FACULDADE DE FARMÁCIA

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Mestrado em Biotecnologia Farmacêutica

Dissertação

CYP2D6 genetic variation and predicted metabolic profile in post-cesarean section pain: pharmacogenetic interpretation

Variação genética do *CYP2D6* e a previsão do perfil metabólico na dor pós-cesariana: interpretação farmacogenética

Dissertação apresentada à Faculdade de Farmácia da Universidade de Coimbra, para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia Farmacêutica, realizada sob a orientação científica da Professora Doutora Maria Manuela Monteiro Grazina (Faculdade de Medicina da Universidade de Coimbra).

Rosa Quinta, 2014

"Life is made up of small pleasures. Happiness is made up of those tiny successes. The big ones come too infrequently. And if you don't collect all these tiny successes, the big ones don't really mean anything."

Norman Lear (1922 – present)

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Rosa Quinta

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ABBREVIATIONS

A	Adenine
Å	Ångström
ADRs	Adverse Drug Reactions
AS	Activity Score
ASA	American Society of Anaesthesiologists
C	Cytosine
CEIBA.FP	Consortium of the Ibero-American Network of Pharmacogenetics and Pharmacogenomics
CSF	Cerebrospinal Fluid
CI	Confidence Interval
COMT	Gene coding for the enzyme Cathecol-O-methyltransferase
CNS	Central Nervous System
CNVs	Copy number variations
CYP450	Cytochrome P450
CYP2D6	CYP2D6 enzyme
CYP2D6	Gene coding for CYP2D6 enzyme
DA	Dopamine
DNA	Dexoribonucleic Acid (N=A,T, G, C)
EM	Extensive Metabolizer
FDA	Food and Drug Administration
G	Guanine
GWAS	Genome-Wide Association Studies
IM	Intermediate Metabolizer
kb	kilobase
L-DOPA	L-3,4-dihydroxypheyllanine
mg	miligrams
M3G	Morphine-3-glucuronide
M6G	Morphine-6-glucuronide
N	Number of alleles
n	number of subjects
NADH	Nicotinamide Adenine Dinucleotide

ng	nanograms
nm	nanometers
OPRM1	gene coding for miu-opioid receptor 1
p	*p-value* (statistical analysis)
PCR	Polymerase Chain Reaction
PGE	Prostaglandine
PGx	Pharmacogenetics and Pharmacogenomics
PM	Poor Metabolizer
qRT-PCR	Real Time PCR
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic Acid
RNase P	Ribonuclease P
SNPs	Single Nucleotide Polymorphisms
T	Thymine
Taq	Termostable DNA polymerase (*Thermus aquaticus*)
µl	microliter
UM	Ultrarapid Metabolizer
χ^2	Chi-square (test in statistical analysis)
WBC	White blood cells
wt	*wild type*
6FAM dye-MGB	6-carboxyfluorescein dye-dihydrocyclopyrroloindole tripeptide minor groove binder
5-HT	5-hydroxytryptamine (serotonine)
5-HT3	5-hydroxytryptamine 3 receptor
5-MT3	5-methoxytryptamine

RESUMO

Desde 1959, o papel da Farmacogenómica na sociedade tem-se revelado cada vez mais importante. Nesta área, têm particular interesse as variações genéticas localizadas em genes envolvidos na resposta a fármacos, de modo a inferir a dose certa a administrar. Deste modo a definição "medicina personalizada" tem sido um dos objetivos da farmacogenómica, sobrevalorizando cada vez mais a máxima, "*the right dose of the right drug to the right person*". No entanto, os efeitos adversos a fármacos são a causa de uma percentagem elevada dos internamentos a nível mundial e poderiam ser facilmente reduzidos recorrendo a testes de farmacogenómica.

O corpo humano está exposto a um elevado número de xenobióticos, incluindo uma enorme variedade de componentes farmacêuticos. Deste modo, de forma a reduzir os efeitos adversos que poderiam advir desta exposição, o organismo humano desenvolveu mecanismos enzimáticos de destoxificação, sendo as enzimas da família citocromo P450 um exemplo disso, em particular a CYP2D6.

A CYP2D6 é uma das enzimas mais amplamente estudadas e intervém na destoxificação de xenobióticos. Para além disso, tem uma elevada relevância no metabolismo hepático de agentes terapêuticos como é o caso de antidepressores, neuroléticos, antiarrítmicos, analgésicos, entre outros, também tem um papel relevante no metabolismo de substâncias endógenas, tais como percursor da dopamina. Relativamente aos analgésicos, destaca-se o exemplo da morfina, utilizada no presente estudo.

A morfina é o opióide mais usado para o tratamento da dor e o seu efeito analgésico está associado a alguns efeitos secundários associados, como é o caso de náuseas, vómitos e prurido. A morfina atua pela ativação do recetor miu opióide e a dopamina é libertada no núcleo accumbens em consequência desta ação. Para além disso, salientam-se algumas diferenças no grau de diminuição de dor após a sua administração e foi descrito que 10-15% das mulheres sofrem de dor crónica persistente após cesariana.

Deste modo, foi realizado este estudo numa população de puérperas caucasianas submetidas a cesariana e às quais foram administradas 2,5mg de morfina. Assim, foi efetuada uma interpretação farmacogenética para avaliar a previsão do perfil

metabólico com base nas variantes genéticas do *CYP2D6* e a sua relação com os *scores* clínicos de dor e efeitos secundários associados à morfina, anteriormente mencionados. Para isso, foi realizada a determinação do número de cópias do gene *CYP2D6* e efetuada a genotipagem para as variantes alélicas *CYP2D6*1*, **2*, **3*, **4*, **5*, **6*, **10*, **17* e **41*, através de Real-Time PCR. Os parâmetros avaliados foram a dor (medida após, 4, 8 e 12 horas e quantificada numa escala de 0 a 10) e o aparecimento de prurido.

Após a análise estatística dos resultados, concluiu-se que os haplótipos que resultam na ausência e redução da função da enzima estão positivamente correlacionados com elevados *scores* de dor. Este facto está positivamente associado à diminuição da síntese de dopamina pela via da tiramina. Consequentemente, a ativação da via de transdução do sinal que controla a dor e o efeito analgésico poderá estar reduzida, levando ao aumento da dor após cesariana.

Este trabalho contribui para um melhor entendimento de como as variantes alélicas do *CYP2D6* podem afetar a dor, aumentando o conhecimento acerca do tratamento associado à analgesia pós-parto (cesariana).

***Palavras-chave*:** *CYP2D6*; Farmacogenómica; Cesariana; Dor; Analgesia

SUMMARY

Since 1959, the role of the Pharmacogenomics in society has become increasingly important. In this area, genetic variations located in genes involved in drug response have particular interest, in order to infer the right dose levels. Thus a "personalized medicine" definition has been an objective of pharmacogenomics, overvaluing the paradigm, "the right dose of the right drug to the right person." However, adverse drug effects are the cause of a high percentage of admissions worldwide and it could be easily reduced using pharmacogenomics testing.

The human body is exposed to a large number of xenobiotics, including a huge variety of pharmaceutical compounds. Thus, in order to reduce the adverse effects that could arise from this exposure, the human body has developed a detoxification enzyme mechanisms, and the cytochrome enzymes P450 family are an example, in particular CYP2D6.

The CYP2D6 is one of the most widely studied enzymes and it is involved in the detoxification of xenobiotics. In addition, it has a high relevance in hepatic metabolism of therapeutic agents such as antidepressants, neuroleptics, antiarrhythmics, analgesics, among others, also playings an important role in the metabolism of endogenous substances such as dopamine precursor. Regarding analgesics, it stands out the example of morphine used in the present study.

Morphine is the most commonly used opioid for the treatment of pain and its analgesic effect is associated with some side effects such as nausea, vomiting, and pruritus. Morphine acts by activation of miu opioid receptor and dopamine is released in the nucleus accumbens as a result of this action. In addition, we highlight a few differences in the degree of reduction of pain after administration and it was described that 10-15% of women suffer from chronic persistent pain after cesarean section.

This study was conducted in a population of Caucasian parturient undergoing cesarean section and who were administered with 2.5mg of morphine. Therefore, a pharmacogenetics interpretation was conducted to evaluate the prediction of metabolic profile based on genetic variants of *CYP2D6* and its relationship with clinical scores for pain and side effects associated with morphine, previously mentioned. The determination of the CYP2D6 gene copy number and the genotyping for allelic variants

*CYP2D6*1*, **2*, **3*, **4*, **5*, **6*, **10*, **17* and **41* by Real-Time PCR. The evaluated parameters were pain (measured after 4, 8 and 12 hours and quantified in a 0-10 scale) and the appearance of pruritus.

After statistical analysis of the results, it was found that haplotypes that result in the absence or reduced function of the enzyme are positively correlated with high scores for pain. This fact is positively associated with dopamine synthesis decreased by tyramine's pathway. Consequently, the activation of the signal transduction pathway that controls pain and analgesic effect may be reduced, leading to increased pain after cesarean section.

This work contributes to a better understanding of how the allelic variants of CYP2D6 may affect pain, increasing knowledge about treatment associated with postpartum analgesia (cesarean).

***Key-words*:** *CYP2D6*; Pharmacogenetics; Cesarean; Pain; Analgesia

BACKGROUND

1. History

Physicians have long been aware of the subtle differences in the responses of patients to medication.

In the last century, the physiologist Archibald Garrod develops the concept of "chemical individuality" (1902-1909). It was the first time that the concept of genetic variations might modulate variability in drug effects was proposed[1]. Then, in the 1950s others developments took place. In 1956, the association of primaquine-induced hemolysis with glucose-6-phosphate-dehydrogenase deficiency in erythrocytes was discovered. In 1957 twin studies indicated polygenic influences on the pharmacokinetics of numerous drugs[2].

The term pharmacogenetics was coined in 1959, and the first textbook was published in 1962, before methods for studying DNA sequence variation were available[3]. Then, the first time that the "pharmacogenetics" term appears in the literature was in 1959 by Friedrich Vogel[4].

The field of pharmacogenetics had new developments in the 1970s, when Vesell and colleagues demonstrated that plasma half-lives of many drugs were less divergent among monozygotic twin pairs than dizygotic twin pairs[2]. Over 50 years, examples of novel drug effects exaggerated responses or lack of effectiveness of drugs, as a manifestation of inherited individual traits have been observed. More recently, developments have broadened pharmacogenetic approaches to include novel genomic techniques with introduction of the term pharmacogenomics in the 1990's with emergence of the Human Genome Project[5].

In 2005, the first case of fatal respiratory depression in a breastfed 13-day-old neonate whose codeine-prescribed mother was a Cytochrome 2D6 (CYP2D6) ultrarapid metabolizer (UM) was described[6]. Neonatal post-mortem blood contained a high concentration of morphine, and breast milk morphine concentration was four fold higher than any previous literature report. The death was attributed to opioid intoxication as a result of almost two week exposure to higher than expected morphine dose excreted into breast milk of an ultrarapid metabolizing mother. The breastfeeding mother herself also experienced sedation and constipation and needed to halve her codeine (3-methilmorphine) dose accordingly. As a result of this case, the

United States Food and Drug Administration (FDA) and Health Canada have issued public health advisories and a proposed labelling update with the caution that codeine may not be safe during breastfeeding for infants whose mothers are *CYP2D6* UMs[6].

There are many examples in literature stressing out the importance of genetics influence in drug response. Scientists are increasingly learning more about the interaction between drugs and human genetics and the paradigm "the right dose of the right drug to the right person" is even more overvalued. This is one of the goals of pharmacogenomics and personalized medicine[7].

2. Pharmacogenetics and pharmacogenomics

Findings from the Human Genome Project results made it clear that 99.9% of the information in the genomes containing the estimated 20,000 human genes, is identical from one person to the other 20,000. The differences in the remaining 0.1% of genes present in the human cells are keys to individuality, including the influence in an individual's susceptibility to certain health problems or determine how one reacts to different treatments[8,9].

In a large patient population, a medication that is proven efficacious in many patients often fails to work in some other subjects. Furthermore, when it does work, it may cause serious side effects, even death, in some patients. Factors that cause variations in drug response are multifold and complex, some of which involve fundamental aspects of human biology, because a drug response directly affects well being and survival[10].

Pharmacogenomics, takes use of a genome wide approach to study the entire spectrum of genes involved in drug response, for providing the base for a more rational approach for prescription drugs[11,12].

This concept refers to the complex interactions of genes across the genome and includes identifying candidate genes and polymorphisms, correlating these polymorphisms with possible therapies, predicting drug response and clinical outcomes, reducing adverse events and dosing of therapeutic drugs on the basis of genotype[13].

In summary, pharmacogenetics focuses on a single gene while pharmacogenomics studies multiple genes[14].

One goal of pharmacogenomics is to customize drugs for defined sub-populations of patients, and eventually, perhaps tailor therapies for specific individuals (Figure 1)[13] with specific genetic characteristics.

