in the metabolic *ratios*, which varied about 10- and 6-fold for M3G and M6G-to-morphine *ratios* respectively and 2-fold for M3G/M6G *ratio* (Table 1; Figure 1B). Patient 2 (haplotype *UGT2B7*2*) received the lower dose of morphine and had a better pain control. Besides a lower M6G-to-morphine *ratio*, this patient also has a lower M3G-to-morphine and M3G/M6G *ratios*. M3G seems responsible for some adverse reactions and to counteract the analgesic effect of morphine (Christrup, 1997; Holthe *et al.*, 2002). Therefore, a M3G-to-morphine *ratio* higher in Patient 1 can also be a significant factor to explain the different analgesic effect. As drug administration and blood collection were made around the same hour, the major differences observed can have a genetic cause.

Taking the data altogether, Patient 1 presents some genetic differences that can help to understand the outstanding differences in morphine requirements and pain intensity. Being a heterozygous for *OPRM1* and *COMT* SNP, this patient is more likely to have a decreased analgesic effect with morphine and increased pain intensity. In addition, differences in *UGT2B7* may be

part of the cause for variability in morphine and metabolites concentrations and *ratios*. The different roles played by each compound may also account for different pain intensities and morphine requirements.

This report describes the genetic study of outlier cases as an opportunity to analyze the influence of some SNP in nociception and morphine requirements. However, some confounding factors cannot be forgotten. The baseline pain severity before morphine treatment is unknown, as also the response to neuropathic specific medicines (gabapentin, prednisolone). The different pathology and gender can also influence pain control. Nevertheless, both patients were in-patients of Palliative Care Unit with advanced metastatic bone disease, which causes severe pain. In addition, the potential interactive effect of other polymorphisms in genes encoding other opioid receptors, transporters, enzymes and modulators /suppressors of pain perception should be tested in the future. Also, the analysis of these SNP in a larger number of individuals may provide more information about this association.

Sincerely yours,

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The authors declare no conflict of interest.

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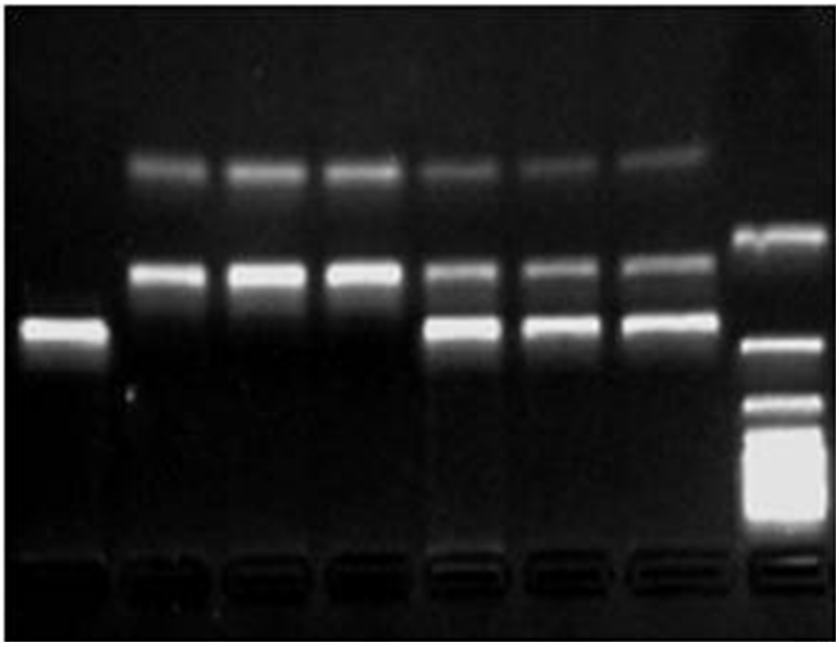
Study IV

Interleukin-1 genotype and circulating levels in cancer patients: metastatic status and pain perception

(Submitted for publication)



Interleukin-1 genotype and circulating levels in cancer patients: metastatic status and pain perception

Running title: Interleukin-1 and cancer-related pain

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Abstract

Proinflammatory cytokines released during inflammation can cause hyperexcitability in pain transmission neurons, leading to hyperalgesia and allodynia. Polymorphisms in interleukin 1 (IL-1) family of genes (*IL1A*, *IL1B*) and in IL-1 receptor antagonist (IL-1Ra, coded by *IL1RN*) may therefore induce alterations in cytokine levels/effects and pain related response. Our purpose was to investigate the influence of polymorphisms in *IL1A/B/RN* on cytokine serum levels and its correlation with pain intensity, performance status, adverse effects, metastases and breakthrough pain in Caucasian cancer patients. Serum IL-1α/β levels of 74 cancer patients were measured by competitive enzyme immunosorbent assay. All patients were also genotyped for the polymorphisms in *IL1A* (rs17561), *IL1B* (rs1143634) and *IL1RN* (rs419598) with Real-Time PCR. Results were then correlated to the appearance of bone or CNS metastases and several pain-related parameters. IL-1β rs1143634 homozygous for T allele were associated with lower levels of IL1-β (p = 0.032, Mann-Whitney test) and presented a trend for lower levels of pain (p = 0.06, Fisher's exact test). Also, IL1-β levels were related with cancer onset status, since a four-fold increase probability of metastatic disease was observed in high IL-1β individuals (OR = 4.074, p = 0.010, Pearson χ2 test). Among the female patients presenting metastatic disease and carriers of the TT genotype we observed a trend to lower levels of IL1-β (p = 0.053, Pearson χ2 test). Our results indicate that genetic variation at IL1-β gene may influence serum levels of IL1-β, with proportional consequences in cancer-related pain.

Keywords: Interleukin-1, cancer-related pain, metastatic disease, polymorphisms, C3954T

Introduction

The primary goal of palliative care remains in adding life quality and, if possible, increase the patient's life time. The World Health Organization treatment guidelines include opioid analgesics as the mainstay for moderate to severe acute pain and chronic cancer-related pain (WHO, 1996). However, the perception of pain varies greatly among patients, which implies wide variations in opioids dosage, pharmacological efficacy and tolerability (Shi *et al.*, 2010; Oliveira *et al.*, 2013). Therefore, it is increasingly important to study the factors that influence cancer-related pain, which is one of the most persistent and incapacitating symptoms of cancer.

In the last years, evidences of a central role of cytokines in pain and hyperalgesia have been described (Shi *et al.*, 2010). Proinflammatory cytokines as interleukins (IL) 1, 2, 6, 8, 15, 18, interferon γ (IFN-γ) and tumour necrosis factor-α (TNF-α) have already demonstrated to interfere in the nociceptive transmission, neuropathic pain and analgesics efficacy (Hutchinson *et al.*, 2008; Kawasaki *et al.*, 2008; Albulescu *et al.*, 2013).

There is a growing body of evidence of the role of IL-1 in pain sensitivity (Watkins and Maier, 2002; Gabay *et al.*, 2011), especially IL-1α (coded by the gene *IL1A*) and IL-1β

(coded by the gene *IL1B*), which exert their actions through IL-1 receptors (IL-1R). The activity of an endogenous competitive inhibitor, IL-1R antagonist (IL-1Ra, coded by *IL1RN*), also seems to be important. The induction of hyperalgesia and allodynia by IL-1β has been extensively reported (Falchi *et al.*, 2001), as well as a decrease in the analgesic efficacy of morphine (Shavit *et al.*, 2005; Mika *et al.*, 2008). Moreover the blockade of IL-1 signalling by IL-1Ra was shown to diminish allodynia, hyperalgesia and the development of neuropathic pain symptoms (Mika *et al.*, 2008; Gabay *et al.*, 2011), as well as to enhance morphine analgesia (Shavit *et al.*, 2005). IL-1β is also capable of evoking the production of other proinflammatory cytokines as IFN-γ, TNF-α and IL-6 (Mika *et al.*, 2013), which can also contribute to pain sensitivity. However, the role of IL-1α in pain is still a matter of debate and this cytokine seems to have an antinociception role in pain under inflammatory conditions (Mika *et al.*, 2008).

As single nucleotide polymorphisms (SNP) in cytokine genes have been shown to alter their expressions or functions (Qian *et al.*, 2010; Lacruz-Guzman *et al.*, 2013), and taking into account the important role of IL-1α, IL-1β and IL-1Ra in pain sensitivity, correlation between SNP, serum levels and clinical data can produce valuable information for cancer-related pain treatment. Given the previous reports concerning the possible association with inflammation, pain and cancer (Zabaleta *et al.*, 2006; Yilmaz *et al.*, 2010; Lozano-Ondoua *et al.*, 2013; Mika *et al.*, 2013; Wu *et al.*, 2013), we studied the influence of the SNP *IL1A G*4845T, *IL1B* C3954T and *IL1RN T*2018C in IL-1α and IL-1β serum levels, and its correlation with the appearance of bone or CNS metastases and to several pain-related parameters, namely, pain intensity including breakthrough pain, opioid dose requirements, adverse effects associated with opioid therapy, performance status, age, and gender.

Methods

Ethics commitment

All data were obtained with the informed consent of the participants prior to their inclusion in the study, according to Helsinki Declaration principles. The study was also approved by the Hospital (Portuguese Institute of Oncology - Porto) Ethical Internal Commission.

Subjects

We conducted a hospital-based study, analyzing 74 Caucasian individuals admitted in the Portuguese Institute of Oncology, Porto, Portugal, between 2010 and 2012. All the patients were in-patients from the Palliative Care Unit-Network or followed for pain consultation and were recruited according to the criteria: expected survival above 1 month, with at least 1 week of oral or subcutaneous opioid treatment for cancer-related pain, must read and write, not in confusional state and without renal or hepatic dysfunction. Data concerning time to adverse effects associated with opioid therapy (fatigue, pruritus, anorexia, perspiration, nausea and vomiting, diarrhea, xerostomia, cough, dyspnea, insomnia, drowsiness, nervousness, sadness and confusion), time to switch for another pain-relief regimen due to inadequate analgesia or

intolerable side effects, overall survival time, cancer diagnosis, age, gender and ethnicity were obtained from clinical files. Daily opioid doses were collected from the patients' ward charts and were re-expressed as oral morphine equivalents (OMEQ) as previously described (Cepeda *et al.*, 2010).

Assessments

Pain was measured daily, through evaluation of average and maximal pain during the last 24 h using a numeric 11-point scale, where 0 represents "no pain" and 10 means "worst pain possible" (Klepstad *et al.*, 2002). Patient's side effects associated with opioid therapy were assessed daily through a 5-point scale: "no", "mild", "moderate", "intense", and "maximum" (Aaronson *et al.*, 1993). Patients' functional status was evaluated by the Eastern Cooperative Oncology Group (ECOG) performance status scale (Oken *et al.*, 1982).