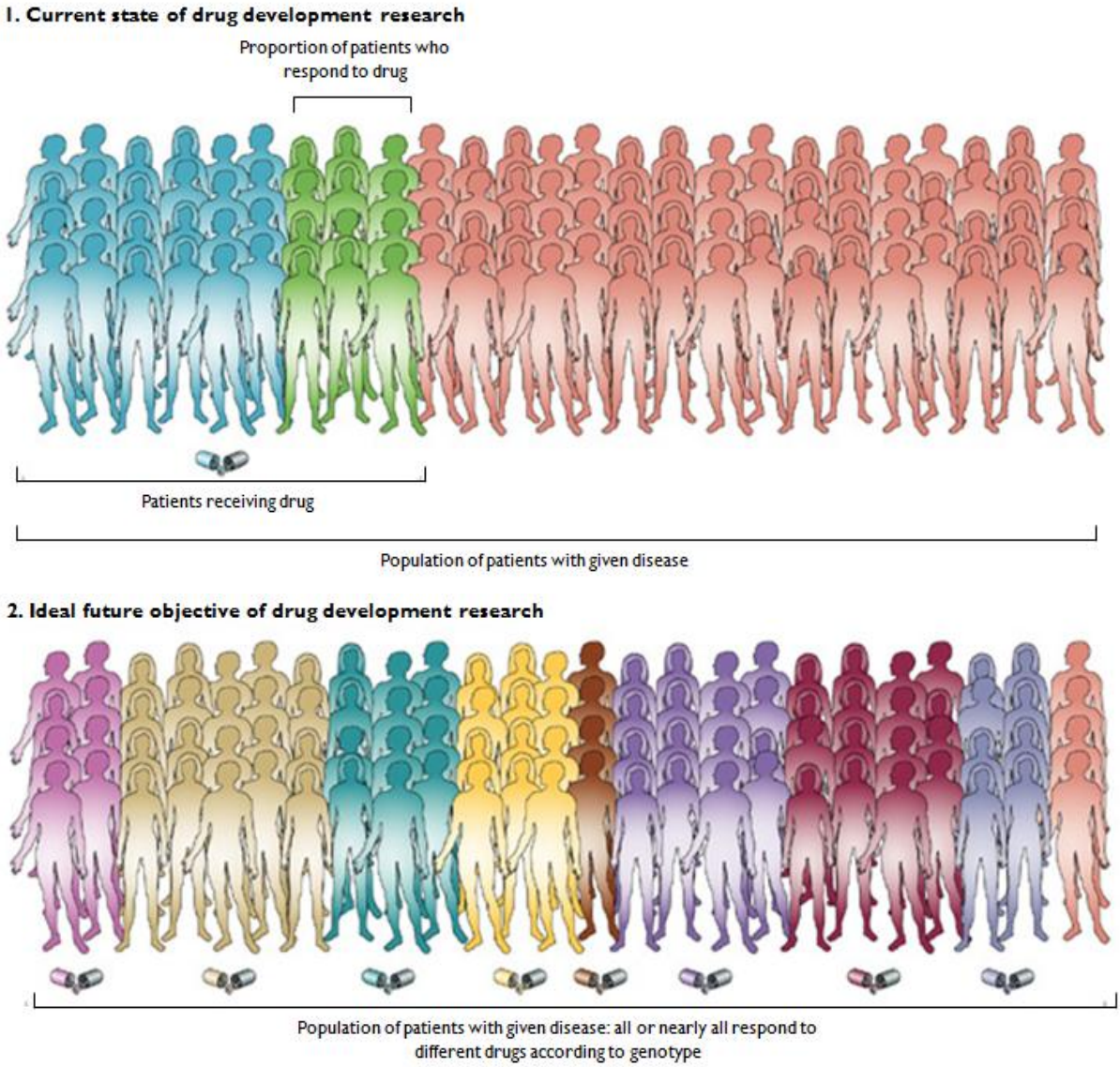


Figure 1 – An ultimate clinical goal of pharmacogenomics. 1. At present, only a limited number of patients are treated with a specific drug for any given disease due to adverse events and not all respond. 2. Pharmacogenomics allows 'tailor' therapies, so that, all patients who have a given disease will receive different drugs on adjusted doses, responding to therapy with less risk of adverse events[13] (Adapted from Issa, 2002).

So that, pharmacogenetics and pharmacogenomics (PGx) have a potential role in reducing Adverse Drug Reactions (ADRs) that have been reported to be the cause for drug withdrawal after marketing, hospital admissions, death in hospitalized patients and to be the fourth leading cause of death in developed countries ahead of pulmonary disease, diabetes, AIDS, pneumonia, accidents, and automobile deaths. The costs associated with ADRs may radically escalate the cost for healthcare[14,15].

Pharmacogenomics is a highly attractive field of research, which has been recently stimulated by multi-omics technologies[16]. In fact, Genome-Wide Association Studies (GWAS) may help to identify multiple variants that might incrementally affect response to a particular drug at the same time[17].

A substantial improvement may be expected over the next few decades through development of procedures, their medical availability, and through reduction of the methods costs, which will allow effective screening of a patient for genes that control his or her response to a given drug (Figure 2), influencing ADRs.

Presently a document is on public discussion about incorporation of Pharmacogenetics in clinical trials[18].

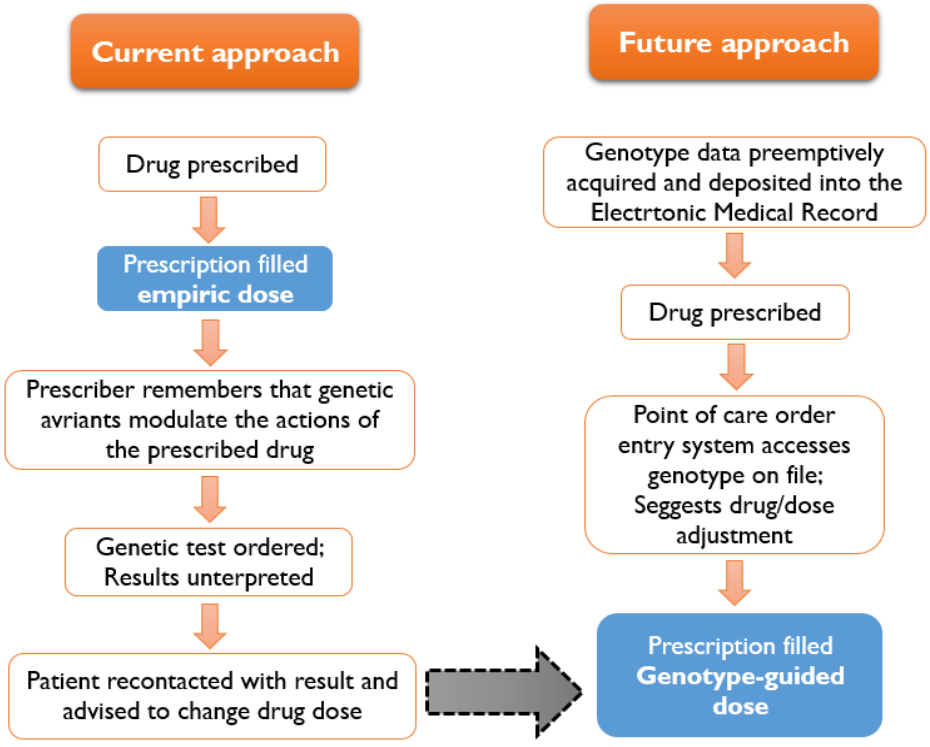


Figure 2 – Contrasting approaches to incorporating genomic information into prescribing. Current practice vs future approach[3] (Adapted from Roden *et al*, 2011).

3. Adverse Drug Reactions (ADRs)

Pharmacogenetics is the science that understanding of the role that an individual's genetic variations play in xenobiotics aims the response, including drugs, as well as what side effects are likely to occur. It is important to regulate this subject in order to minimize the ADRs, make decisions based on risk/benefit criteria, and allow the rational use of drugs based on other criteria[8]. So, this science starts with the observation of an unexpected drug response and continues for finding a genetic cause for that effect.

The ADRs are unpredictable, even among individuals who are receiving the same therapeutic regimen[19]. When chosing a drug, physicians need to consider the risk of adverse events, or any detrimental, unintended consequence of administering a drug at indicated clinical doses[9]. ADRs caused more than 2 million hospitalizations including 100,000 deaths per year in the United States, according to data from 2006. The ADRs could be due to multiple factors such as disease determinants, environmental and genetic factors[12].

Many drug responses appear to be genetically determined and the relationship between genotype and drug response may have a very relevant diagnostic value. Moreover, the genetics of the drug metabolizing enzymes plays a critical role for understanding interindividual differences in drug response and ADRs[20].

Interindividual variability in drug response, including ADRs, is a consequence of multiple factors, including genomics, epigenomics, environment and a patient's characteristics, such as gender, age and/or concomitant medication[16] (Figure 3).

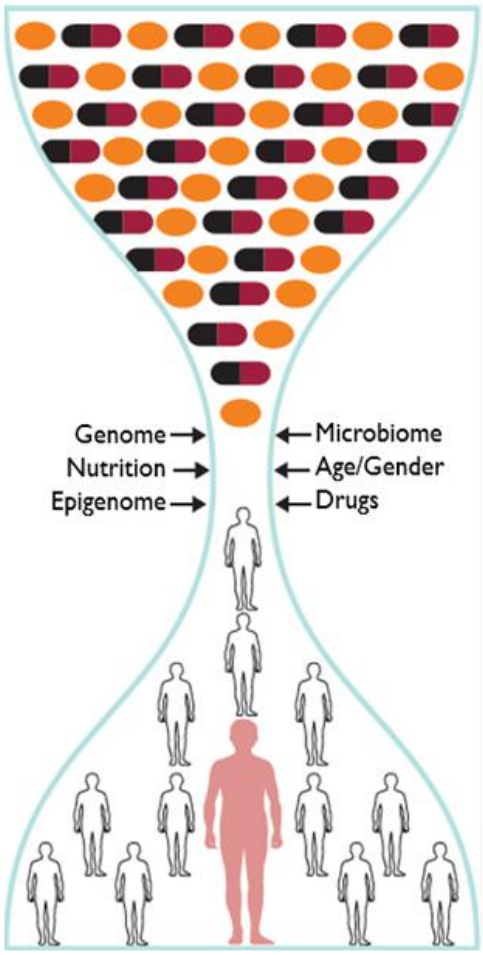


Figure 3 – Interindividual variation in drug response is the consequence of a combination of genetic and environmental factors as well as patient's individual characteristics[17] (Adapted from Schwab, 2012).

Although individualized medications remain as a challenge for the future, the pharmacogenetic approach in drug development should be still continued. If it becomes a reality, it delivers benefits to improve public health and allow genetically subgroup diseases thereby avoiding adverse drug reactions[12].

In figure 4, the progression of ADRs' notification in Portugal is represented, showing the increase in past years.

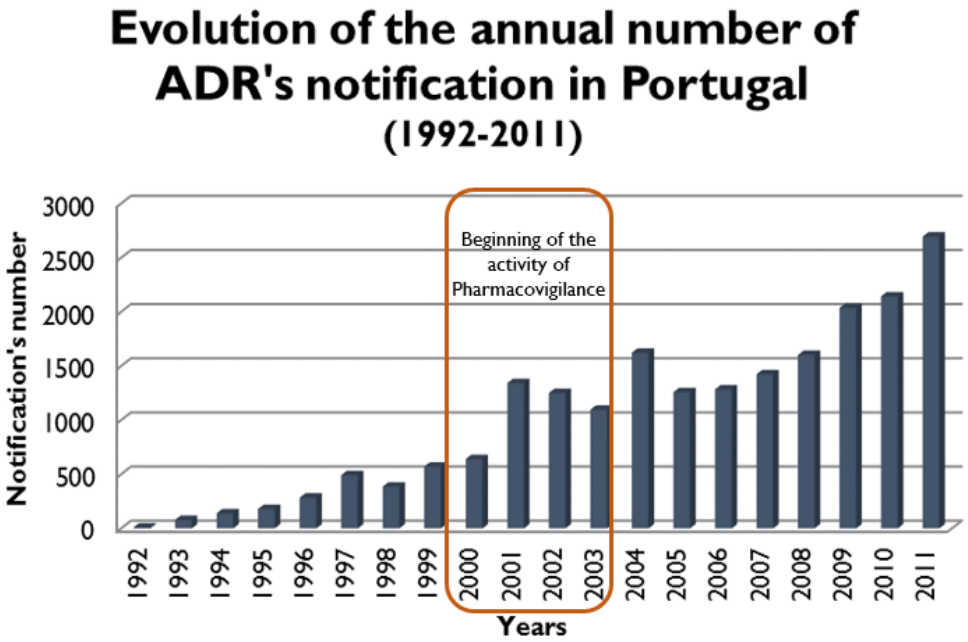


Figure 4 – Evolution of the annual number of ADRs reports (1992-2011). Pharmacovigilance started in 2000. This contribute to the increase of notification's number per year. The number of notifications in 2011 is 4 times greater than in 2000[21] (Adapted from INFARMED, 2011).