Blood samples and pharmacogenetic analysis

Blood samples were collected by venipuncture to EDTA tubes after stable analgesic opioid doses were achieved. Genomic DNA was extracted from peripheral blood samples by using QIAMP DNA Blood Mini kit (QIAGEN®), according to the manufacturer's protocol.

All genotypes were determined by direct allelic discrimination in the ABI Prism Real Time PCR System 7300 and Taqman™ Allelic Discrimination. Genotyping of *IL1A* (rs17561), *IL1B* (rs1143634) and *IL1RN* (rs419598) were performed as previously described (Gordon *et al.*, 2008). Probe sequences for VIC/FAM are described in Supplementary Table 1. Allelic discrimination PCR reactions were carried out in 6 µL volumes using 2.5 µL of TaqMan® Universal PCR Master Mix (2×), 0.125 µL of 40× assay mix, 2.375 µL of sterile H_2O and 1 µL of genomic DNA. Amplification of DNA was carried out on an ABI 7300 using the following conditions: 95ºC for 10 min, followed by 45 cycles of 95ºC for 15s and 60ºC for 1 min. Data capture and analysis were performed through the ABI 7300 Real Time PCR System (Applied Biosystems) and the Sequence Detection Systems software (Applied Biosystems version 1.2.3). Quality control included the use of non template controls in all runs and blind replicate genotype assessment on 10 % of the samples. Concordance was consistently observed among duplicates.

Cytokines quantification

Serum cytokines levels were quantified using commercially available enzyme immunosorbent assay kits (Biolegend® Human IL-1α/β ELISA MAX™ Deluxe) in accordance with the manufacturer's instructions.

Statistical analysis

Data analysis was performed using the computer software Statistical Package for Social Sciences (SPSS) for Windows (version 18.0) and GraphPad Prism® for Windows (version 5.0). Pain evaluation was divided in non-severe (0-3) and severe (4-10). Cancer diagnosis status was divided into four groups according with the frequency: breast, prostate, multiple myeloma and others. Furthermore, two groups of patients were defined according with IL-1β levels: low (IL-1β < 5 pg/mL) and high (IL-1β ≥ 5 pg/mL). The patients were also

divided according with the presence of metastatic disease and gender. Differences in proportions were evaluated by univariate comparisons of genotype frequencies using the Pearson χ2 test, Fisher's Exact test and Mann-Whitney test and a $p < 0.05$ was considered statistically significant. The result of the metastatic disease in high IL-1β individuals was also confirmed by logistic regression, adjusted to age, gender, stress and mood state.

Results

Patients

Seventy four patients receiving chronic opioids for cancer-related pain were admitted in this study (Table 1). No statistically significant association was found between the patients' characteristics and the genotype groups of *IL1A* and *IL1RA* SNP (data not shown). The intensities of other symptoms and adverse effects associated with morphine therapy such as fatigue, pruritus, anorexia, perspiration, nausea and vomiting, diarrhea, xerostomia, cough, dyspnea, insomnia, drowsiness, nervousness, sadness and confusion were also similar among all groups (data not shown).

Genotype distribution

IL1RA rs419598 evidenced a distribution of: 47.8 % TT, 46.4 % TC and 5.8 % CC. Regarding *IL1A* rs17561, genotype frequencies were: 63.5 % GG, 33.8 % GA and only 2.7 % AA. For *IL1B* rs1143634, genotype frequencies were: 64.9 % CC, 32.4 % CT and 2.7 % TT. In a second examination, the CT group of the *IL1B* rs1143634 SNP was analyzed together with the CC. Allele frequencies and the results of the X^2 test showed no significant departure from Hardy-Weinberg equilibrium.

IL1B genotype and correlation to cytokine levels, pain intensity, metastases and cancer diagnosis status

When comparing the different *IL1B* genotypes with IL-1β serum levels it was possible to observe that patients with TT genotype had the lower levels (2.12 ± 0.37 pg/mL) and the presence of the C allele was related with an increase in IL-1β levels (5.76 ± 0.58 pg/mL for CT genotype and 5.68 ± 0.47 pg/mL for CC genotype). Analysing the TT individuals *vs.* presence of C allele, significant differences were found (5.71 ± 0.36 (CC + CT) *vs*. 2.12 ± 0.37 pg/mL (TT) $p = 0.032$, Mann-Whitney test; Figure 1). Also, regarding pain intensity, we found higher maximum levels of pain in the carriers of C allele (5.44 ± 0.35 for C allele carriers and 2.00 ± 0.12 for TT genotype), with 75.8 % of the C allele carriers presenting severe maximum pain ($p = 0.06$, Fisher's Exact Test; Figure 1).

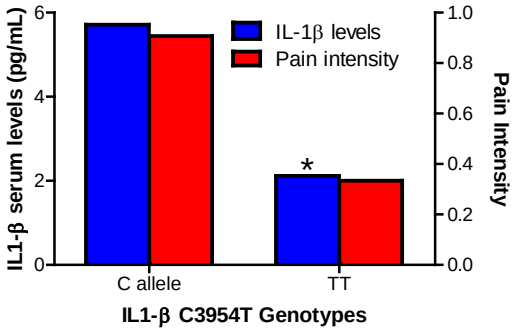

Figure 1. IL1-β C3954T polymorphism *vs.* IL1-β serum levels and pain intensity. Mann-Whitney test ($p < 0.05$). Significant differences ($p = 0.032$) were found between genotypes and IL1-β levels.

Table 1: Patients' data.

Variable	Patients (n = 74)	Variable	Patients (n = 30)[a]
Gender		Pain category	
Male	28	Visceral pain	3
Female	46	Nociceptive pain	7
Age	61.54 ± 12.83	Neuropathic pain	6
Tumor		Nociceptive + Neuropathic pain	3
Lung	4	Mixed pain	11
Urologic	3	OMEQ (mg/24 h)	181.41 ± 37.93
Breast	32	Breakthrough pain	
Prostate	12	Yes	19
Multiple Myeloma	8	No	11
Female reproductive organs	3	Rescue opioid (breakthrough pain)	
Other	12	No	13
Metastasis		Morphine	15
No	19	Tramadol	1
Liver	6	Methadone + Morphine	1
Bone	28	OMEQ (mg/24 h) for breakthrough pain	48.60 ± 27.48
CNS	3		
Lung	7		
Non visceral metastases (unknown location)	15		
Visceral metastases (unknown location)	18		
Pain Intensity	4.30 ± 2.33		
Maximum Pain	5.35 ± 2.81		
Other	5		
ECOG	2.28 ± 1.34		

[a]Variables accessible only for 30 patients.

All numbers are absolute numbers or mean ± SD. No statistically significant differences were observed between groups. Categorical data were analyzed using the chi-square test. CNS, central nervous system; ECOG, Eastern Cooperative Oncology Group; OMEQ, Oral Morphine Equivalents.

Serum IL1-β levels were also correlated with cancer diagnosis status and we were able to separate our patients into two groups, one with lower levels of IL-1β (2.85 ± 0.35 pg/mL) and other with higher cytokine levels (6.77 ± 0.67 pg/mL), the latter including breast, prostate and multiple myeloma. In the high IL-1β group, 83 % of the patients presented metastatic disease, in which a four-fold increase of the metastatic disease probability was observed (p = 0.010, Pearson $\chi 2$ test, Figure 2). This result was also confirmed by logistic regression, adjusted to age, gender, stress and mood (p = 0.016). Between the metastatic female patients, carriers of the TT genotype presented a trend to lower levels of IL-1β (6.67 ± 0.52 pg/mL for carriers of the C allele, 2.26 ± 0.60 pg/mL for allele T homozygous; p = 0.053, Pearson $\chi 2$ test; Figure 3). No additional statistically significant

associations ($p > 0.05$) were found between *IL1B* rs1143634 SNP and other patient's characteristics, symptoms or adverse effects (data not shown).

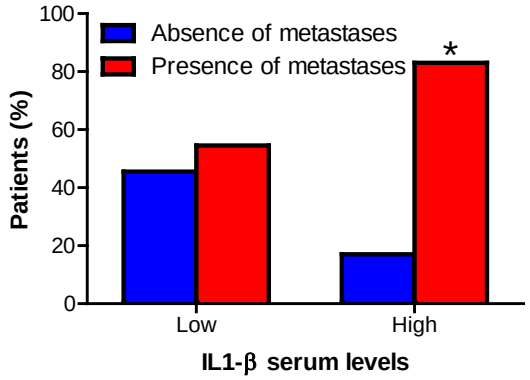

Figure 2. Two classes of IL1-β serum levels *vs.* presence of metastatic disease. Pearson χ2 test ($p < 0.05$). Significant differences ($p = 0.010$) were found between the two groups with a four-fold increase of the metastatic disease probability in high IL-1β individuals.

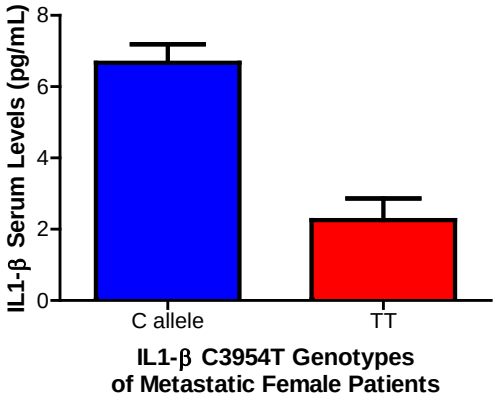

Figure 3. IL1-β serum levels *vs* IL1-β C3954T polymorphism among metastatic female patients. Carriers of the TT genotype presented a trend to lower levels of IL-1β ($p = 0.053$, Pearson χ2 test). Mean ± SEM.

Discussion

The present study analyzed SNP in the major elements of the IL-1 family and provides novel insights regarding a significant influence of *IL1B* C3954T polymorphism on cytokine serum levels, pain intensity, metastases and cancer diagnosis status, while the SNP related to *IL1A* and *IL1RN* have no statistically significant association with patient's characteristics, metastases, OMEQ, adverse effects or pain sensitivity.

Expression of IL-1 family is altered in inflammatory conditions, influencing pain perception (de Oliveira *et al.*, 2011; Mika *et al.*, 2013), with IL-1β being especially involved in the proinflammatory effect. It is known that IL-1β is expressed in nociceptive dorsal root ganglion neurons (Copray *et al.*, 2001), astrocytes and microglia, and it is one of the first cytokines formed in inflammatory processes, simultaneously with TNF-α. These two cytokines lead to the synthesis of other several inflammation effectors (Watkins and Maier, 2002), releasing and activating important substances for pain perception, like substance P and calcitonin-gene related peptide (de Oliveira *et al.*, 2011). IL-1β also activates B1 and B2 bradikinin receptors, induces cyclooxygenase-1 (COX-1), COX-2, prostaglandin E2 (PGE2), nitric oxide synthase (NOX) and matrix metalloproteases (MMPs), increases the activity of N-methyl-D-aspartate (NMDA) receptor and inhibits γ-aminobutyric acid (GABA) and glycine mechanisms, leading to thermal, chemical, mechanical and inflammatory hyperalgesia (Buvanendran *et al.*, 2006; Cunha *et al.*, 2007; de Oliveira *et al.*, 2011; Paz Aparicio *et al.*, 2011; Burada *et al.*, 2013). All these

proinflammatory and nociceptive properties have been highlighted by intrathecal administration of IL-1β and IL-1 receptor antagonists, leading to hyperalgesia and decreased allodynia, respectively (Sweitzer *et al.*, 2001; Sung *et al.*, 2004).

The *IL1B C*3954T (rs1143634) SNP is a silent polymorphic alteration in exon 5 (Phe105Phe) that has been related with several inflammatory diseases (Zabaleta *et al.*, 2006; Cimaz *et al.*, 2007; Solovieva *et al.*, 2009; Paz Aparicio *et al.*, 2011) and, like IL-1β, with pain (Mika *et al.*, 2013), more specifically with cancer-induced bone pain (Lozano-Ondoua *et al.*, 2013). Additionally, a decreased analgesic effect of morphine by IL-1β has been described (Shavit *et al.*, 2005), though no correlation was found between the SNP C3954T and opioid consumption (Bessler *et al.*, 2006). In this study, we observed higher intensity of pain in carriers of the C allele, associated with higher serum levels of IL-1β in the same group of individuals. This fact adds to previous data and indicates a special vulnerability of these patients to cancer-related pain. Other studies have also evaluated the relation among genetic variation, inflammation status and serum levels of IL-1β, but the correlation of the SNP C3954T and serum levels of IL-1β in pain-treated cancer patients was now disclosed for the first time. A recent study by Lacruz-Guzmán and collaborators correlated the rare allele T with lower serum levels of IL-1β (Lacruz-Guzman *et al.*, 2013), which was in agreement with previous studies (Santtila *et al.*, 1998; Tolusso *et al.*, 2006). Despite contradictory results concerning the association of this polymorphism with serum levels of IL-1β, the SNP C3954T has also been associated with lower C-reactive protein (CRP) concentration in healthy individuals (Eklund *et al.*, 2003) and end-stage renal disease patients (Maruyama *et al.*, 2005) carriers of the T allele. These findings are consistent with lower IL-1β levels, which reinforces the importance of results, showing the opposite pattern for the C allele.

In this study, we also observed different levels of serum IL-1β according to cancer diagnosis status, with patients diagnosed with breast, prostate cancer and multiple myeloma presenting the highest levels, and a correlation between the levels of the cytokine, the degree of metastatic disease and carriers of the C allele. In fact, in our study population, the individuals with higher levels of serum IL-1β presented a four-fold increase of the metastatic disease probability. Corroborating our findings, the association of increased levels of IL-1β in cancer was previously described, especially in tumour proliferation, metastasis and resistance (Liu *et al.*, 2006; Albulescu *et al.*, 2013; Burada *et al.*, 2013). In addition, several studies report a role of IL-1β in pathogenesis and metastatic disease in prostate, breast cancer and multiple myeloma (Eiro *et al.*, 2012; Vangsted *et al.*, 2012; Liu *et al.*, 2013), through direct proliferative effects, activation of inflammation and angiogenesis signalling (Saijo *et al.*, 2002) and especially through induction of MMPs (Eiro *et al.*, 2012). Considering our results, these events may be exacerbated in carriers of the C allele, and therefore these patients may require further clinical attention in the disease progression and associated pain.

The present study also analyzed the influence of genetic variation in IL1A and IL1RN. *IL1A* G4845T (rs17561) SNP leads to a

nonsynonymous mutation (Ala114Ser), which was already related to inflammatory conditions (Berger *et al.*, 2002), as well as to pain and cancer (Sigurdson *et al.*, 2007; Yilmaz *et al.*, 2010). However, no correlation among the different genotypes, serum levels and clinical data were found in this study. The same results were obtained with *IL1RN* T2018C (rs419598) SNP. This polymorphism has been related to colorectal (Burada *et al.*, 2013) and gastric cancer (Crusius *et al.*, 2008), and to inflammatory conditions (Wu *et al.*, 2013), but no variation was found in the present study.

This study has some limitations, such as heterogeneity of study population and a reduced number of individuals involved. Nevertheless, the agreement of the biochemistry, molecular biology and clinical data demonstrated a consistent association between IL-1β genotypes serum levels, pain intensity and metastatic disease. Also, the potential interactive effect of other polymorphisms in genes encoding other inflammatory effectors (IL-2, IL-6, IL-10,TNF-α) was also examined and does not seem to influence the studied clinical parameters (data not shown). Furthermore, no correlation between the levels of IL-1β and TNF-α and IL-6 was found (data not shown). Therefore, this preliminary report encourages the analysis of a larger number of individuals, to provide more information about this association, along with the analysis of other SNP in IL-1α/β/Ra and the quantification of IL-1Ra.

Conclusions

Pain is a complex trait and the influence of genetics in pain sensitivity and efficacy of analgesics is an ongoing challenge. Our results suggest that IL-1β C3954T SNP can affect IL-1β serum levels and maximum pain intensity in cancer pain patients and that IL-1β is associated with cancer proliferation, confirming the role of this cytokine as a pain effector and cancer biomarker.

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Author Disclosure Statement

No competing financial interests exist.

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Supplementary Tables

Supplementary Table 1: Probe sequences for VIC/FAM

SNP	VIC/FAM sequences
IL1RA (rs419598)	ATCTGAGGAACAACCAACTAGTTGC[C/T]GGATACTTGCAAGGACCAAATGTCA
IL1α (rs17561)	ACATTGCTCAGGAAGCTAAAAGGTG[A/C]TGACCTAGGCTTGATGATTTCTAAA
IL1β (rs1143634)	CATAAGCCTCGTTATCCCATGTGTC[G/A]AAGAAGATAGGTTCTGAAATGTGGA

IL, Interleukin; IL1RN, Interleukin 1 receptor antagonist; SNP, Single Nucleotide Polymorphism.

Study V

Morphine glucuronidation increases its analgesic effect in guinea-pigs

(Submitted for publication)

Morphine glucuronidation increases its analgesic effect in guinea-pigs

Running title: Morphine metabolism and analgesia

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Abstract

Morphine is extensively metabolized to the neurotoxic morphine-3-glucuronide (M3G) and the potent opioid agonist morphine-6-glucuronide (M6G). Due to the different roles of both metabolites, interindividual variability and co-administration of drugs that interfere with metabolic enzymes may lead to differences in analgesic response. The aim of the study was to investigate the repercussions of administration of an inducer (2,3,7,8-tetrachlorodibenzo-*p*-dioxin, TCDD) and an inhibitor (ranitidine) of glucuronidation in the morphine metabolism and consequent analgesic effect, using guinea pigs as a suitable animal model. Thirty male Dunkin-Hartley guinea pigs were divided in six groups: control, morphine, ranitidine, ranitidine + morphine, TCDD and TCDD + morphine. After previous exposure to TCDD and ranitidine, morphine analgesic effect was assessed by an increasing temperature hotplate test (35 – 52.5 °C), during 60 min after morphine administration. Then, blood was collected and plasma morphine, M3G and M6G were quantified by liquid chromatography with diode array and electrochemical detection. Animals treated with TCDD presented faster analgesic effect and 75 % reached the cut-off temperature, comparing with only 25 % in morphine group. Animals treated with ranitidine presented a significantly lower analgesic effect, compared with morphine group ($p<0.05$). Moreover, significant differences between groups were found in M3G levels and M3G/morphine *ratio* ($p<0.001$ and $p<0.0001$), with TCDD animals presenting the highest values for M3G, M6G, M3G/morphine and M6G/morphine, and the lowest value for morphine. The opposite was observed in the animals treated with ranitidine. Our results indicate that modulation of morphine metabolism may result in variations in M3G and M6G concentrations, leading to different analgesic responses to morphine, in an animal model that may be used to understand and improve morphine effect in clinical practice.

Keywords: Morphine, morphine-3-glucuronide, morphine-6-glucuronide, morphine metabolism, pain assessment.

Introduction

Morphine is one of the first-line drugs for pharmacological treatment of severe postsurgical and moderate-to-severe acute and chronic cancer-related pain (WHO, 1996). However, the set of adverse effects associated with morphine and the high interindividual variability of morphine dosage, efficacy and tolerability (Aubrun *et al.*, 2003; Ross *et al.*, 2005; Shi *et al.*, 2010; Oliveira *et al.*, 2013) are important limitations to its therapeutic effectiveness. Pain perception and response to analgesic medications are complex processes that involve multiple pathways, such as neurotransmission, inflammation, drug metabolism and drug transport, among others (Carpenter and Dickenson, 2002). Therefore, several hypotheses have been raised to explain morphine's analgesic variability, including genetic variation of opioid receptors, transporters and metabolizing enzymes (Belfer *et al.*, 2004; Lötsch and Geisslinger,

2006; Kadiev *et al.*, 2008; Kasai *et al.*, 2008; Jannetto and Bratanow, 2010; Kleine-Brueggeney *et al.*, 2010; Muralidharan and Smith, 2011).

Variability in morphine metabolism can particularly account for different analgesic effects. Morphine undergoes extensive human hepatic metabolism, especially by UDP-glucuronosyltransferase 2B7 (UGT2B7), producing two main metabolites, morphine-6-glucuronide (M6G) and morphine-3-glucuronide (M3G) (Carrupt *et al.*, 1991). M6G is a potent opioid receptor agonist with higher analgesic activity than morphine (Carrupt *et al.*, 1991; Osborne *et al.*, 1992). On the other hand, M3G has no opioid action and it seems to cause adverse effects, namely hyperalgesia / allodynia and neurotoxicity, and to exert a functional antagonistic effect, decreasing morphine analgesia (Carrupt *et al.*, 1991; Christrup, 1997; Holthe *et al.*, 2002). Since M6G has been ascribed as an important mediator of the analgesic effect of morphine (Klepstad *et al.*, 2000; Penson *et al.*, 2005), it has been postulated that the 6-glucuronidation probably increases the analgesic effect, in spite of concomitant M3G formation. However, the correlation of morphine metabolism and M6G concentration with analgesic effect is still a matter of controversy (Osborne *et al.*, 1992; Portenoy *et al.*, 1992; van Dongen *et al.*, 1994; Klepstad *et al.*, 2000; Quigley *et al.*, 2003; Penson *et al.*, 2005; Ing Lorenzini *et al.*, 2012; Gretton *et al.*, 2013), due to the variety of drugs and substrates of UGT that can interfere in M3G and M6G formation during therapy (Wittwer and Kern, 2006), and therefore the real effect on analgesic efficacy of morphine metabolism inhibition and induction is still unknown.