4. Opioids and pain treatment

Clinicians are well aware of the large and unpredictable interindividual variability in pain perception and sensitivity to analgesia.

Pain is a major public health issue, and it is the most common reason for physician consultation in the United States. However, despite a wide variety of pharmacological agents available on the market, many people cannot achieve optimal analgesia, and inadequate treatment remains a major cause of suffering and disapproval in pain therapy[22].

4.1. Mechanisms mediating pain

In order to understand nociception, it is essential to understand the mechanism behind it and only then is it possible to specifically target the source of the pain stimulus[23].

Pain and inflammatory stimuli result in a series of diverse effects, including pain transduction, sensitization of central nervous system and peripheral nerve endings[23].

Nociceptors, or receptors of pain, do not have a continuous function under normal activity, but, when stimulated upon pain stimuli or when tissue irritation or injury occurs, they respond with a magnitude relevant to the degree of the stimulus[23].

Epidural analgesia with a combination of local anesthetics and opioids is an excellent multimodal method for better analgesia and enhanced recovery. Epidural analgesia should not be considered as a single generic entity because many factors like the congruency of catheter insertion location to site of surgical incision, type of analgesic regimen whether local anesthetic or opioids, and also the type of pain assessments which can be either at rest or dynamical. Epidural analgesia, regardless of analgesic agent, location of catheter placement, and type and time of pain assessment, provided better postoperative analgesia compared with parenteral opioids[23].

However, there is a central role for dopamine (DA) neurotransmission in modulation of pain perception and analgesia. Opioid system activity is related to pain processing and has also been linked with DA neurotransmission. The DA and opioid systems may work together in the context of pain processing, with the opioid system responding rapidly to noxious stimuli, which in turn promotes DA release.

In addition to the anatomical overlap between brain regions associated with pain processing and those that comprise the DA system, there is also a substantial overlap between the cognitive and emotion-associated factors that influenced by DA neurotransmission and the cognitive and affective factors that influence the experience of pain. Dopaminergic neurotransmission has an important influence on outcome prediction, attention, response inhibition, and motivation, as well as affective symptoms associated with anxiety and depression[24].

The epidural administration of morphine in obstetrics aims to promote analgesia in women undergoing cesarean section. The figure 5 is a general representation of

nociception postpartum pain, and it is possible verify that, opiate drugs (morphine), which act primarily in the central nervous system, block the transfer of pain signals from the brain to the spinal cord. Thus, morphine exerts its analgesic effect by binding to presynaptic miu-opioid receptor in sensory neurons, mimetizing the action of endorphins. Consequently, there is hyperpolarization of the neuron and reducing the release of neurotransmitters, particularly dopamine and serotonin by blocking the perception of pain signals[25].

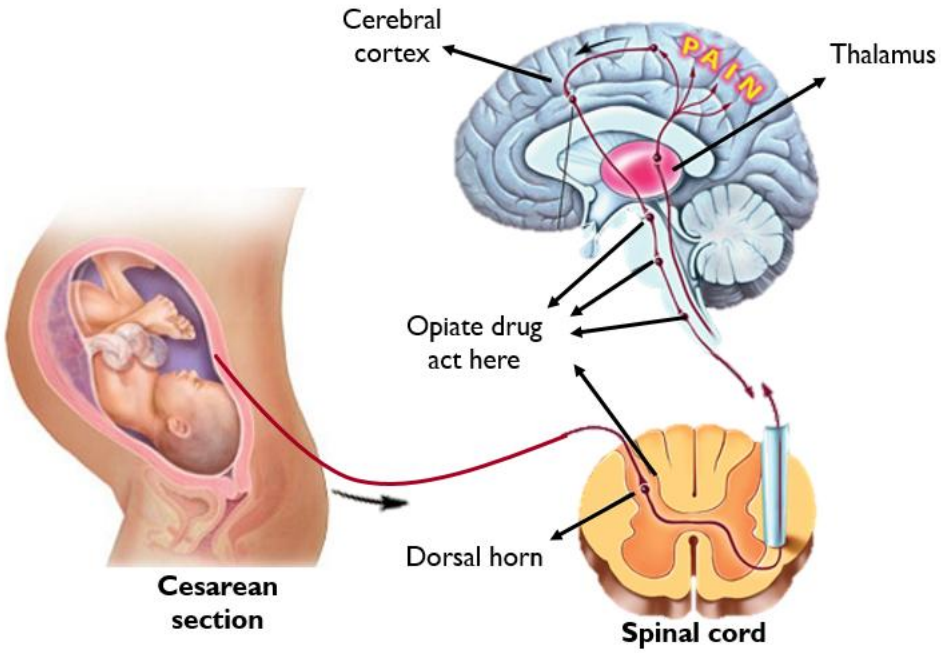


Figure 5 – Representation of a pain nociception mechanism[15] (Adapted from Gasche *et al*, 2004).

Pain relief can be attained by the conventional pharmacological option of administering opioids, such as morphine.

Morphine has been the analgesic drug of choice for anesthesia for decades. The adverse effects of such opioids are quite common and patients frequently have nausea, vomiting, pyrexia, pruritus and hypotension[23].

Pain relief by epidural and spinal anesthesia or combined spinal-epidural anesthesia have found major success in obstetric procedures because of the major advantage over general anesthesia and thus the parturient can stay awake during a cesarean section[23].

In the presently accepted definition, 'Pain' is described as an unpleasant sensory and emotional experience associated with real or potential tissue damage, or described in terms of such damage – IASP, International Association Study of Pain[23,26,27].

Pain is also considered (EFIC) as a private perception that arises to the conscious brain, typically in response to noxic stimuli, but sometimes also in its absence.[28]

Pain perception is a diverse set of complex perceptual events (including neurotransmitters and hormones) that are characterized by their unpleasant or distressing nature[29,30].

In the clinical setting, subjective patient self-reported pain severity ratings that encompass both nociceptive (pain) stimulus intensity and the patient's response to the stimulus are used to guide analgesic drug treatment, as there are no objective "pain tests" that can be readily applied at the bedside or in the primary care physician's office[31].

Nociception is the physiologic component of pain and includes transduction, transmission and modulation of nociceptive stimuli. If nociceptive stimuli be determined, several neuroendocrine responses occur, and a hiperexcitability state of the periferic and central nervous system is already installed[26].

The sensation of pain produced by an acute insult such as trauma or surgery is an adaptive response characterized by guarding behavior to facilitate healing and is an important survival mechanism. Globally, the prevalence of pain is about 15–20%, in adults, and not only adversely affects patient quality of life but it also imposes a high socioeconomic cost encompassing work days lost, reduced productivity and increased healthcare costs. So, the use of opioids in pain management requires careful dose escalation and empirical adjustments based on clinical response and the presence of side effects or ADRs[31,32].

Pain severity ratings and the analgesic dosing requirements of patients with apparently similar pain conditions may differ considerably between individuals. Contributing factors include those of genetic and environmental origin with epigenetic mechanisms that enable dynamic gene-environment interaction, more recently implicated in pain modulation[31].

Indeed, more than 350 candidate pain genes have been identified as potentially contributing to heritable differences in pain sensitivity. A large number of genetic association studies conducted in patients with a variety of clinical pain types or in humans exposed to experimentally induced pain stimuli in the laboratory setting, have examined the impact of single-nucleotide polymorphisms in various target genes on pain sensitivity and/or analgesic dosing requirements[22,31].

Pharmacogenomic approaches offer insight into the genetic variables that can affect drug uptake, transport, activation of its target, metabolism, interaction with other medications and excretion. The use of pharmacogenomics in patients requiring pain management can lead to more efficient opioid selection, dose optimization and minimization of ADRs to improve patient outcome[32].

Hence, in the past 15 years, a big effort has been directed to identification of genetic factors that may explain inter-individual differences in pain sensitivity and analgesic drug dosing requirements[31].

The effect of genotype on the pharmacokinetics and pharmacodynamics of analgesic drugs has also been assessed in numerous genetic association studies. Such studies have generally been designed to assess the influence of single-nucleotide polymorphisms (SNPs) in candidate pain genes encoding receptors and ion channels implicated in pain modulation, and/or the effect of genes encoding drug metabolizing enzymes and transporters on analgesic drug pharmacokinetics[31].

The "genetic architecture of human pain perception" has been proposed to include rare deleterious genetic variants and more common genetic polymorphisms as mediators of human pain perception and clinical pain phenotypes. For example, the differences between men and women resulting from evolutionary pain-modulation processes, which afford women a greater sense of awareness of potential environmental threats to offer heightened protection to their offspring[33].

4.2. Morphine and analgesia

Opioids are indicated for the treatment of moderate to severe pain. Morphine is the main alkaloid of *Papaver somniferum* and is used in therapeutics as a potent analgesic[34] to control moderate and severe pain[31,32].

According to Zhu *et al*, 2005, human organism is capable to produce morphine. Human white blood cells (WBC), specifically polymorphonuclear cells, have the ability to synthesize morphine. The WBC also express CYP2D6, enzyme capable of synthesizing morphine from tyramine, norlaudanosoline, and codeine. Morphine can also be synthesized by another pathway, via L-3,4-dihydroxyphenyllanine (L-DOPA). Finally, they showed that WBC release morphine. These studies provided evidence that the synthesis of morphine by various animal tissues is more widespread than previously thought, including human immune cells. Moreover, another pathway for morphine synthesis exists, via L-DOPA, demonstrating an intersection between DA and morphine pathways. WBC can release morphine into the environment to regulate themselves and other cells, suggesting involvement in autocrine signaling since these cells express the μ3 opiate receptor subtype[35].

Worldwide patterns of opioid consumption differ depending on cultural, educational, and economical factors, in addition to characteristics inherent to health care infrastructure and accessibility. The top 20 countries with the highest consumption of morphine miligram equivalents per capita (in 2008), are listed in figure 6. In these countries, particularly the United States and Canada, opioids are more commonly administered to pregnant and lactating women for the treatment of acute pain[36].

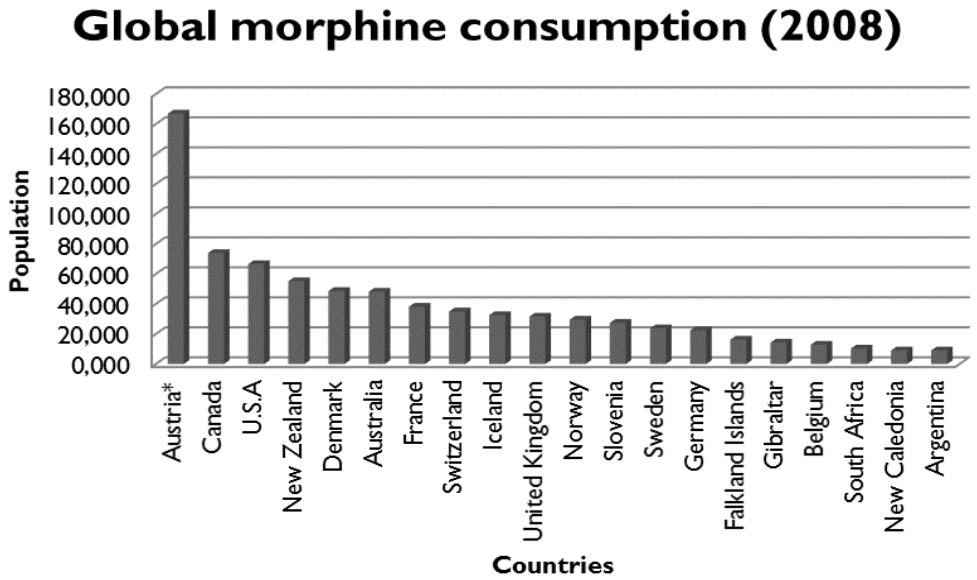


Figure 6 – Global Morphine Consumption, 2008: mg/capita – Top 20 Countries[36] (Adapted from Madadi *et al*, 2012) [* *Austria uses morphine in treatment of drug dependence*.].

Morphine has been widely used to treat severe pain. Besides the classic side effects that comes from epidural morphine administering, the most common ADRs are pruritus, nausea and vomiting[37]. The incidence of pruritus after epidural morphine ranges from 1% to 90%, nausea from 7% to 60%, and vomiting from 17% to 50%. In women receiving epidural morphine to treat post cesarean section pain, 58%-93% complained of pruritus and 16%-59% of nausea and vomiting (5%-10%)[38].