Although several species can metabolize morphine, remarkable interspecies differences have been found in the urinary excretion and site-selective glucuronidation of morphine (Kuo *et al.*, 1991). On the other hand, the guinea-pig presents a M3G:M6G *ratio* of 4:1 (Kuo *et al.*, 1991; Aasmundstad *et al.*, 1993), very similar to the *ratio* described for humans (Yue *et al.*, 1990; Andersen *et al.*, 2002; De Gregori *et al.*, 2012), and therefore represents a suitable animal model to clarify the influence of morphine glucuronidation in the resulting analgesic effects. A number of compounds are known to interfere significantly with metabolic enzymes, thereby influencing drug metabolism. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is a potent halogenated aromatic hydrocarbon that exerts its biological and toxic responses through binding to the aryl hydrocarbon receptor (AhR) (Santostefano *et al.*, 1998). In addition to many other effects, TCDD can induce several isoforms of cytochrome P450, UGT and glutathione-S-transferase in humans and rodents, including guinea pigs (Münzel *et al.*, 1999; Fletcher *et al.*, 2001; Münzel *et al.*, 2003; Collier *et al.*, 2006; Erichsen *et al.*, 2008). Therefore it can be used to induce morphine metabolism. Besides morphine metabolism induction, its inhibition could also be of therapeutic interest. In this particular case, *in vitro* experiments with guinea pig cells have shown that ranitidine may differentially inhibit morphine glucuronidation, causing higher inhibition of the production of M3G than that of M6G (Aasmundstad and Morland, 1998). Interactions of ranitidine with morphine effect and metabolism have also been described in mice (Suh *et al.*, 1996) and humans (McQuay *et al.*, 1990; Aasmundstad

and Storset, 1998), yielding a reduced serum M3G/M6G *ratio*.

The lack of a good analgesic response in some patients, the variability of the relative amount of glucuronides formed and uncertainty of their contributions on the total analgesic effect prompted us to formulate a controlled study of both induction, using TCDD, and inhibition, using ranitidine, of morphine metabolism and pain assessment in an adequate animal model, the guinea-pig.

Methods

Ethics commitment

All experimental procedures followed the regulations of local authorities in handling laboratory animals, as well as the European Directive 2010/63/EU and the ethical guidelines for the study of pain in experimental animals (Zimmermann, 1983). The study was also approved by the Ethical Internal Commission of Faculty of Medicine of University of Porto / São João Hospital.

Reagents and Standards

Commercially formulations of morphine (morphine sulfate, MST® 10 mg) and ranitidine (ranitidine hydrochloride, Zantac® 25 mg/mL) were obtained in a local pharmacy. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) was obtained from Sigma-Aldrich (St. Louis, MO). Morphine was dissolved in saline solution and TCDD in corn oil (Merck - Darmstad, Germany) for the intraperitoneal (IP) administrations. For the quantification of morphine and metabolites, standards of morphine hydrochloride, M3G hydrochloride and M6G hydrochloride were obtained from Lipomed (Arlesheim, Switzerland). Phenacetin (internal standard, IS), triethylamine, sodium dodecyl sulfate and hydrochloric acid were obtained from Sigma-Aldrich (St. Louis, MO). Methanol, acetonitrile, sodium dihydrogen phosphate and phosphoric acid were obtained from Merck (Darmstad, Germany). OASIS® weak cation exchange (WCX) cartridges, 60 mg, 3 mL were obtained from WATERS (Milford, MA) and Bond Elut® C18 cartridges, 100 mg, 1 mL were obtained from Agilent. All chemicals and reagents were of analytical grade or from the highest available grade.

Animals and Experimental Design

Animals

Thirty male Dunkin-Hartley guinea pigs (Harlan Laboratories, Spain) weighing 250–300 g were used. Animals were kept under constant photoperiod conditions (12-hour alternating light-dark cycles) at 22 °C and 40-50 % relative humidity with food and water *ad libitum*. In order to minimize fear-motivated behaviors, all animals were handled daily and habituated to all testing procedures before the onset of the experiments. In all behavioral tests, the evaluator was unaware of the animal's experimental group.

Experimental protocol

Thirty animals were randomly distributed in six experimental groups (n = 5): (i) Control (C); (ii) Morphine (M); (iii) Ranitidine (R); (iv) Ranitidine + Morphine (RM); (v) TCDD (T); (vi) TCDD + Morphine (TM) (Table 1). After the period of habituation, the experimental protocol was held for 3 days (Figure 1 and Table 1). The enzymatic inducer was administered twice, 48 and 24 h before the behavioral assessment, whereas the inhibitor

was administered three times (48, 24 and 2 h before the hot plate test). Behavioral assessment was performed immediately before and 15, 30, 45 and 60 min after saline or morphine administration. Morphine (10 mg/kg), TCDD (1 µg/kg) and ranitidine (200 mg/kg) doses were defined according to the literature (Collier *et al.*, 1961; Flecknell, 1984; Olster, 1994; Orishiki *et al.*, 1994; Enan *et al.*, 1996) and all solutions were administered IP between 9 and 11 A.M..

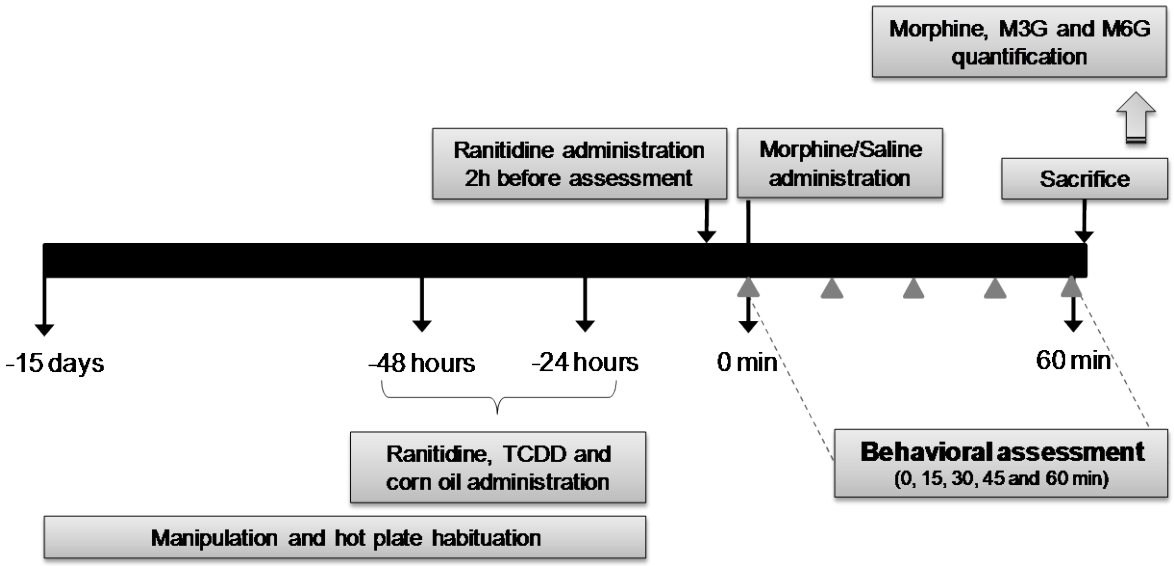

Figure 1. Schematic representation of the experimental protocol. All drugs were intraperitoneally administrated, between 9 and 11 AM. M3G, morphine-3-glucuronide; M6G, morphine-6-glucuronide; TCDD, 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin.

Table 1. Treatment groups for the experimental protocol

Treatment group	-48 and -24 hours	-2 hours	0 min
Control	Corn oil	–	Saline
Morphine	Corn oil	–	Morphine 10 mg/kg
Ranitidine	Ranitidine 200 mg/kg	Ranitidine 200 mg/kg	Saline
Ranitidine + Morphine	Ranitidine 200 mg/kg	Ranitidine 200 mg/kg	Morphine 10 mg/kg
TCDD	TCDD 1 µg/kg	–	Saline
TCDD + Morphine	TCDD 1 µg/kg	–	Morphine 10 mg/kg

TCDD was dissolved in corn oil.

Assessment of hot plate thermal analgesia

The hot-plate test was performed in a computer-controlled hot/cold plate analgesia meter (Bioseb, Vitrolles, France). The animals were placed on a metal surface (16.5 cm×16.5 cm), surrounded by a plexiglass box (36.5 cm height). The initial surface temperature was 35 °C and a cut-off temperature of 52.5 °C was defined, to prevent tissue damage. After a short adaptation period (20-30 s), an increasing thermal gradient of 9 °C/min was applied. This heating rate was chosen in order not to cause unnecessary stress in the animals (maximal assay duration *ca*. 2 min, as previously described) (Tjolsen *et al.*, 1991). The temperature (in °C) to elicit genitalia licking was recorded (Leite-Panissi *et al.*, 2001).

Sample collection

Immediately after the end of the behavior assessment, anesthesia was induced with isoflurane. Animals were placed in the *decubito supino* position and the thorax was opened by two lateral transversal incisions and one central longitudinal incision. Blood was collected from the heart, with heparinized needles, into EDTA containing tubes and then centrifuged (2500×*g*, 4 °C, 10 min). Plasma was aliquoted in eppendorf vials and stored (−80 °C) until analysis.

Quantification of morphine and metabolites

Plasma quantification for morphine and metabolites was performed according to the method previously validated and described (Oliveira et al, submitted elsewhere). Briefly, morphine, M6G, and M3G were extracted by two-step solid-phase extraction (SPE) and plasma concentrations were analyzed by high performance liquid chromatography (HPLC) with sequential diode-array and electrochemical detection. For the extraction, 30 µL of the internal standard phenacetin at 10 mg/mL were added to 1.5 mL of plasma. The sample was then transferred to C18 cartridges, which have been previously conditioned with 2 mL of methanol and 2 mL of phosphate buffer (10 mM, pH = 9.5). The cartridge was then washed with 2 mL of phosphate buffer (10 mM, pH = 9.5) and eluted with methanol with 0.5 % of triethylamine. After drying the samples with a nitrogen stream, they were reconstituted with 1 mL of 80 % acetonitrile and transferred into a WCX cartridge, previously conditioned with 4 mL of acetonitrile. After washing the cartridge with 4 mL of acetonitrile, the compounds were eluted with 1.5 mL of 80 % methanol containing 0.05 M HCl. The eluate was dried in a Labconco® evaporator. Samples were reconstituted with 50 µL of mobile phase.