Pruritus is a side effect caused by epidural morphine. Besides pruritus there are some more side effects, like nausea, vomiting, respiratory depression and difficulty in micturition. These undesirable effects, although with low morbidity rate, bring discomfort to patients and may prolong hospital stay. Different treatments have been proposed to prevent or treat these effects. Naloxone, opioid antagonist drug, would be the best choice and can reverse the side effects of morphine. However its use has been associated to lower analgesia quality, in addition to undesirable cardiovascular effects in some cases. Propofol has also been suggested as therapeutic option, but its effectiveness against itching is controversial and cost is high. More recently, ondansertron, a serotonin antagonist, is being successfully used to treat cholestasis and morphine-induced itching[39].

Epidural morphine produces better analgesia than intravenous morphine, but it way produce more severe pruritus. The exact mechanisms underlying pruritus pathophysiology remain poorly understood. However, medullary dorsal horn activation may be related to pruritus[40], according to a study conducted with 58 women.

Pruritus may be generalized but is more likely to be localized to the face, neck, or upper chest. Severe pruritus is rare, occurring in only about 1% of patients. On the face, it is more common on trigeminal-nerve innervated areas, indicating cerebrospinal fluid (CSF) migration and interaction with the nucleus of this nerve, which in turn has opioid receptors also present on its nervous roots. This rostral movement of opioids toward baseline cisterns helps the access to the trigeminal nucleus. Some other mechanisms, suggested for itching, are based on histamine, tachycinine and interleukin release, substances which promote itching by themselves, but which have pro-inflammatory activity, being able to sensitize nervous terminations and lead to itching. In addition, 5-HT3 (serotonin) may be an itching regulating substance by directly acting on its receptors, mechanism observed in patients with cholestasis[37,39].

Histamine and compounds release peripheral histamine from mast cells and are the primary experimental itch mediators in humans. A specialized itch pathway has been identified, with specific mechano-insensitive C. fiber primary afferents and spinothalamic projection neurons in lamina I of the dorsal horn, both responding to histamine application. However, non-histaminergic pruritic mediators are known (e.x. proteases), and antihistamines are ineffective in many pruritogenic diseases and models.

Although pruritus is now unanimously considered a distinct sensation from pain, there are undoubtedly interactions between the two. Conversely, pain inhibition via local anesthesia intensifies histamine-induced itch, and pruritus is a common and problematic side effect of pain treatment with u-opioid agonists[41]. However, animal studies were the basis for the concept of an "itching center" located below the spinal cord and indicating that the trigeminal nucleus would be involved in this mechanism[37].

Serotonin is a powerful pruritic agent in mice, but there are probably important differences between rodents and humans in the relative involvement of histamine and serotonin in the mediation of pruritus[41].

In the era of opioid therapy, pharmacogenomic studies to guide opioid-based analgesic regimens are flourishing. Among the numerous candidate genes that have been considered important in opioid response, the CYP family of enzymes, the μ-opioid receptor gene (*OPRM1*, p.118 A>G) and the Cathecol-O-methyltransferase gene (*COMT*, p.158Val>Met) have been extensively reviewed. However, most drug effects are determined by the interaction of several polymorphisms that influence the pharmacokinetics and pharmacodynamics of medications, including inherited differences in drug targets (ex. receptors) and drug availability (ex. drug-metabolizing enzymes and transporters). This interplay may result in polygenic determinants that involve numerous potential combinations of drug-metabolism, drug-transporters and drug-receptor genotypes with corresponding drug-response phenotypes yielding a wide-range of therapeutic indexes (efficacy/toxicity ratios) for a given drug[33].

4.2.1. Opioid metabolism

Opioid metabolism by CYP450 enzymes and enzymes that regulate glucuronidation to produce metabolites influence the concentration of drug available at

the effect site and therefore its clinical efficacy. Genes encoding enzymes responsible for these metabolic pathways exhibit considerable polymorphism resulting in wide variability in inter-individual responses to some opioids[42].

The clinical effects of the weaker opioids codeine, dihydrocodeine, tramadol, oxycodone, and hydrocodone rely upon the formation of more potent hydroxyl metabolites (such as morphine, dihydromorphine, and oxymorphone) by a metabolic pathway (Figure 7) mediated via the cytochrome P450 enzyme CYP2D6[36,42].

Morphine is further metabolized into morphine-6-glucuronide (M6G), morphine-3-glucuronide (M3G) and normorphine; both morphine and M6G display opioid activity[33]. Additionally, in a smaller extent, morphine is also metabolized by CYP2D6[35].

Opioids have been widely used within the fields of anesthesia and acute and chronic pain for many years, and their use is characterized by large inter-patient variations in dosage requirements. Although many factors can influence pain perception and sensitivity, some of the variable clinical response to opioids can be explained by genetic heterogeneity in factors affecting both the pharmacodynamic and the pharmacokinetic behavior of these drugs[42].

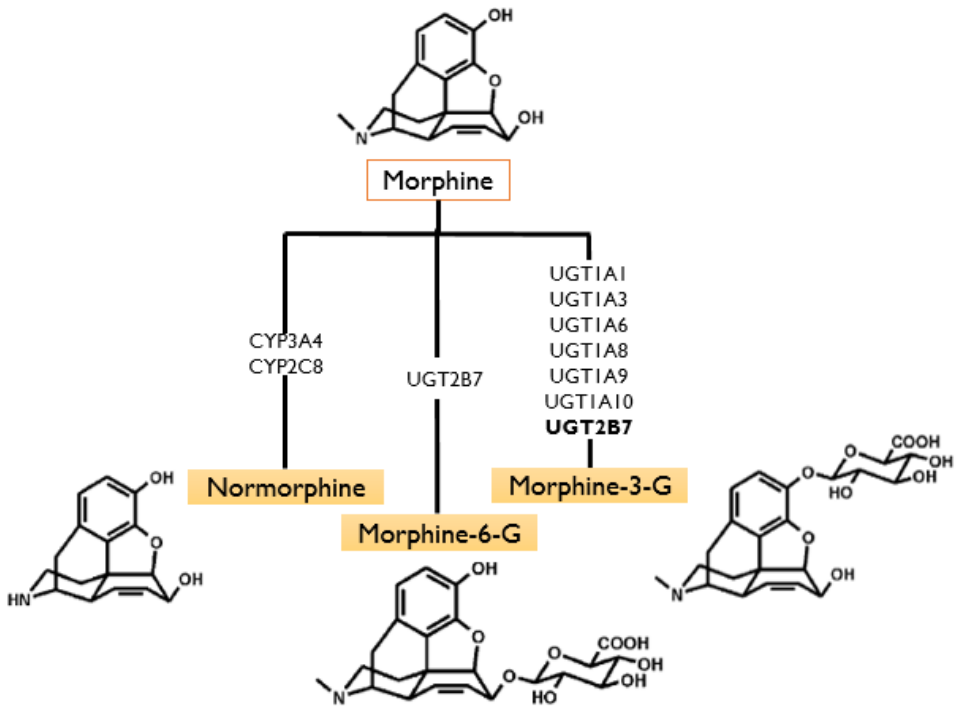


Figure 7 – Major metabolic pathways of morphine in humans with the isoenzymes involved[33,34] (Adapted from Maurer *et al*, 2006 and Vuilleumier, 2012).

5. Detoxification metabolism and xenobiotics

The human body is exposed to a high number of xenobiotics during lifetime, including a variety of pharmaceutical drugs and food components, and has developed complex enzymatic mechanisms to detoxify these substances (Figure 8). These mechanisms exhibit significant individual variability, and are affected by environment, lifestyle, and genetic influences[43].

Therefore, human body is capable of managing exposure to toxic substances, using a complex systems of detoxification enzymes that minimize the potential damage of xenobiotics.

The hypothesis that xenobiotics consumed by animals are transformed to water-soluble substances and excreted through the urine was first put forth in the late 18th century. Later, on 1947, R.T. Williams defined the field of detoxification in his monograph "Detoxification Mechanisms". This biochemist proposed that these non-reactive compounds could be biotransformed in two phases: functionalization, which uses oxygen to form a reactive site, and conjugation, which results in addition of a water-soluble group to the reactive site[44].

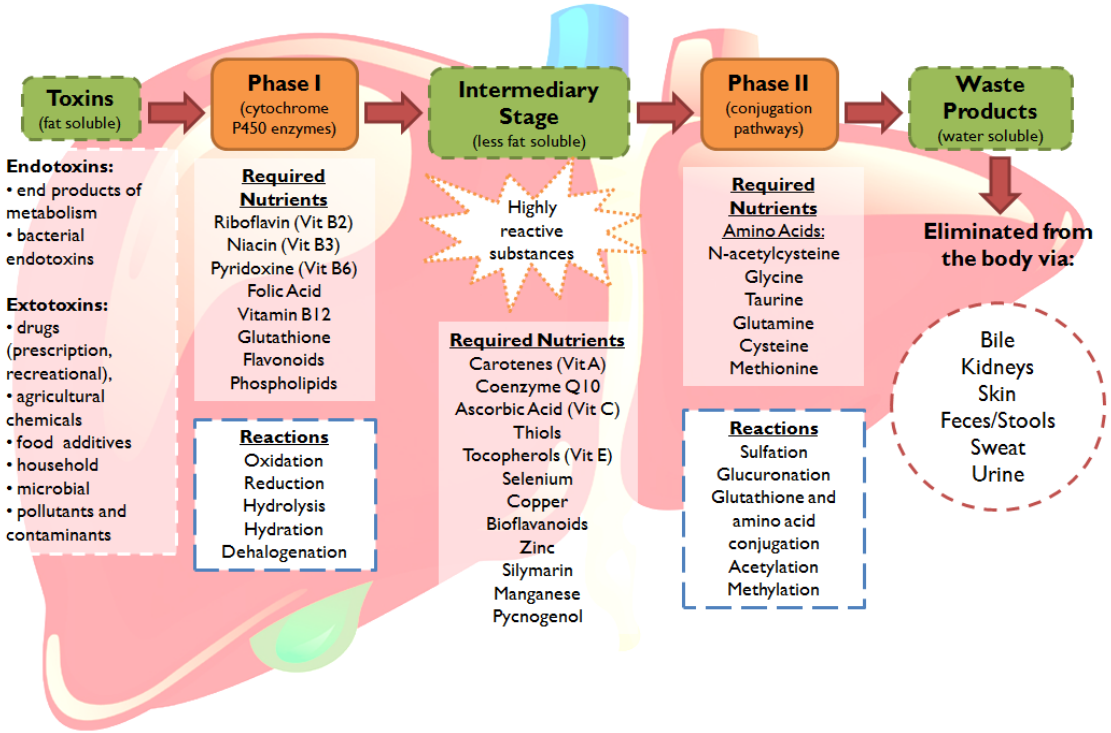


Figure 8 – Liver detoxification pathways in the metabolism of xenobiotics and endogenous components[43] (Adapted from Liska, 1998).

These two steps, functionalization and conjugation, are termed Phase I and Phase II detoxification, respectively:

- **The Phase I system**: composed mainly of cytochrome P450 (CYP450) supergene family of enzymes. It is generally the first enzymatic defense against foreign compounds and endobiotics also, including end products of metabolism and bacterial endotoxins. Most drugs are metabolized trough Phase I biotransformation (Table 1). In a typical Phase I reaction, a CYP450 enzyme uses oxygen and NADH as a cofactor to add a reactive group, such as a hydroxyl radical. As a consequence of this step in detoxification, reactive molecules, which may be more toxic than the original molecule, are produced. If these reactive molecules are not further metabolized by Phase II conjugation, they may cause damage to lipids proteins, RNA, and DNA, within the cell. In table 1, the common genetic variants in Phase I drug metabolizing enzymes and some selected drugs substrates, are presented.

- **The Phase II system**: Phase II conjugation reactions generally follow Phase I activation, resulting in a metabolite that has been transformed into a water-soluble compound that can be excreted through urine, bile or stool. Several types of conjugation reactions are present in the body, including glucuronidation, sulfation and glutathione and amino acid conjugation[43,45]. However, there are xenobiotics and endobiotics like hormones, that are metabolized through this phase first.

Table 1 – Overview of common genetic variants in Phase I drug metabolising enzymes[45] (Adapted from Pinto *et al*, 2011).