Samples (40 µL) were injected in a HPLC Waters® 2690 system and analytes were separated using a Waters Spherisorb® ODS2 reversed-phase column (250 mm × 4.6 mm × 5 µm) and 0.01 M potassium phosphate buffer:acetonitrile (85:15 v/v) containing 0.04 mM sodium dodecyl sulfate as the mobile phase. Detection of M3G was performed in a diode-array Waters® 996, at 210 nm. Detection of M6G and morphine was performed at Coulochem® II 5200A, with 0.200 V for cell 1, 0.350 V for cell 2 and 0.400 V for guard cell.

Statistical analysis

Data analysis was performed using the computer software GraphPad Prism® for

Windows (version 5.0). All data obtained from behavior assessment and morphine and metabolites quantification were expressed as mean ± SEM. Differences between groups were evaluated with one-way or two-way ANOVA followed by the Bonferroni's *post hoc* test. Statistical significance was fixed at $p<0.05$ for all analyses.

Results

General observations

Animals subjected to TCCD or ranitidine treatments showed no weight reduction or abnormal signs throughout the study (data not shown). After morphine administration, animals became more prostrated than their respective saline controls (at least at the end of 60 min), though the onset of this prostration was remarkably faster in animals undergoing treatment with TCDD. For groups C, R, RM and T, a n = 4 was used due to atypical behavior or treatment-unrelated death of four animals.

Analgesic response

Baseline hot-plate threshold temperatures were recorded before morphine or saline administration and then every 15 minutes afterwards, until the end of the experiment. Since baseline threshold temperatures were 2 – 3 °C higher in ranitidine-treated animals than in other groups, the variations of temperature threshold relative to baseline thresholds were used for analysis, rather than the absolute threshold values (Figure 2).

The analgesic effect of morphine amounted to a > 5 ºC increase in threshold temperature, after 60 min. This effect was reduced to *ca*. 3.5 ºC in animals subjected to ranitidine treatment ($p<0.05$). On the other hand, TCDD-treated animals showed a sharper onset of the analgesic effect than both other morphine-treated groups.

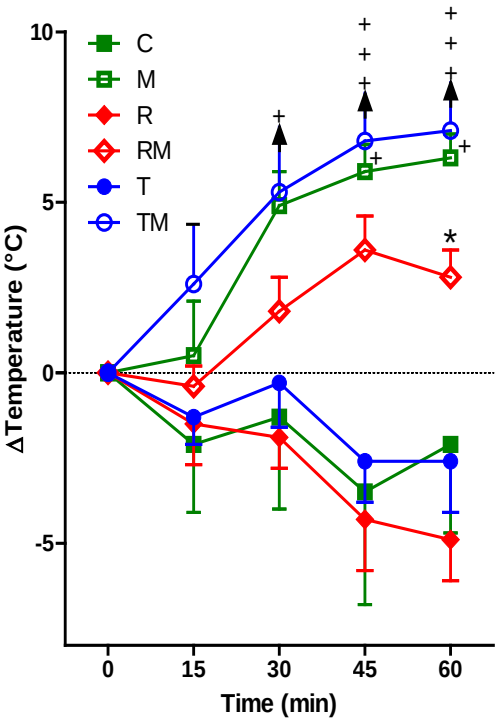

Figure 2. Differences between threshold hot plate temperature at each time-point after morphine/saline administration and basal threshold temperature (ΔT) for the different treatment groups (Mean ± SEM). Comparisons between groups were performed by repeated measures ANOVA (*$p<0.05$ *vs*. morphine group). In groups M and TM, some animals were withdrawn from the hot plate after reaching the cut-off temperature without behavioral response. This information is represented in the graph as a (+) for each of those animals. M, morphine group; RM, ranitidine + morphine group; T, TCDD group; TM, TCDD + morphine group.

These results demonstrate a more intense analgesic effect in the TM group, although a parametric statistical analysis cannot be used to show significant differences between TM and M groups due to the unavailability of

effective threshold temperatures for the animals which showed no discomfort upon reaching the cut-off temperature. No significant changes in threshold temperatures were observed in any control group.

Plasmatic concentrations of morphine and metabolites

Plasmatic concentrations of morphine, M3G and M6G are shown in Figure 3. The highest morphine concentrations were found in RM group and the lowest in TM (199 ± 42 ng/mL (RM) *vs.* 161 ± 17 ng/mL (M) *vs.* 96 ± 13 ng/mL (TM). Conversely, M3G and M6G levels were highest in TM animals and lowest in the RM group, which strongly supports alterations in morphine metabolism: 1009 ± 181 ng/mL (RM) *vs.* 1791 ± 372 ng/mL (M) *vs.* 3793 ± 389 ng/mL (TM) for M3G and 203 ± 48 ng/mL (RM) *vs.* 224 ± 91 ng/mL (M) *vs.* 466 ± 70 ng/mL (TM) for M6G. The differences in the concentrations were also evidenced after the calculation of the metabolic *ratios* (Figure 4), with TM group presenting the highest M3G/morphine and M6G/morphine *ratios* and RM the lowest. Significant differences between groups were found for M3G concentration (p<0.001) and M3G/morphine *ratio* (p<0.0001). M3G/M6G ratios were also calculated, with RM group presenting the lowest value (6.5 ± 0.5 (RM) *vs.* 10.4 ± 1.8 (M) *vs.* 8.4 ± 0.7 (TM)), but no statistically significant differences were found.

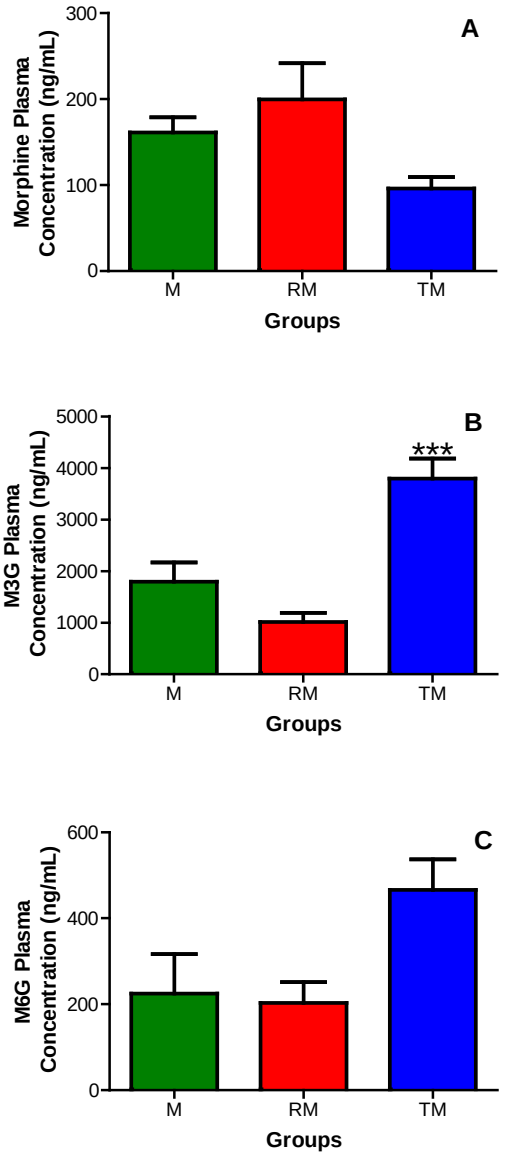

Figure 3. Plasma morphine and metabolites concentration (Mean ± SEM). A – Plasma concentration of morphine (ng/mL); B - Plasma concentration of M3G (ng/mL). Significant differences were found between groups (***p<0.001, TM *vs.* M/RM treatments); C - Plasma concentration of M6G (ng/mL). M3G, morphine-3-glucuronide; M6G, morphine-6-glucuronide; M, morphine group; RM, ranitidine + morphine group; TM, TCDD + morphine group.

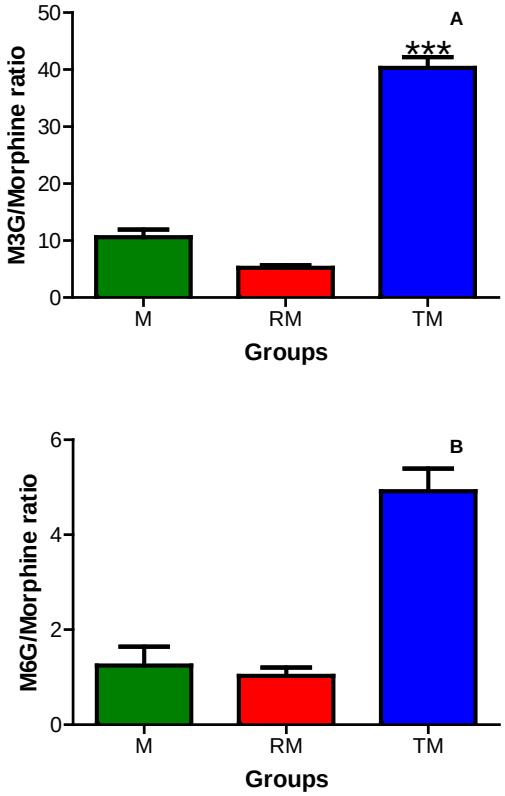

Figure 4. Metabolic concentration *ratios* (Mean ± SEM). A - M3G/Morphine. Significant differences were found between groups (***p<0.0001, TM *vs.* RM/M groups) B - M6G/morphine. M3G, morphine-3-glucuronide; M6G, morphine-6-glucuronide; M, morphine group; RM, ranitidine + morphine group; TM, TCDD + morphine group.

Discussion

The present study analyzed the influence of morphine metabolism in its analgesic efficacy and provides novel insights for a possible association of metabolism induction and inhibition with metabolites concentration and consequently different analgesic effects. Upon morphine administration, TCDD-treated animals (TM) presented higher thermal thresholds in behavioral assessment, lower morphine and higher M3G and M6G plasma concentrations and higher metabolite/morphine *ratios* than morphine-only treated animals (M), although with similar M3G/M6G *ratio* between M and TM groups. On the other hand, in ranitidine-treated animals (RM) the morphine analgesic efficacy was significantly lower than in TM and M groups, plasmatic morphine values were higher and M3G and M6G were lower than in other morphine-treated groups. Also, values for M3G/morphine and M3G/M6G *ratios* were the lowest in ranitidine-treated animals, though the M6G/morphine *ratio* was very similar to the M group.

TCDD, a well known dioxin, binds to AhR and, in the presence of the nuclear factor erythroid 2-related factor 2 (Nrf2), induces the gene expression of many enzymes involved in drug metabolism, including glucuronidation enzymes (Buckley and Klaassen, 2009; Yeager *et al.*, 2009). Thus, TCDD-exposure is expected to increase the production of both major metabolites, M3G and M6G. The latter has been subject of several studies, with controversial results. Some reported its antinociceptive action and importance for pain control (Osborne *et al.*, 1992; Portenoy *et al.*, 1992; Klepstad *et al.*, 2000), while others reported no correlation between M6G concentrations and pain perception or side-effects severity (Tiseo *et al.*, 1995; Quigley *et al.*, 2003).

In our work, M3G levels and M3G/morphine *ratios* were significantly increased in TM animals, confirming the success of TCDD-induction of morphine metabolism in guinea pigs. M6G concentration and M6G/morphine *ratio* were also tendentially increased, although this result was not statistically significant. Furthermore, TCDD-treated animals presented a faster and more marked analgesic effect than other groups, with 75 %

of TM animals reaching the temperature cut-off value at 45 and 60 minutes post-morphine injection. Taking all data into account, it is possible to hypothesize that a higher metabolite production rate led to a better and faster analgesic effect, probably through the potent opioid action of M6G. This metabolite presents a lower affinity to μ-opioid than morphine, but a higher efficacy, together with a lower affinity to κ-opioid receptor, which might explain its analgesic activity with reduced tendency to opioid-related adverse effects (Kilpatrick and Smith, 2005; Dorp *et al.*, 2008). In addition, the pharmacokinetic profile of M6G is very different from morphine, which in part might explain the slower onset of M6G effect but of longer duration than morphine (6-fold longer), causing adequate and long-lasting pain relief (Suh *et al.*, 1996; Kilpatrick and Smith, 2005; Ing Lorenzini *et al.*, 2012), but further work is required to fully explain the differences between morphine and M6G. Despite the reported M3G toxicity and the higher levels of this metabolite as a result of the induction protocol, no deleterious M3G effects were detected in our single morphine administration protocol. In fact, the proximity of the values of the M3G/M6G *ratio* in TM and M groups indicates that the analgesic potency of M6G prevails over the hyperalgesic effect of M3G. However, chronic administration and/or induction protocols would require further studies.

Unlike morphine and ranitidine, TCDD can produce diverse toxic effects including a lethal wasting syndrome whose hallmark is suppressed hepatic gluconeogenesis (Enan *et al.*, 1996). Guinea pigs are particularly sensitive, presenting the lowest LD50 for this dioxin among rodents (Korkalainen *et al.*, 2001). Nevertheless, the selected dose (1 μg/kg), already tested in this animal model (Enan *et al.*, 1996; Enan *et al.*, 1998) and described to cause significant weight reduction only after 7-14 days after a single-dose administration, did not cause body mass loss or any apparent change in the activity and social interaction in our animals, during the evaluated period.

The effects of morphine metabolism inhibition by ranitidine were also evaluated in our study. Previous studies have suggested that ranitidine may interfere with morphine metabolism, especially in M3G production, by differential inhibition of UGT isoforms (McQuay *et al.*, 1990; Aasmundstad and Morland, 1998; Aasmundstad and Storset, 1998). According to these studies, this drug could cause not only an increase in plasmatic morphine levels but also a decrease in M3G/M6G concentration *ratios* through decreased M3G and, sometimes, increased M6G levels. In our study, ranitidine decreased morphine metabolism in guinea pigs, leading to higher morphine, lower M3G and M6G values and lower M3G/M6G *ratios* than the other morphine-treated groups. Behaviorally assessed, these animals showed significantly lower morphine analgesic effect than the other morphine-treated groups (M and TM). Our results corroborate the differential inhibition of morphine metabolism. In fact, although both M3G and M6G were diminished, M3G levels were more affected, leading to a lower M3G/M6G *ratio* than the M group. However, despite this slightly differential inhibition, the analgesic effect was not improved, which may be explained by the decreased levels of M6G. Aasmundstad and collaborators (Aasmundstad and Morland, 1998) also

reported lower *in vitro* M3G/M6G concentration *ratios* by increasing ranitidine concentration. Additionally, they observed that the concentration of morphine and ranitidine can affect the rate formation of both metabolites, obtaining a reduced formation rate of M3G and M6G by increasing ranitidine concentration and a less restrained inhibition effect when higher doses of morphine were used (McQuay *et al.*, 1990) In another study only serum, but not urinary, M3G/M6G *ratios* were altered by ranitidine in humans (Aasmundstad and Storset, 1998), which may be due to alternative excretion pathways, including the biliary excretion. Further studies are required to achieve a higher differential inhibition and improve morphine analgesic effect, through decreasing M3G formation while maintaining M6G levels.

Guinea pig was the selected species for this study due to a theoretical production of a M3G/M6G average *ratio* of 4:1, similar to humans (5-8.5:1) (Yue *et al.*, 1990; Kuo *et al.*, 1991; Aasmundstad *et al.*, 1993; Andersen *et al.*, 2002; De Gregori *et al.*, 2012). We obtained a higher plasmatic *ratio* in morphine-treated animals (10:1) one hour after administration. However, higher ratios have also been reported in guinea-pig (6.3 ± 1.8) (Lawrence *et al.*, 1992). In addition, the reported *ratios* were calculated based on the urinary concentrations of M3G and M6G (24-hour urine) and, especially, on *in vitro* experiments, with several and different sampling times, which may explain the slight differences observed.

Differences in the physicochemical properties and hydrophobicity of the drugs used to induce (TCDD) and inhibit (ranitidine) the morphine metabolism required the use of different vehicles for their administration: TCDD (highly hydrophobic) was dissolved in corn oil, while ranitidine (hydrophilic) was administered in an aqueous solution. Therefore, the experiment would require 8, instead of 6 experimental groups (morphine- and saline-treated TCDD, ranitidine, TCDD-vehicle control, and ranitidine-vehicle control). Furthermore, since we used a commercially available injectable formulation of ranitidine, we did not have an adequate vehicle to use. However, since no changes on pain thresholds were detected upon corn oil or aqueous saline administration, we decided to use only corn oil-treated controls, thus reducing the number of guinea pigs used in the study as suggested by the Ethical Commission. Another uncontrolled-for manipulation was the third ranitidine administration, two hours before morphine/saline administration and behavioral evaluation. This could (at least partially) explain the higher baseline threshold temperatures of R and RM groups.

Our results, in a controlled *in vivo* model, have shown that inhibition and induction of morphine metabolism can influence morphine analgesic efficacy. Furthermore, the induction/inhibition animal model developed seems to be promising for future studies concerning morphine metabolism, due to the similarity of glucuronidation processes, as compared to humans, and the availability of inducers and/or inhibitors of glucuronidation, as clearly demonstrated. In clinical practice, the variability of morphine metabolism, efficacy and adverse effects contributes to a reduced pain control and quality of life. An improved knowledge of the mechanisms behind the modulatory influences on morphine

metabolism may help the understanding of pharmacokinetic interactions of co-administered drugs and allow the manipulation of the production of morphine's metabolites, thus overcoming the therapeutic constraints related to genetic variability and providing a better pain control and quality of life.

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Author Disclosure Statement

No competing financial interests exist.

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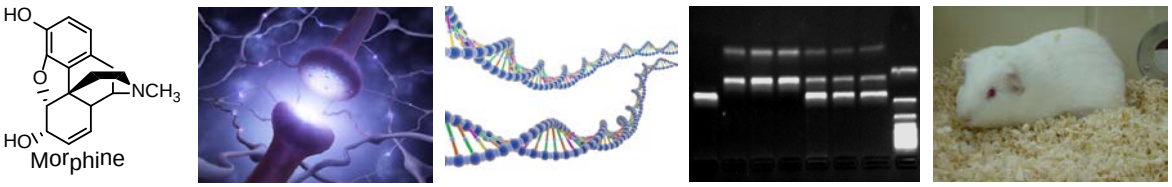
CHAPTER IV

INTEGRATED DISCUSSION



4.1 Integrated discussion

Inter-individual variability of opioids is well known by physicians when treating chronic cancer and non-cancer pain. The scientific community believes that this unpredictable variation might be related with genetic factors, especially SNPs in important molecules, as receptors, enzymes and endogenous transmitters. The present thesis aimed to explore the role of genetic variants in the analgesic effect of opioids, especially morphine, in order to understand and improve the analgesic efficacy in a foreseeable future, particularly in cancer pain patients. To achieve this objective we developed a strategy that included i) the recruitment of patients from Palliative Care Units; ii) a revision of the most studied and influent SNP (Chapter 1); iii) the analysis of the polymorphisms, by choosing SNPs that are involved in the several steps of opioid action and pain processing, together with the quantification of morphine and metabolites; and iv) to develop an animal model for the study of morphine metabolism and its implication in the analgesic efficacy.

In the early stages of this work we focused in the recruitment of cancer pain patients in IPO-Porto, considered the limiting step of the work, accordingly with the criteria selection referred in Study II-IV. The recruitment was conducted through the course of the work but due to the narrow criteria, the need to complete a questionnaire, the small size of the Palliative Care Units and especially the patients' status, only 100 samples were collected. From these, complete pain and healthcare questionnaires were available only for 75 patients and complete information about opioid administration (opioid, regular dose, dose for breakthrough pain) and other drugs administered concomitantly were only obtained for 30 patients. Taking this into account, besides morphine, as originally planned, we extended the work to patients under treatment with other opioids. Also, the selection of the SNPs had to be made carefully in order to include representative variants in opioids pharmacodynamics and pharmacokinetics (receptor, metabolizing enzyme and transporters) and in pain modulators (COMT and cytokines).

Along with the recruitment, the developing of the quantification method for morphine and major metabolites was initiated, as it was essential to human and animal studies. After several attempts in gas chromatography coupled with mass spectrometry, a HPLC method with diode array and electrochemical detection was developed and validated as can be seen in Study I. Despite the several methods available for morphine and metabolites, the presented low-cost methodology proved to be very specific, sensitive, precise and accurate, not only for plasma samples, the most common matrix in human clinical studies along with serum, but also for five other matrices, including *postmortem*. The developed technique was a very important step as it enabled the determination of

morphine, M3G and M6G in plasma of patients and guinea pigs in the following studies. In addition, this technique will also permit ongoing and further studies aiming to quantify morphine, M3G and M6G in *postmortem* samples collected from opioids-related deaths, namely in whole blood, urine, liver, kidney and brain. The less positive point of this technique is the volume of sample required, mainly plasma (1.5 mL). However, due to the limits of detection and quantification achieved it is generally possible to dilute the sample. In patients with chronic administration of morphine, as is the case of our sampling, values of metabolites are generally higher than those of morphine, especially M3G (mean values for morphine, M6G and M3G: 42.9 ng/mL, 63.5 ng/mL and 1026.8 ng/mL, respectively) and for all would be possible to use at most 750 µL of sample (limits of quantification for morphine, M6G and M3G in plasma: 1.2 ng/mL, 3.2 ng/mL and 8.5 ng/mL, respectively).