CYP2D6				
Important Variants	**Variations**	**Consequence of Variation**	**Effect on Enzyme Activity**	**Some selected drugs acting as substrates**
CYP2D6*2	c.1457C>G (rs#1135840)	Silent mutation	Normal activity	Amitriptyline Atomoxetine Carvedilol

Important Variants	Variations	Consequence of Variation	Effect on Enzyme Activity	Some selected drugs acting as substrates
CYP2D6*3	c.775delA (rs#35742686)	Frame shift mutation, premature codon stop	Absence activity	Chlorpromazine Citalopram Clomipramine Clozapine Codeine Debrisoquine Dextromethorphan Flecainide Fluoxetine Gefitinib Haloperidol Imipramine Imaprotiline Metoprolol **Morphine** Nortriptyline Paroxetine Risperidone Tamoxifen Thioridazine Timolol Tramadol
CYP2D6*4	c.506-1G>A (rs#3892097)	Splicing defect	Absence activity	
CYP2D6*5	*CYP2D6* deleted	Whole gene deletion	Absence activity	
CYP2D6*6	c.454DelT (rs#5030655)	Frame shift mutation	Absence activity	
CYP2D6*10	c.100C>T (rs#1065852)	p.Pro34Ser	Reduced activity	
CYP2D6*17	c.320 C>T (rs#28371706)	p.Thr107Ile	Reduced activity	
CYP2D6*41	c.985+39G>A (rs#28371725)	Splicing defect	Reduced activity	

6. CYP450 enzymes

The CYP450 enzymes comprise a ubiquitous and very large superfamily of hemeprotein monooxygenases that metabolize physiologically important compounds vital to life of most organisms from protists to plants and mammals. They catabolize and detoxify not only xenobiotics compounds, including drugs, procarcinogens and carcinogens, but also participate in synthesis of steroid hormones, cholestrol, bile acids and degradation of endogenous compounds, namely fatty acids and steroids[46].

It is well known that polymorphisms in the *CYP* genes affect the activities and or specificities of the encoded enzymes, which causes differences in the response to drugs that are substrates for these enzymes[47].

Furthermore, CYP450 is also involved in the maternal metabolism of pregnant women. The placenta contains CYP450 enzymes that are capable of metabolizing xenobiotics and endotoxins. N-acetyltransferase, glutathione transferase, and sulphating enzymes are also present. These enzymes can change the activity of drugs as they cross through the fetus. As more drugs are metabolized within the placenta, two effects may occur: the drug is metabolized to an active or an inactive form. During the first trimester, the fetal liver is not able to metabolize drugs using CYP450 enzymes. Once fetal liver development is complete, the fetus has the same enzymes, with the exception of CYP3A4, which is expressed only later in life[48]. It is important to mention that the majority of CYP enzymes are inducible, being expressed in the presence of the substrate.

However, changes in maternal metabolic function during pregnancy are largely unknown and the activity of the cytochrome P450 enzymes needs further study to effectively predict the metabolic rate of metabolized drugs by the liver. Renal function is gradually elevated, leading to increased elimination of water-soluble drugs and metabolites[48].

The major families responsible for the oxidative metabolism of drugs and environmental chemicals are CYP1, CYP2 and CYP3 (Figure 9).

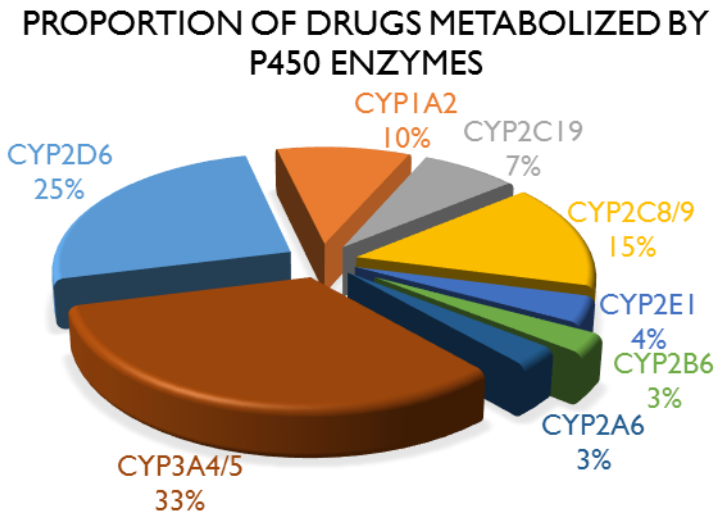


Figure 9 – The most important CYPs of Cytochrome P450[43] (Adapted from Liska, 1998).

6.1. CYP2D6

The CYP2D6, also known as debrisoquine/sparteine hydroxylase, is one of the most important and perhaps the most extensively studied drug metabolizing enzyme in humans[49]. It is responsible for the metabolism of 25-30% of all drugs on the market[20,50,51] such as antidepressants, neuroleptics, antiarrhythmics, analgesics, steroids, antiemetics, anticancer drugs and other xenobiotics[52,53].

Moreover, CYP2D6 substrates are lipophilic bases with a protonable nitrogen atom. The hydroxylation reaction takes place at a distance of 5 or 7 Å from the nitrogen atom. CYP2D6 has a very high affinity for alkaloids[20].

This enzyme, with 497 aminoacids, is encoded by the *CYP2D6* gene, which was the first P450 gene to be characterized at the molecular level and has a high clinical importance[20], because influence pharmacokinetic rate and drug effects[34].

The *CYP2D6* gene is located in chromosome 22, together with the two pseudogenes CYP2D7P and CYP2D8P, which are localized in tandem[20,47,54] (Figure 10).

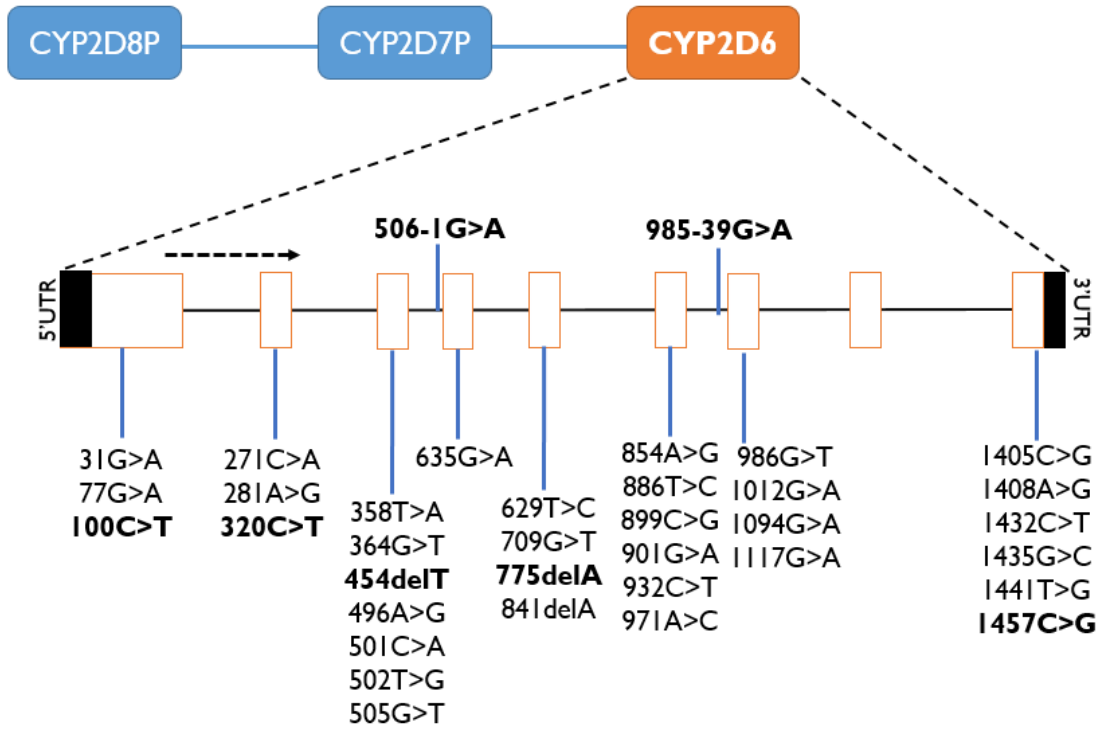


Figure 10 – The *CYP2D6* gene and most common polymorphisms (UTR=untranslated region)[55] (Adapted from Zhou *et al*, 2009).

Approximately 100 different alleles (Allele Nomenclature Database) of the *CYP2D6* gene have been identified, and their frequency varies between ethnic populations[56]. The enzymatic activities in individuals carrying these allelic differences vary from total absence to different degrees of ultrarapid metabolism, which causes considerable variability in the response to certain drugs treatments[31,47]. In addition, the UM phenotype is one explanatory factor for lack of response to antidepressants and decreased levels of several drugs which are CYP2D6 substrates, such as tramadol, antiemetics, venlafaxine, morphine and metoprolol are evident.

This gene is also expressed in small amounts in other tissues such as brain, especially in the midbrain, gastrointestinal tract and lungs[45,57].

It is very interesting that CYP2D6 polymorphisms have some relationship with personality traits (psychic anxiety, psychasthaenia, inhibition of aggression, and socialization) and so that is involved in the metabolism of endogenous psychoactive substrates or products, participating in the metabolism of some neuroactive amine and a possible influence on gender-related factors[58]. In humans, it is known that tyramine is one of the trace amines and is present in the brain, especially in the basal ganglia or limbic systems, which are thought to be related to personality and emotion[59]. Recent *in vivo* and *in vitro* animal studies support *CYP2D6* involvement in the biotransformation of endogenous substances, such as tyramine to dopamine, progesterone to its hydroxylated derivates and 5-methoxytryptamine (5-MT) to serotonin (5HT) (figure 11)[58,60,61].

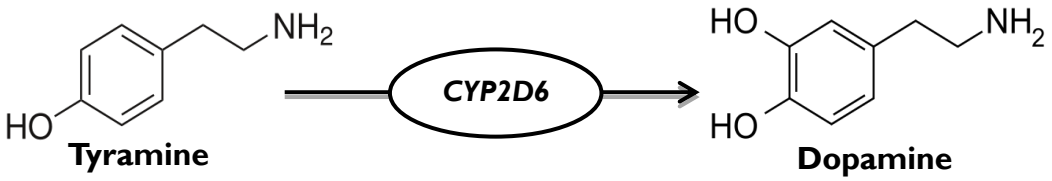


Figure 11 – Synthesis of dopamine by CYP2D6[35] (Adapted from Zhu *et al*, 2005).

Serotonin is implicated in the maintenance of psychological balance, and defects in its production are for example responsible for depression, impulsivity, obsessionality,

aggression, psychomotor inhibition, analgesia, hallucinations, eating disorders, attention and mood. In addiction, serotonin is converted into melatonin, and these two amines control the wake-sleep cycle[62,63].

Additionally, individuals with null CYP2D6 activity (Poor Metabolizers (PMs)) may present a potential reduction in regeneration of serotonin, as well as a slightly elevated dopamine tone due to the inverse correlation between the serotonin and dopamine systems, which may affect psychological process. Dopamine and serotonine are two key players in pain control and response[60].

6.1.1. CYP2D6 alleles

The functional impact of the various *CYP2D6* alleles can be classified into three groups: alleles resulting in increased activity; alleles resulting in decreased or loss of activity and alleles with normal or activity (Table 2).

Table 2 – Variant alleles of *CYP2D6* predicted activity and the metabolic profiles in correlation with genetic variants[64] (Adapted from The Human Cytochrome P450 (CYP) Allele Nomenclature Committee).

Allele Variant	Consequence	Predicted Activity	Phenotypes
***1**	2 functional genes	Normal	EM
***2**			
***3**	lacking functional enzyme due to defective or deleted genes	Null	PM
***4**			
***5**			
***6**			
***10**	1 functional and 1 defective allele but may also carry 2 partially defective alleles	Decrease	IM
***17**			
***41**			
(*1 or *2) x N	more than 2 active genes	Increase	UM

Most of the *CYP2D6* allelic variants are quite rare. Important variants in Caucasian population include *CYP2D6*2*, *CYP2D6*3*, *CYP2D6*4*, *CYP2D6*5*, *CYP2D6*6*, *CYP2D6*10*, *CYP2D6*17* and *CYP2D6*41*[64].

*6.1.1.1. CYP2D6*1*

The **1* allele corresponds to the *wild-type* variant. So, it is the more frequent in the general population, and corresponds to normal enzyme activity[55,65].

*6.1.1.2. CYP2D6*2*

The *2 allele has been described as having no functional impact in the metabolic profile. Enzyme activity is similar to that of the *wild-type* variant. The most relevant SNPs associated to this allele are c.900C>T transition in exon 6, leading to p.Arg296Cys change, and c.1457C>G variation in exon 9, resulting in p. Thr48Ser substitution[55].

*6.1.1.3. CYP2D6*3*

This variant contains a deletion of "A" in position 2,549 of the gene, in exon 5 (c.775delA), leading to a disrupted reading frame. So, this fact would readily explain the absence of CYP2D6 protein and function in PMs due to premature termination of protein synthesis[55].

*6.1.1.4. CYP2D6*4*

The *4 allele is the most common variant allele in Caucasians (allele frequency is 21%). This variant is characterized by one of seven SNPs: c.100C>T in exon 1; c.271C>A, c.281A>G and c.305C>G in exon 2; c.406G>C in exon 3; c.506-1G>A in

intron 3; c.1455 G>C in exon 9, resulting in, respectively, 4 aminoacid substitutions (p.Pro34Ser; p.Leu91Met; p.His94Arg; p.Ser486Thr), one silent variation and one splicing defect contributing to a null debrisoquine hydroxylation *in vivo*[66].

*6.1.1.5. CYP2D6*5*

The *CYP2D6*5* allele, is characterized by a deletion of the entire gene. The homozygous carriers totally lack metabolic activity due to total absence of CYP2D6 protein in liver[47]. It is present in 4% of Caucasian populations and it is considered to result from an unequal crossover between two chromatids, while repeated unequal crossover events of chromatids will lead to gene multiplication[55].

*6.1.1.6. CYP2D6*6*

The *6 allele is defined by c.454delT SNP allele in exon 3, a nucleotide deletion that causes a frame shift of one aminoacid (p.Trp152Glyfs) leading to a total loss of enzyme activity[55].

*6.1.1.7. CYP2D6*10*

CYP2D6*10 is probably the most common *CYP2D6* allele worldwide[20]. This allele has a c.100C>T SNP causing p.Pro34Ser substitution in the proline-rich region near the NH_2 terminal of the protein, which is highly conserved among CYPs of different species and is associated with a low *in vivo* sparteine clearance. The residue P34 may act as a hinge between the hydrophobic membrane anchor and the heme-binding region of the enzyme. The c.100C>T SNP resulted in an unstable protein with significantly reduction of enzyme activity[67].

*6.1.1.8. CYP2D6*17*

*CYP2D7*17* is generally considered to be a reduced function allele[68], and this is the major variant *CYP2D6* allele among Africans[20]. This variant has been classified according to the presence of four SNPs: c.320C>T in exon 2; c.406G>C in exon 3 (a silent SNP); c.884C>T in exon 6; and c.1455G>C in exon 9. Compared with CYP2D6*2, CYP2D6*17, it contains one more substitution (p.Thr107Ile). This change occurs in a region of the β'-helix that is conserved across species, and residue 107 may be involved in substrate recognition. The *17 allele has been associated with decreased debrisoquine hydroxylation in vivo[55].

*6.1.1.9. CYP2D6*41*

The *41 allele has been described as having reduced function. This variant is characterized by one of three main SNPs: c. 886C>A in exon 6; c.1457G>C in exon 9 and c.985+39G>A in intron 6, resulting respectively in one missense substitution, one splicing defect and one aminoacid substitution (p.Ser486Thr)[69].

6.1.1.10. Copy Number Variations (CNVs)

The CNVs are DNA segments at 1kb or larger with a variable number of copies in comparison with a reference genome. Besides, they can have dramatic phenotypic consequences as a result of altering gene dosage, disrupting coding sequences, or perturbing long-range gene regulation[52].

By the invention of new techniques that rapidly allow the analysis of full genomic occurrence of CNVs using Single Nucleotide Polymorphisms (SNP) genotyping arrays and clone-based comparative genomic hybridization, it was recently found that this is a very common phenomenon in the human genome. A total number of 1447 CNVs had been identified in the human genome, covering 360Mb (12% of the genome)[52].

Focus on enzymes involved in xenobiotic metabolism reveals that there are several well-known examples of CNV, such as *CYP2A6* and *CYP2D6*. Initially CNVs were thought to be very rare events but an increased number of functionally active genes was first described for *CYP2D6* and at the moment the maximum identified was 13 gene copies. *CYP2D6* gene can suffer duplications and even multiplications being more frequent in Africans than in Asians and north Caucasians. On the other hand, CNVs may also be deleted (*CYP2D6*5*) at a relatively high frequency in at least one ethnic group. Because of that, *CYP2D6* CNVs affect drug metabolism to a large extent, but do not crucially affect disease susceptibility, so a high frequency of CNVs is permitted[52].

6.1.2. Activity Score (AS)

The activity score is based on extensive genotype/phenotype comparisons and measurements of enzymatic activity and is used to approximate the phenotype for a given genotype in an individual. The scoring system, based on enzymatic activity, assigns a value of one for normal functioning alleles, and values of 0 or 0.5 for null and reduced functioning alleles, respectively. Duplicated alleles have their values counted twice, as determined to be valid in the derivation of the activity score. Phenotypic extensive metabolizers are classified as "high metabolizers" if their activity score is greater than 1.5. Therefore, the sum of the individual allele values formed the AS[70] and it is used to predict metabolic profile.

6.1.3. Metabolic profiles of CYP2D6

The high molecular multiplicity of *CYP2D6* gene, results in four major drug oxidation phenotypes which were termed poor (PM), intermediate (IM), extensive (EM) and ultrarapid metabolizers (UM) (Table 3). These phenotypes are associated with variable drug response, including ADRs, due to increased drug plasma concentration in PMs or therapeutic failure as a result of very rapid drug degradation in Ums[71].

Furthermore, the distribution of PM, IM, EM and UM of *CYP2D6* varies among ethnic groups[72].

6.1.3.1. Poor Metabolizer

This phenotype is characterized by lacking functional enzyme due to defective or deleted genes[52] and so that, PM individuals have no detectable enzymatic activity[32]. This condition can lead to an excessive or prolonged therapeutic effect or drug-related toxicity after a normal dose, conferring a genetic predisposition to drug-induced adverse effects. Moreover, in case of compounds that need to be activated, the PM condition may result in association with decreased response[73]. In Portugal, the frequency of PMs is 6.3%[56].

6.1.3.2. Intermediate Metabolizer

The IMs phenotypes located between the PM and EM, and are usually carrying 1 functional and 1 defective allele but may also carry 2 partially defective alleles[52]. So, IM-classified individuals show decreased enzymatic activity[27] In Portugal, the frequency of IMs is 32.7%[56].

6.1.3.3. Extensive Metabolizer

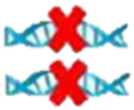
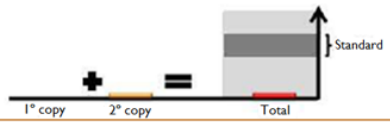
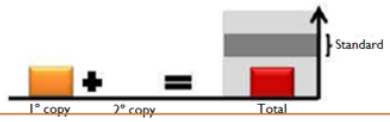
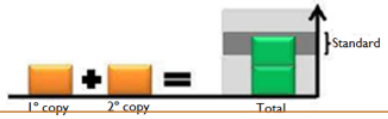
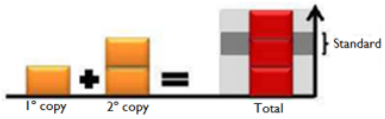
The EMs carry 2 functional genes[52] and they are the most common worldwide and also in the Portuguese population, with a frequency of 56.3%[56]. Therefore EM-classified individuals are characteristic of the normal population and have a wild-type copy of the gene[32].

6.1.3.4. Ultrarapid Metabolizer

This phenotype is characterized by the presence of more than two active *CYP2D6* alleles presenting a higher metabolic activity[52,74]. Thereby, UM-classified individuals typically contain multiple copies of a gene, which results in an increase in

drug metabolism[2]. UMs may have a hydroxylation capacity more than 100 times higher than PMs[60]. In Portugal, the frequency of IMs is 4.7%[56].

Table 3 – CY2D6 variability[56,75] (Adapted from Albuquerque *et al.*, 2013 and Prado *et al.*, 2009).

Genetic code	Enzimatic Activity	Phenotype	Allele	Predicted Activity
		PM (6.3%)*	*3 *4 *6	Null
		IM (32.7%)*	*10 *17 *41	Decreased
		EM (56.3%)*	*1 *2	Normal
		UM (4.7%)*	*>2xN	Increased

§ Portuguese caucasian population; N = number of alleles; PM = Poor Metabolizer; IM = Intermediate Metabolizer; EM = Extensive Metabolizer; UM = Ultrarapid Metabolizer.

Pain perception is one of the most complex quantifiable traits because it encompasses several phenotypes involving the peripheral and central nervous systems, and as a complex trait it is expected to have a polygenic nature shaped by environmental factors such as trauma, lifestyle, and stress. In addition, an important characteristic in determining the pain phenotype is the wide interindividual pharmacologic range in response to drugs. Therefore, not surprisingly, translating pharmacogenetics to clinical practice has been particularly challenging in the context of pain, due to the complexity of this multifaceted phenotype and the overall subjective nature of pain perception and response to analgesia. Yet, with a growing body of evidence demonstrating a strong association between severe acute pain and the risk for persistent pain, identifying individuals with an increased vulnerability to pain, including genetic factors, may allow to substantially improve clinical outcomes[33].

It is reasonable to think, from the text above, that CYP2D6 metabolic profile may influence pain and this is the novel hypothesis that has been explored in the present work.

7. Clinical impact

Estimates reveal that between 25-30% of all drugs in clinical use are metabolized at least in part by CYP2D6. Either they are metabolic inactivate, substrate or metabolic inducers, their clinical efficacy, safety and response will change drastically, according to *CYP2D6* polymorphisms, leading to heterogeneous ADRs[76].

Individuals with multiple gene copies will metabolize drugs more rapidly and therapeutic plasma levels will not be achieved at ordinary drug dosages. On the other hand, individuals lacking functional *CYP2D6* genes metabolize selective CYP2D6 substrates at a lower rate, and the risk for adverse drug reactions is higher[20].

For example, the pro-drug codeine is converted to morphine in the liver by the CYP2D6 enzyme. There are many genetic variations of *CYP2D6*, which affect the extent of this conversion in individuals. This leads to differences in the plasma levels of morphine and different levels of pain relief.

So that, the individuals with *CYP2D6* PM profile have no analgesic effect due to extremely low morphine plasma concentrations. Conversely, increased effectiveness of codeine with sometimes life-threatening opioid intoxication[9] was observed in patients with multiple *CYP2D6* gene copies, but also in neonates whose breastfeeding mothers were genetic *CYP2D6* UMs, consistent with higher rates of conversion to morphine in patients with UM phenotype[76].

Accordingly, due to the importance of *CYP2D6* gene, it is relevant to characterize different populations so that could become a routine as part of an "individualized" drug treatment[54].

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SCIENTIFIC PAPER

To be submitted to AJOG – *American Journal of Obstetrics & Gynecology*

Characterization of *CYP2D6* pharmacogenetics in morphine treatment of post-cesarean section pain: preliminary study

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Condensation:

This study investigates the association between the *CYP2D6* genetic variants defining metabolic profiles and pain scoring and/or the ADR pruritus, after cesarean section. The pharmacogenetic interpretation is discussed in order to stress out the clinical impact of the results.

Short version title:

CYP2D6 genotypes and post-cesarean morphine pain treatment.

Abstract

Objectives: Pharmacogenetic *CYP2D6* analysis, in 55 Portuguese Caucasian adult parturients undergoing analgesia with morphine after cesarean section, including the association with pain scoring and pruritus.

Study Design: DNA was extracted from peripheral blood of 53 Portuguese Caucasian adult parturients. Genetic analysis included allelic discrimination (*CYP2D6*1*, **2*, **3*, **4*, **5*, **6*, **10*, **17* and **41*) and copy number determination with TaqMan probes by Real-Time PCR. Allele duplications were confirmed by long PCR and PCR-Restriction Fragment Length Polymorphism (RFLP). Metabolic profiles were predicted based o genetic data and activity scores. The genotypes and metabolic profiles were correlated with pain scores and the disclosure of the ADR pruritus. The statistical analysis was performed by χ^2 test and results are considered statistically significant if $p<0.05$.

Results: The percentage of Poor Metabolizer, Intermediate Metabolizer, Extensive Metabolizer and Ultrarapid Metabolizer found are 9%, 38%, 46% and 7%, respectively. As expected, EM and IM are the most frequent metabolic profiles, as in Caucasian population. The results reveal a positive association with pain to alleles **2*, **4* and **10* and with pruritus to alleles **2* and **10*.

Conclusions: A positive association was found between CYP2D6 reduce activity and increased pain. It can be hypothesized that if CYP2D6 activity is reduced, tyramine metabolism is decreased, resulting in reduced formation of endogenous dopamine. Consequently the activation of signal transduction pathway that controls neuronal pain and analgesic effect may be reduced, leading to an increase of pain after cesarean.

A positive association was also found between CYP2D6 reduced activity and pruritus. However the exact mechanism of neuroaxial opioid-induced pruritus remains unclear.

Keywords: *CYP2D6*; Pain; Pharmacogenetics; Cesarean section; Labor analgesia.

1. Introduction

Pain is defined as a dynamic and complex process that involves actions at multiple sites ranging from the peripheral nociceptor to the genome of cells within the central nervous system to the patient's psychosocial milieu[32,77,78]. Individual variability to pain response and treatment is influenced by multiple factors including sensitivity to pain, neurochemical factors, age, gender, ethnicity, smoking, alcohol drinking, disease process and genetics.

A significant number of women experience moderate or severe acute post-partum pain after vaginal and cesarean deliveries. Furthermore, 10-15% of women suffer chronic persistent pain after cesarean section[79]. Therefore, are the mainstay for treatment of pain[80] and are used to provide analgesia with substantial interindividual variability in efficacy[81].