In Study II, the influence of selected SNPs was studied in the samples of 30 Caucasian cancer patients. The first SNPs analyzed were related with pharmacodynamics (*OPRM1*), pharmacokinetics (*ABCB1*) and pain sensitivity (*COMT*), and daily opioid doses were re-expressed as oral morphine equivalents (OMEQ). An association between COMT Val(108/158)Met genotypes and OMEQ was found, with patients carrying Met allele related with higher opioid requirements, although no significant associations were found concerning *OPRM1* and *ABCB1* polymorphisms. The obtained results were in accordance to our expectations, as carriers of Met allele were already correlated with lower enzymatic activity, higher pain sensitivity, lower µ-opioid system activation during sustained pain, higher affective ratings of pain and a more negative internal affective state (Zubieta *et al.*, 2003; Jensen *et al.*, 2009; Mobascher *et al.*, 2010; Loggia *et al.*, 2011). However, controversial information was already reported, concerning the correlation of Val(108/158)Met SNP and opioid doses, with the Met allele being associated with lower opioids requirements, due to compensatory increase of µ-opioid receptor density and binding potential (Chen *et al.*, 1993; Zubieta *et al.*, 2003; Rakvåg *et al.*, 2005; Reyes-Gibby *et al.*, 2007; Rakvag *et al.*, 2008). In fact, there is an increased expression of µ-opioid receptor at baseline, but during sustained pain they have a decreased activation of the µ-opioid system (Zubieta *et al.*, 2003; Ross *et al.*, 2008). Thus, an association of the Met allele with higher pain sensitivity and opioid requirement during sustained pain seems to be more consistent. These results also emphasize the importance of non-opioid systems in pain processing and opioids analgesic effect. In fact, higher levels of catecholamines and modulation of adrenergic receptors were already related to inhibition of morphine analgesia and hyperalgesia (Khasar *et al.*, 1999; Kolesnikov *et al.*, 2011), and $\beta_{2/3}$-adrenergic antagonists can block pain sensitivity induced by COMT inhibition (Nackley *et al.*, 2007; Tchivileva *et al.*, 2010; Chu *et al.*, 2012). The reason for finding differences in

the population for Val(108/158)Met SNP but not for the other SNPs analyzed may be due to a special feature of the polymorphism itself: in a Caucasian population, the alleles have a similar frequency, which helps to overcome the small number of patients. In fact, a sampling of only 30 cases is sufficient for 80% power and 90% confidence interval, for the Val(108/158)Met SNP.

When analyzing the patients with a global approach, small differences may not be revealed. Therefore, a detailed and individual analysis was performed, focusing in patients with the higher and lower opioid doses. Besides the referred SNPs, additional variants affecting OATP1A2 and UGT2B7 were analyzed, without significant results in the overall sampling (data not shown), together with morphine, M3G and M6G quantification. Reviewing all the patients, the individual with the higher opioid dose was receiving 800 mg/day of morphine (Patient 1, low responder to morphine) and the patient with the lower opioid dose was controlled with 20 mg/day of morphine. Both patients presented bone metastatic disease, a painful condition, and were under similar treatment (morphine and adjuvant drugs). However, Patient 1 required a higher dose of morphine and still complained of lack of analgesic effect and breakthrough pain. Genetic differences were then analyzed and results are described in Study III. Firstly, the individual approach confirmed the previous obtained result for *COMT* Val(108/158)Met, with Patient 1 carrying the Met allele. Secondly, genetic variants in two additional molecules were pointed out, μ-opioid receptor and UGT2B7. Genetic variants in *OPRM1* were already correlated with morphine requirements, especially SNP A118G, with individuals carrying the A allele requiring lower doses of morphine to achieve a good and controlled analgesic effect (Klepstad *et al.*, 2004; Reyes-Gibby *et al.*, 2007; Sia *et al.*, 2008; Tan *et al.*, 2009), as in the case of Patient 2. Concerning UGT2B7, controversial results have been reported in relation to the linked SNPs C802T (His268Tyr) and T801A and its influence in UGT2B7 activity (Holthe *et al.*, 2002; Hirota *et al.*, 2003; Sawyer *et al.*, 2003; Saeki *et al.*, 2004; Ross *et al.*, 2005; Levesque *et al.*, 2007; Parmar *et al.*, 2011). While Patient 1 was a homozygous T801C802 (His268; *UGT2B7*1*), Patient 2 was a homozygous A801T802 (Tyr268; *UGT2B7*2*), probably with lower glucuronidation capacity (Parmar *et al.*, 2011). Accordingly, Patient 2 presented lower M3G/morphine and M6G/morphine *ratios*. As M3G and M6G have different and opposing pharmacologic activities, differences in morphine metabolism can lead to alterations in morphine analgesic activity. However, the real consequences of morphine metabolism variations in patients chronically administered with morphine are still unknown. In these specific cases, it seems that genetic variants in Patient 1 may lead to higher pain sensitivity, higher morphine requirements and altered

metabolism, helping to explain the difference between daily morphine doses and lack of analgesic effect.

This case series also highlight some difficulties related with the study population, as the heterogeneity of the diagnosis and lack of previous clinical history, especially concerning drugs and doses, baseline pain severity before opioid treatment, titration of opioids and response to adjuvant drugs. Therefore, conclusions about tolerance or hyperalgesia cannot be taken. These difficulties were also present in Studies II and IV. Nevertheless, despite the different diagnosis, these two Patients had some characteristics that helped overcome the limitations, as similar conditions of mental and physical status, similar painful metastatic disease and being under treatment with the same opioid, but with different analgesic responses. Then, individual and more detailed analysis, as this case series presented, can contribute to evidence genetic differences that might otherwise go unnoticed, especially in such heterogeneous population.

After the preliminary results of *COMT* in Study II and *COMT*, *OPRM1* and *UGT2B7* in Study III, genetic variants in cytokines were analyzed (Study IV). Several polymorphisms were analyzed in important pro- and anti-inflammatory molecules [IL-1α, IL-1β, IL-1 receptor antagonist (IL-1Ra), IL-2, IL4 receptor (IL-4R), IL-6, IL-10, TNF-α and IFN-γ]. A significant association between *IL1B* C3954T SNP and cytokine serum levels, pain intensity, metastases and cancer diagnosis status was observed. IL-1β is expressed in nociceptive neurons of the dorsal root and, together with TNF-α, is one of the first cytokines to be released after injury, leading to the synthesis of several other inflammatory effectors, as cytokines, chemokines, prostanoids, neurotrophins, NO, kinins, lipids, ATP and members of the complement pathway. Also, this cytokine originates inhibition of GABA and glycine mechanisms, activation of bradikinin receptors and increase of AMPA and NMDA activity (Buvanendran *et al.*, 2006; Cunha *et al.*, 2007; de Oliveira *et al.*, 2011; Paz Aparicio *et al.*, 2011; Burada *et al.*, 2013). All these actions lead to thermal, chemical, mechanical and inflammatory hyperalgesia. Additionally, an interference with morphine analgesia has been described (Shavit *et al.*, 2005; Mika *et al.*, 2008).

Concerning the polymorphism C3954T, we found that carriers of C allele were related with higher pain intensity and higher serum levels of IL-1β. Additionally, patients diagnosed with breast, prostate cancer and multiple myeloma presented the highest levels of the cytokine, with a four-fold increase of the metastatic disease probability. Although IL-1β lead to the release of other pro-inflammatory cytokines, levels of IL-6, IL-8 and TNF-α were not correlated with IL-1β levels. These negative results may be due to the low number of individuals and some difficulties in cytokines quantification, especially TNF-α,

where the majority of patients had levels below the quantification limit of the test. Despite heterogeneity of the population and the small number of individuals (n = 75), the results are consistent and there is a high degree of agreement of the biochemistry, molecular biology and clinical data. More sensitive methods and an increase in the number of patients might reveal other differences within the population. Also, as IL-1α and lL-1Ra may have different roles in pain, IL-1Ra quantification should also be done in the future.

Finally, the last study of this thesis was performed in an animal model (Study V). As it was already mentioned, and suggested in Study III, alterations in morphine metabolism might lead to different analgesic efficacy, either by drug interactions or genetic variations, but its relation is still unknown. Usually, an increase in drug metabolism may lead to a decrease of drugs effect; however morphine originates two pharmacologically active metabolites, with antagonic actions, making the outcome unpredictable. Thus, an attempt was made to study the influence of induction and inhibition of morphine metabolism in its analgesic effect in the guinea pig, reported as the best animal model to study morphine metabolism due to the similarity with human metabolic *ratio* (Yue *et al.*, 1990; Andersen *et al.*, 2002; De Gregori *et al.*, 2012). The first observation of this study was the effective animal model developed. The metabolic *ratios* were in fact close to those obtained in human, the behavioral assessment (hot-plate test) was adequate and differences between the three groups (induced, inhibited and regular metabolism) were noticed. This animal model can then be used for several further acute/chronic studies of morphine metabolism modulation and analgesic effect, helping to understand morphine pharmacokinetics and its implication in the clinical practice.

Second, and concerning the obtained results, it was possible to observe that the induction of morphine metabolism with TCDD led to higher metabolites concentration and metabolic *ratios* and higher thermal thresholds in behavioral assessment, while the metabolism inhibition assay with ranitidine led to opposite results. TCDD is well-known for its induction properties, especially enzymes involved in drug metabolism (Buckley and Klaassen, 2009; Yeager *et al.*, 2009), but also for its high degree of toxicity in guinea pigs, which could be an influent factor in the behavioral assessment performed. Nevertheless, TCDD toxicity at the selected dose (1 µg/kg) is not relevant in short periods of time as the one used in this assay (three days) (Enan *et al.*, 1996; Enan *et al.*, 1998). The inducting effect of TCDD was demonstrated in this study, as well as a relation between an increase in morphine metabolism and an enhanced analgesic effect after a single administration of morphine, with 75 % of the TCDD-treated animals reaching the temperature cut-off value.

Concerning ranitidine, the chosen inhibitor, its effect on morphine metabolism has already been suggested, leading to a higher inhibition of M3G formation than M6G (McQuay *et al.*, 1990; Aasmundstad and Morland, 1998; Aasmundstad and Storset, 1998). A differential inhibition of morphine metabolism could be potentially beneficial for patients under treatment with morphine, highlighting the analgesic effect of morphine and M6G and reducing the neurotoxic and hyperalgesic effect of M3G. A slightly differential inhibition was observed in our experiment, but the overall metabolism was inhibited leading to lower metabolic *ratios* and significantly lower analgesic effect. In view of previous studies, the chosen morphine and ranitidine concentration may influence the inhibition effect and the rate formation of both metabolites (Aasmundstad and Morland, 1998) , suggesting the need of further studies to achieve a higher differential inhibition that may improve the analgesic effect instead of decreasing it, as it was observed.