Morphine is the foremost opioid used for pain relief in the opiate family and has a high affinity and intrinsic activity for the miu-opioid receptor[82]. This drug is metabolized into morphine-6-glucuronide (M6G), normorphine and morphine-3-glucuronide; both morphine and M6G display opioid activity[33,34,52,83].

In current clinical practice, morphine is used for the management of acute and chronic pain and in analgesia of parturients undergoing cesarean. This opioid induced vasodilatory response and appeared to be mediated or modulated by both opioid receptor and histamine-receptor-sensitive pathways[84].

Over the past two decades, epidural and spinal opioids management to provide labor analgesia and treat postoperative pain have been increased[82]. Morphine is the main alkaloid of *Papaver somniferum* and is used as a potent therapeutic analgesic. It is also an opioid agonist, highly specific for miu-receptors located in the central nervous system and smooth muscle.

In pregnancy, morphine is used to alleviate pain associated with parturition[82]. This opioid activates the pathway mediate by opioids receptor in midbrain and terminates in the nucleus accumbens to end GABA inhibition of dopaminergic neurons, causing an increase in dopamine release, essential for pain control[85].

However, there is a large inter-individual variability in efficacy of and tolerance to opioids, namely onset of side effects like pruritus, nausea and vomiting.

Understanding the metabolism of opioid analgesics is important for studying of different sensitivities to analgesic therapy. The diversity of responses is due to several factors, mentioned above. Differences in the cytochrome P450 (CYP450) metabolizing capacity, caused by genetic polymorphisms, have a huge impact in individual response and in the development of adverse effects to drug therapy[20].

The CYP450 system is a superfamily coded by, so far known, 56 functional genes[86], including the *CYP2D6* that is located at the chromosome 22q13.1[87] and consists of nine exons with an open reading frame of 1,491 base pairs encoding 497 amino acids[55]. Besides, this highly polymorphic gene is responsible for the metabolism of about 25% of all drugs on the market and its polymorphisms significantly affect the metabolism of about 50% of the drugs[31,56,71,88].

The *CYP2D6* enzyme has been found in liver, heart and brain tissues and is also responsible for the oxidation metabolism of various drugs and endogenous substances, such as the tyramine and 5-hydroxytryptophan as precursors of dopamine and serotonin, respectively in brain [62,76].

Currently, there are more than 100 different allelic *CYP2D6* gene variants described to date on the Human Cytochrome P450 Allele Nomenclature Database and the number of alleles is still growing. In table 4, some examples of these alleles, influencing metabolic profiles, are described.

Because the high variability of *CYP2D6* gene, the enzyme activity can be classified as resulting in four major phenotypes that are described in table 4[64]. Note that the Ultra Metabolizer (UM) has more than 2 active functional genes[89].

Table 4 – Allelic variant and predicted metabolic profile.

Allele	***2**	***3**	***4**	***6**	***10**	***17**	***41**
Predicted activity	Normal	Null			Decreased		
Phenotypes	EM	PM			IM		

EM (Extensive Metabolizer), PM (Poor Metabolizer), IM (Intermediate Metabolizer).

However, populations with different ethnic origins, the pattern of *CYP2D6* polymorphisms and phenotype differs dramatically. The frequency of CYP2D6 UMs, EMs, IMs and PMs is approximately 3–5%, 70–80%, 10–17% and 5–10%, respectively, in Caucasians[55].

The clinical relevance of CYP2D6 genotyping to predict analgesic outcomes is still relatively unknown. In a recent pilot study, the relationship between CYP2D6 genotype, post-cesarean pains, codeine consumption, and side effects were evaluated. The two extremes in CYP2D6 genotypes seemed to predict pain response and/or adverse effects[33].

The present study aimed to analyze the *CYP2D6* genetic variations and copy number for predicting metabolic profiles in a population of Portuguese Caucasian adult parturients submitted to analgesia with morphine after cesarean section. Addictionally, an association analysis of CYP2D6**1*,**2*,**3*,**4*,**5*,**6*,**10*, **17* and **41* variants that modulating the activity of the enzyme and the efficacy of pain relief and emerging of pruritus, was also performed.

2. Material and Methods

Subjects

Blood samples were collected from 55 Portuguese Caucasian adult parturients followed at *Centro Hospitalar* e *Universitário de Coimbra* (mean age 32±4, range was 21–42 years).

All women were treated with 2.5mg of morphine for analgesia after cesarean section.

After surgery, pain, pruritus, nausea and vomiting were evaluated. To classify pain, was used an increasing scale of 0 to 10, was used in which 0 is absence of pain and 10 is the worst pain possible. For pruritus, it was used a 3 point scale, were 0=none, 1=mild, 2=moderate and 3=severe; nausea and vomiting was evaluated as "presence" or "absence". In terms of calculations, it was considered pain less or equal to 3 and more than 3. For pruritus, it was considered only the presence or the absence. The first score of pain and pruritus was measured immediately before administration of morphine, by epidural, and reassessment was performed after 4, 8

and 12 hours. Every woman was continuously monitored in the first 12 hours and hemodynamic parameter settings (pulse oximetry, respiratory frequency, pain scores, nausea, vomiting, pruritus, respiratory depression, urinary retention and level of consciousness) were evaluated.

This study had the approval of the University Hospitals of Coimbra Ethics Committee, following the Tenets of Helsinki Declaration, and informed consent was obtained from all participants.

Inclusion Criteria

Parturients older than 18 years; single or multiple pregnancies; cesarean section with regional anesthesia and American Society of Anesthesiologists (ASA) I/II.

Exclusion Criteria

Parturients who presented severe or chronic cardiovascular disease caused by pregnancy; patients with history of opioids consumption and pruritus during pregnancy; patients with allergy to local anesthetics or opioids and with contraindication for regional anesthesia.

DNA Extraction

The DNA used was extracted from lymphocytes of peripheral blood, using a standard phenol-chloroform method, followed by ethanol precipitation. Extracted DNA samples were stored at 4°C before use. The DNA concentration was determined by measuring the optical density at 260nm and quality was checked by calculation of optical densities ration at 260/280nm (NanoDrop, Thermo Fisher Scientific, Inc., Wilmington, USA).

CYP2D6 Genotyping by TaqMan Drug Metabolism Assays

All subjects were genotyped for **2* (rs1135840, c.1457C>G), **3* (rs35742686, c.775delA), **4* (rs3892097, c.506-1G>A), **6* (rs5030655, c.454DelT), **10* (rs1065852, c.100C>T) **17* (rs28371706, c.320 C>T) and **41* (rs28371725, c.985+39G>A) alleles,

using TaqMan Drug Metabolism Genotyping Assays (Applied Biosystems, Foster City, CA, USA). These SNPs allow to determine the *CYP2D6* alleles **1*, **2*, **3*, **4*, **5*, **6*, **10*, **17* and **41* and were selected according to its predicted effect on metabolic activity: *CYP2D6*1/*2* – normal; *CYP2D6*3/*4/*5/*6* – null; *CYP2D6*10/*17/*41* – decreased.

Genetic analysis were performed as previously described[56].

CYP2D6 Copy Number Analysis

CYP2D6 copy number variations were evaluated according to the standard procedure of the CEIBA consortium for the study of Population Pharmacogenetics in Iberoamerican Populations using TaqMan Copy Number Assay (Applied Biosystems, Foster City, CA, USA), as previously described[56].

Samples were run on Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA).

All samples were examined in triplicate and the average copy number values were determined, only for the samples presenting assay confidence above 95%. Data were analyzing using CopyCaller v.2.0 software version 2.0 (Applied Biosystems).

Whenever there was only one *CYP2D6* copy, due to deletion of the entire *CYP2D6* gene, the sample was characterized as *CYP2D6*5*[90].

For detection of the *CYP2D6*1*, **2*, **4* or **10* duplication alleles, DNA samples were analyzed by PCR and PCR-RFLP, as previously reported[44].

CYP2D6 genotype and theoretically predicted metabolic groups

The relationship between *CYP2D6* genotype and theoretically predicted metabolic groups was evaluated assigning a value concerning each variant *CYP2D6* allele, based on the "activity score" system, according to the strategy described previously[56,68,72]. The activity score is used to approximate the phenotype for a given genotype in an individual[70]. For the *wild-type* alleles (**1* and **2*) the scoring system assigned a value of 1, whereas for the null variant alleles (**3*, **4*, **4*xN, **5* and **6*) it was considered the value of 0; for the decreased variant alleles (**10*, **10*xN, **17* and

41) it was assigned the score of 0.5 for each active gene; the multiplication *CYP2D6*1*xN or *2xN alleles were scored with 2 points.

Statistical Analysis

Statistical analysis was performed with χ^2 or Fisher's exact tests, using contingency tables with software GraphPad Prism 5 (GraphPad Software , San Diego, CA, USA)[91]. A $p<0.05$ was considered as statistically significant.

For the calculation of adequate population size needed for this work, we have used the with a confidence level of 94% [103].

For the calculation of adequate population size needed for this work, according to the Portuguese population CENSUS95 we have used software Raosoft® Sample size calculator (Raosoft, Inc., WA, USA)[92], with a confidence level of 89.15%.

A minimum of 55 individuals were required for this study, taking into account the Portuguese population size (~10 million). The lower and upper limits of the 89% CI, for the proportions obtained in genotype determination, were calculated with the software VassarStats© (NY, USA)[93].

Enzyme hidroxylation capacity groups

Theoretical enzyme hydroxylation capacity prediction was based on genotype and was divided in four categories, as previously described[56] UM (more than 2 active alleles), EM (2 active alleles), IM (1 active allele) and PM (zero active alleles). Functionality of alleles was determined according to data available at the *CYP2D6* Allele Nomenclature Database[64].

3. Results

CYP2D6 genotype frequencies and copy number determination

All the *CYP2D6* allelic frequencies of the in the studied population (Table 5) are in accordance to those predicted by Hardy-Weinberg law, with exception of the *CYP2D6*17* and *CYP2D6*41* allele variant, possibly due to the scarcity of these rare variants in this population, which can cause deviations to the Hardy-Weinberg equilibrium. The percentage of individuals with no *CYP2D6* active alleles is 9.1% and with multiple active copies is 7.2% (Table 5).

Table 5 – Frequencies of *CYP2D6* genotypes among a Portuguese Caucasian adult parturients.

Genotype	Active Genes	Activity Score	n	Frequency	95% CI
***1/*1**	2	2	16	0.291	0.187 - 0.421
***1/*2**	2	2	1	0.018	0.003 - 0.096
***1/*4**	1	1	13	0.236	0.143 - 0.363
***1/*5**	1	1	1	0.018	0.003 - 0.096
***1/*6**	1	1	1	0.018	0.003 - 0.096
***1/*41**	2	1.5	4	0.073	0.028 – 0.172
***1x2/*1**	>2	>2	2	0.036	0.010 - 0.123
***1x3/*1**	>2	>2	2	0.036	0.010 - 0.123
***2x2/*4**	2	2	1	0.018	0.003 - 0.096
***2/*2**	2	2	3	0.055	0.018 – 0.148
***3/*4**	0	0	1	0.018	0.003 - 0.096
***4/*10**	1	0.5	1	0.018	0.003 - 0.096
***4/*17**	1	0.5	1	0.018	0.003 – 0.096
***4/*4**	0	0	4	0.073	0.028 - 0.172
***4/*41**	1	0.5	1	0.018	0.028 – 0.172
***41/*41**	1	1	3	0.055	0.018 – 0.148

Abbreviations: CI, confidence interval; n, number of subjects

CYP2D6 allelic variants frequencies

The most frequent alleles was *wild type* (*wt*) allele **1* (0.509). The frequency of allele **2* and **4* are 0.064 and 0.236, respectively. The alleles **3*, **5*, **6* **10* and **17* were found with the same frequencies (0.009) and allele **41* was found with frequency

of 0.100. The duplicated alleles were observed with a frequency of 0.045 for *1xN or for *2xN (Table 6).

Table 6 – Frequencies of *CYP2D6* variant alleles among the Portuguese Caucasian adult parturients.

	Pregnant Women (n=55)		
	Frequency	**n**	**95% CI**
***wt* (*1)**	0.509	56	0.417 - 0.600
***2**	0.064	7	0.031 - 0.125
***3**	0.009	1	0.001 - 0.049
***4**	0.236	26	0.166 - 0.323
***5**	0.009	1	0.001 - 0.049
***6**	0.009	1	0.001 - 0.049
***10**	0.009	1	0.001 - 0.049
***17**	0.009	1	0.001 - 0.049
***41**	0.100	11	0.056 - 0.170
Multiplications: (*1 or *2) x N	0.045	5	0.019 - 0.102

Abbreviations: CI, confidence interval; n, number of subjects; N, number of alleles; *wt*, *wild type*.