This *in vivo* study can help to understand the role and importance of M6G in analgesia, which has not always been consistent (Osborne *et al.*, 1992; Portenoy *et al.*, 1992; Tiseo *et al.*, 1995; Klepstad *et al.*, 2000; Quigley *et al.*, 2003). Our results allowed us to hypothesize that after a single morphine administration, a higher rate of metabolites formation can provide a better analgesic effect, probably due to M6G. Also, the toxic effects of M3G, such as hyperalgesia, were not detected. However, the results may be different in chronic administration, where in a situation of very high levels the hyperalgesic effect of M3G may be predominant. This is probably the case of Patient 1 of the case series reported in this thesis (Chapter 5). Then, continuous modulation studies in the animal model with new acute and chronic administration protocols are required.

The study of morphine pharmacokinetics seems extremely promising in order to improve its analgesic effect, especially by understanding the role of each compound to the final effect. Besides the very well-known analgesic effect of morphine and M6G by binding to μ-opioid receptors, other effects have poorly understood mechanisms, especially the hyperalgesic effect of M3G. During persistent pain, several sensitizing agents are released, as cytokines. However, morphine and its metabolites can also influence the release of sensitizing agents, becoming a “vicious cycle”, which now must also be taken into account when studying opioids variability, as can be seen in Figure 16.

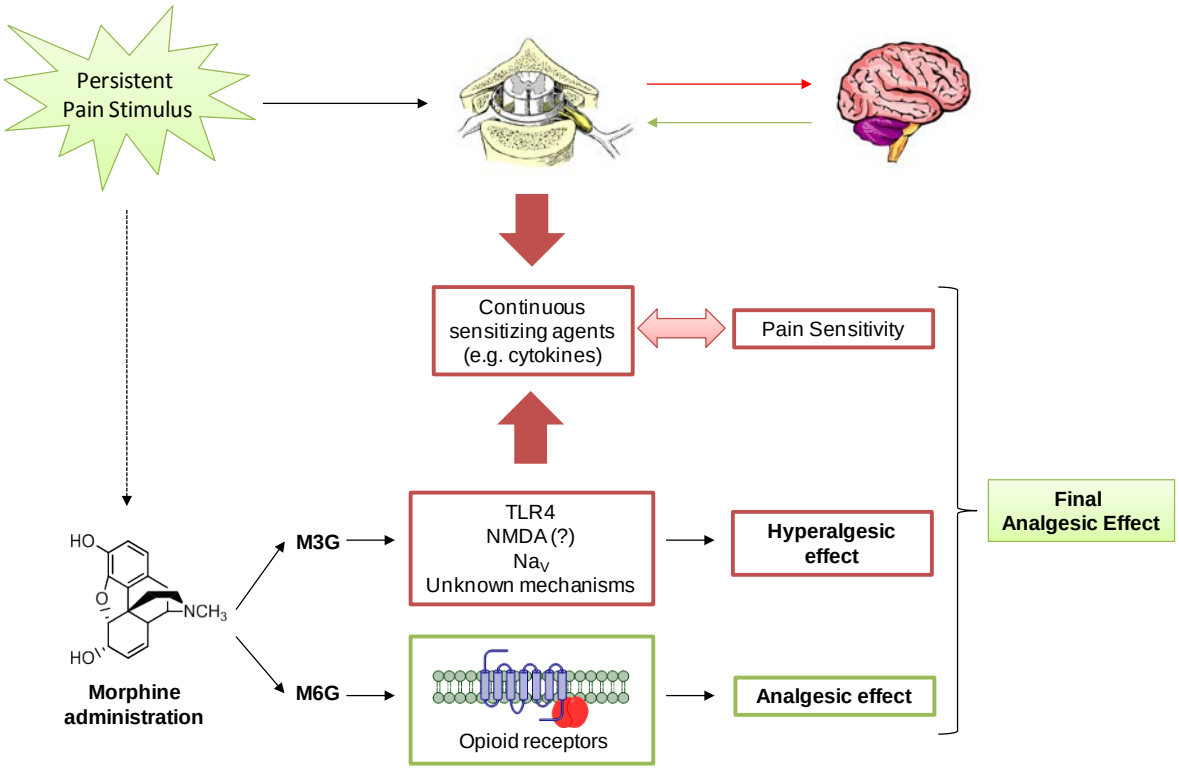


Figure 16. Global approach of morphine variability: variations in pain sensitivity and morphine pharmacodynamics and pharmacokinetics can lead to different final analgesic effects. M3G, morphine-3-glucuronide; M6G, morphine-6-glucuronide; Na_V, voltage-gated sodium channels; NMDA *N*-Methyl-D-Aspartate; TLR4.

It was already reported that morphine induces pro-inflammatory glial activation that can be related to a reduction in the analgesic effect, adverse effects and development of tolerance and dependence (Hutchinson *et al.*, 2010). Recently, this pro-inflammatory response was suggested to be (at least, partially) via toll-like receptor 4 (TLR4), leading to up-regulation or release of pro-inflammatory cytokines (IL-1β, IL6, TNF-α) (Raghavendra *et al.*, 2002; Hutchinson *et al.*, 2010; Lewis *et al.*, 2010; Wang *et al.*, 2012). Also, M3G seemed to cause pain enhancement and hyperalgesia via TLR4 and IL-1β and enhanced Na_V channels in sensory neurons, while M6G was devoid of those properties (Hutchinson *et al.*, 2010; Lewis *et al.*, 2010; Due *et al.*, 2012). Agonist-activation of TLR4 can also enhance the release of CGRP and sensitize the TRPV1 receptor, which are involved in pain transmission and sensitization (Chapter 1) (Due *et al.*, 2012), but the overall consequences of TLR4 activation by M3G are still unknown. All this mechanisms help to understand the very important role of M6G in acute morphine administration, as demonstrated in the study of Study V, and the hyperalgesic role that M3G can evidence in chronic administration, as hypothesized in the case series of Study III. Additionally,

Patient 1 of this case series also presented higher IL-6 and TNF-α (data not shown), which could be due to the very high dose of morphine and subsequent formation of M3G.

The results obtained during this thesis highlight the important role that genetic variation in pain mechanisms can have in cancer-related pain relief. Moreover, it is necessary to realize the importance of observing all the results individually but also integrating them in a global view (Figure 16), analyzing SNPs linked to several phases of pain processing, in the same population, and performing additional *in vivo* studies that can replicate certain phenotypes, in order to obtain an overall perspective and predict the final analgesic effect.

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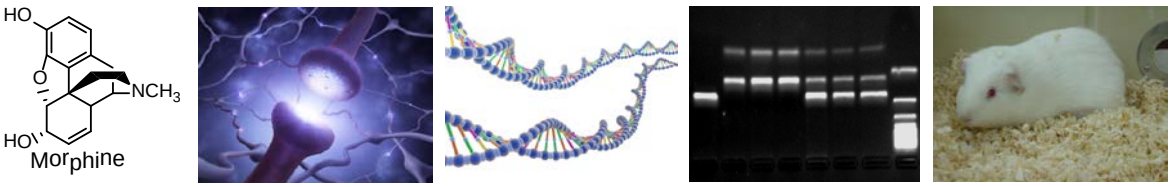
CHAPTER V

CONCLUSIONS



After an overall analysis of this thesis results, several conclusion can be drawn:

a) The construction of an accurate database for cancer pain patients was initiated, with clinical history and pain questionnaire;

b) A simple, sensitive, precise and accurate method for the quantification of morphine, M3G and M6G in several *antemortem* and *postmortem* matrices was developed;

c) Exploratory studies were made, based on several SNPs in important genes as opioid receptors (*OPRM1*), metabolizing enzymes (*UGT2B7*), transporters (*MDR1*) and pain modulators (*COMT* and several cytokines). The important contribution of non-opioid systems to opioid requirements was concluded, based on the influence of *COMT* genetic variation;

d) The influence of proinflammatory mediators was also observed, with genetic variation in IL1B being correlated with cytokine levels, pain intensity and cancer diagnosis status, suggesting this cytokine as a pain effector and cancer biomarker;

e) The relevance of case reports/series was also evidenced as an important tool to unveil masked differences and formulate new hypothesis in the population. Also, with the individual analysis, the contribution of genetic variants in μ-opioid receptor and UGT2B7 was observed, as well as the importance of additional morphine and metabolites quantification;

f) A successful animal model was developed, allowing the study of morphine metabolism and behavioral assessment;

g) Induction and inhibition of morphine metabolism was correlated with morphine analgesic effect:

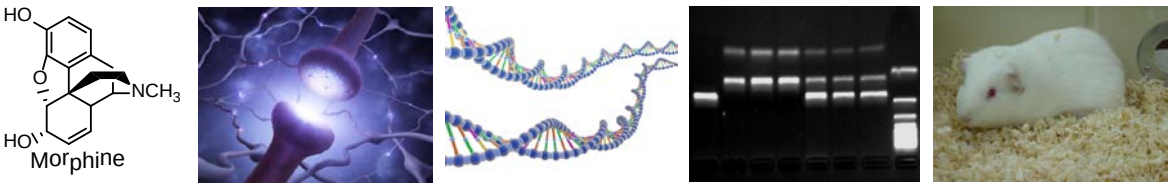
   - TCCD inductive effect led to an improve of the analgesia, after a single morphine administration, highlighting the important role of M6G on pain relief;

- Differential inhibition was slightly obtained with ranitidine, but the overall metabolism inhibition was predominant, diminishing morphine analgesic effect after single administration.

i) The modulation of morphine metabolism has shown to influence its analgesic effect in guinea pigs, suggesting the importance of genetic variants or co-administered drugs that can alter morphine analgesic effect and the importance of this developed model for further studies in order to improve morphine analgesia in clinical practice.

CHAPTER VI

FUTURE PERSPECTIVES



Future studies are required in order to confirm and understand these initial results. Regarding human clinical studies, the recruitment of patients must continue, as a larger number of individuals are necessary to confirm the preliminary positive and negative results and to allow multiple testing along the several SNPs. New polymorphisms should also be analyzed, especially those related with catecholaminergic and serotoninergic systems, morphine metabolism and other pain modulators, as TRP channels. Concerning the developed animal model, its future implications are attractive. Metabolites quantification in animals' organs should be performed, along with a more detailed study of the inhibition and induction mechanisms. Further new acute and chronic studies should be performed to understand the roles of each metabolite, which ultimately could represent a new independent drug. Additionally, new and promising drugs could be tested in order to modulate morphine metabolism and achieve a differential inhibition or induction or to enhance morphine analgesia by diminishing pain sensitivity (e.g. drugs that can modulate the catecholaminergic system).

Pain transmission and perception along with opioids action are very complex traits. Continuous research can lead to a better understand of the interindividual variability in response to opioids and how to improve the pain management, selecting the best opioid and dose adjustment to the therapy. Finally, the aim would be to improve patients' quality of life by applying a tailored-pain treatment.