Metabolic Profiles

We have determined the frequencies of theoretical metabolic profiles in a Portuguese population of Caucasian adult parturients (Figure 12). The EM and IM profiles are more frequent in this population, as expected from other studies[55], 46% and 38%, respectively; the percentages of PM and UM are 9% and 7%, respectively. As we expected by comparison with population of women with fertile age (until 47 years old), it is considered statistically not significant (p=0.7513). However, in this population, the percentage of PMs and UMs was lower than Portuguese Caucasian adult parturients (5% and 8%, respectively) (Figure 13).

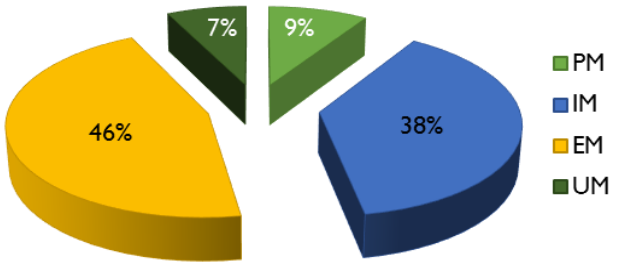

Figure 12 – Frequencies of metabolic profiles in a Portuguese Caucasian adult parturients. Abbreviations: PM, Poor Metabolizer; IM, Intermediate Metabolizer; EM, Extensive Metabolizer; UM, Ultrarapid Metabolizer.

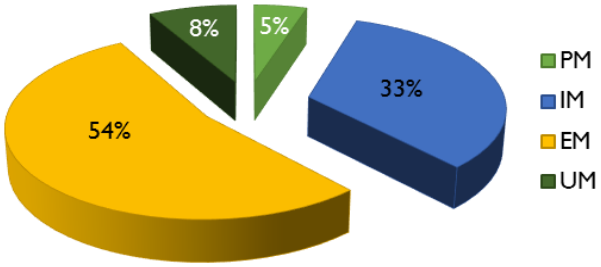

Figure 13 – Frequencies of metabolic profiles in a women Portuguese population (fertile age until 47 years). Abbreviations: PM, Poor Metabolizer; IM, Intermediate Metabolizer; EM, Extensive Metabolizer; UM, Ultrarapid Metabolizer.

Metabolic profiles according to the age of women, is presented in table 7.

Table 7 – Sample characterization.

	PM	IM	EM	UM
Portuguese Caucasian women controls (n=53)	3	21	27	2
Portuguese Caucasian parturients (n=55)	5	21	25	4
Maternal age (yrs):				
Median	33	32	32	31
Range	26-42	24-38	29-41	21-37

Abbreviations: PM, Poor Metabolizer; IM, Intermediate Metabolizer; EM, Extensive Metabolizer; UM, UltraRapid Metabolizer; n, number of subjects; yrs, years.

Women population vs parturients population

The table 8 is representative of the comparison between Portuguese Caucasian adult women and a Portuguese Caucasian adult parturients. For alleles **6* and **17*, it

comparison was not possible to perform because these allelic variants are absent in this population. The *41 allele variant was not determined in the normal population.

Table 8 – Comparison between Portuguese Caucasian Adult Parturients and a fertile population of Portuguese Caucasian Adult Women[56]. (Data from Albuquerque *et* 2013)

Populations	Alleles		Parturients Women	n	95% CI	Fertile Women§	n	95% CI
n			55			53		
Normal Alleles	*1		0.509	56	0.417 - 0.600	0.368	39	0.282 - 0.463
	*2		0.064	7	0.031 - 0.125	0.340	36	0.257 - 0.434
Defective Alleles	*3		0.009	1	0.001 - 0.049	0.028	3	0.010 - 0.080
	*4		0.236	26	0.166 - 0.323	0.189	20	0.127 - 0.274
	*5		0.009	1	0.001 - 0.049	0.019	2	0.005 - 0.066
	*6		0.009	1	0.001 - 0.049	0	0	0
Reduced activity alleles	*10		0.009	1	0.001 - 0.049	0.038	4	0.015 - 0.093
	*17		0.009	1	0.001 - 0.049	0	0	0
	*41		0.100	11	0.056 – 0.170	-	-	-
Multiplication Duplication	*1xN or *2xN	*1x2/*2 ou *1/*2x2 *1/*1x2 *2x3/*2 *2/*2x2	0.045	5	0.019 - 0.102	0.019	2	0.005 - 0.066

§Women between 15 and 47 years old

Abbreviations: CI, confidence interval; n, number of subjects; N, number of alleles.

Association genetic analysis

Pain

Samples of this study were classified according two groups of pain scoring: lower or equal to 3 and above 3 in all the measurements performed. Then it was analyzed the genotypic and allelic frequency according to different groups of pain. It was verified that in two genotype analysis, the results are only statistically significant for *2, *4 and *10. However, for allelic analysis, the results are not significant (Table 9).

Parturients with haplotype *CYP2D6*2* and that have pain above 3, 55% are heterozygous (CG) and in those that have pain less or equal to 3, 61% are homozygous to *wt* allele (CC).

Parturients with haplotype *CYP2D6*4* and pain higher than 3, are mainly heterozygous (GA) 52%; and those that have pain less or equal than 3, 11% are homozygous to *wt* allele (AA).

Finally, in parturients with haplotype *CYP2D6*10*, that have pain greater than 3, 48% are heterozygous (CT) and in those that have pain less or equal than 3, 75% are homozygous for *wt* allele (CC). All of the 3 *CYP2D6* haplotypes presented are statistically significant for differences between groups (p=0.007).

Due to the rare occurrence of the variants *CYP2D6*3*, *6 and *17,* the statistical analysis was not performed for these cases.

On the other hand, according to figure 14, its possible conclude that 56% of IMs+PMs present pain above 3 comparing with the other 36% of IMs+PMs parturients that present pain below 3.

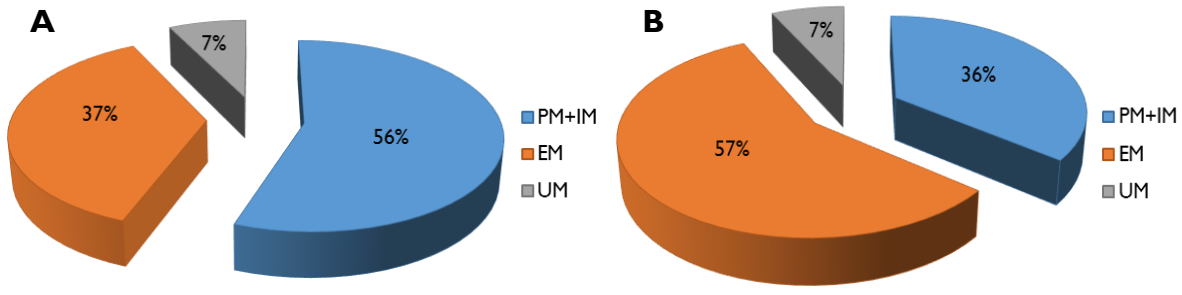

Figure 14 – Frequencies distribution of metabolic profiles according to development of pain. A-Pain>3; B-Pain≤3.

Pruritus

Samples of this study were classified according to the presence or absence of pruritus for any time of measurement after morphine administration. Then the possibility of analyzing the genotype and allele frequencies according to the two different groups. It was verified that results concerning *2, *4* and *10* alleles are statistically not significant for genotype analyzes; however for allele analyzes, the results are statistical significant for *2 and *10*; *p-value* is borderline for *4* (Table 9).

From the parturients with haplotype *CYP2D6*2* and with pruritus, 39% are homozygous for *wt* allele (CC) and in those without pruritus, 57% are homozygous for *wt* allele (CC).

Parturients with haplotype *CYP2D6*4* and with pruritus, are mainly homozygous (GG) 44%; and 67% without pruritus, are homozygous (GG).

Finally, in parturients with haplotype *CYP2D6*10*, having pruritus, 45% are homozygous for *wt* allele (CC) and in those without pruritus, 70% are homozygous (CC).

Due to the scarce number of the variants for *CYP2D6*3*, *6* and *17,* the statistical analysis was not performed in these cases.

According to figure 15, there are more EMs parturients with pruritus than without pruritus (46% and 50%, respectively).

Concerning nausea and vomiting, it was not possible to perform statistical analysis for none of the variants, because almost all results were nulls.

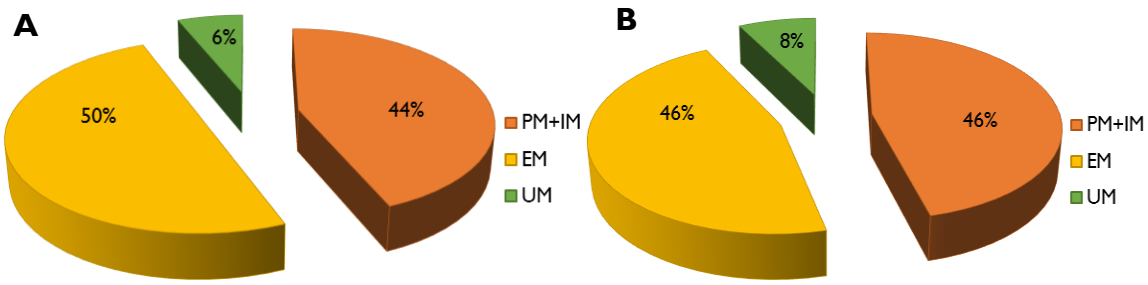

Figure 15 – Frequencies distribution of metabolic profiles according to development of ADR pruritus. A-Presence of pruritus; B-Absence of pruritus.

Table 9 – Genotypic and allelic frequency.

Genotype	Combined Allele	Predicted Activity	N	Pain ≤ 3	Pain >3	Presence of Pruritus	Absence of Pruritus	n	Allele	Pain ≤ 3	Pain >3	Presence of Pruritus	Absence of Pruritus
c.1457C>G (rs1135840)													
GG	*2	Normal	55	5 (0.18)	1 (0.03)	5 (0.28)	1 (0.02)	110	**G**	16 (0.29)	17 (0.31)	16 (0.44)	17 (0.23)
GC				6 (0.21)	15 (0.56)	6 (0.33)	15 (0.41)						
CC				17 (0.61)	11 (0.41)	7 (0.39)	21 (0.57)		**C**	40 (0.71)	37 (0.69)	20 (0.56)	57 (0.77)
p			***p***	****0.020***		****0.019***			***p***	*0.839*		****0.027***	
c.775delA (rs35742686)													
AA	*3	Null	55	27 (0.96)	27 (1.00)	17 (0.94)	37 (1.00)	110	**A**	55 (0.98)	54 (1.00)	35 (0.97)	74 (1.00)
ADel				1 (0.04)	0 (0.00)	1 (0.06)	0 (0.00)						
DelDel				0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)		**Del**	1 (0.02)	0 (0.00)	1 (0.03)	0 (0.00)
p			***p***	–		–			***p***	*1.000*		*0.327*	
c.506-1G>A (rs3892097)													
GG	*4	Null	55	21 (0.75)	12 (0.44)	8 (0.44)	25 (0.67)	110	**G**	46 (0.82)	38 (0.70)	23 (0.64)	61 (0.82)
GA				4 (0.14)	14 (0.52)	7 (0.39)	11 (0.30)						
AA				3 (0.11)	1 (0.04)	3 (0.17)	1 (0.03)		**A**	10 (0.18)	16 (0.30)	13 (0.36)	13 (0.18)
p			***p***	****0.011***		*0.098*			***p***	*0.180*		*0.054*	
c.454delT (rs5030655)													
TT	*6	Null	55	27 (0.96)	27 (1.00)	17 (0.94)	37 (1.00)	110	**T**	55 (0.98)	54 (1.00)	35 (0.97)	74 (1.00)
TDel				1 (0.04)	0 (0.00)	1 (0.06)	0 (0.00)						
DelDel				0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)		**Del**	1 (0.02)	0 (0.0)	1 (0.03)	0 (0.00)
p			***p***	–		–			***p***	*1.000*		*0.327*	

Genotype	Combined Allele	Predicted Activity	N	Pain ≤ 3	Pain >3	Presence of Pruritus	Absence of Pruritus	n	Allele	Pain ≤ 3	Pain >3	Presence of Pruritus	Absence of Pruritus
c.100C>T (rs1065852)													
CC	*10	Decrease	55	21 (0.75)	13 (0.48)	8 (0.45)	26 (0.70)	110	**C**	45 (0.80)	39 (0.72)	2(0.61)	62 (0.84)
CT				3 (0.11)	13 (0.48)	6 (0.33)	10 (0.27)						
TT				4 (0.14)	1 (0.04)	4 (0.22)	1 (0.03)		**T**	11 (0.20)	15 (0.28)	14 (0.39)	12 (0.16)
p			***p***	*****0.007***		****0.034***			***p***	*0.363*		**0.016*	
c.320 C>T (rs28371706)													
CC	*17	Decrease	55	28 (1.00)	26 (0.96)	18 (1.00)	65 (0.97)	110	**C**	50 (1.00)	52 (0.96)	36 (1.00)	72 (0.97)
CT				0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)						
TT				0 (0.00)	1 (0.04)	0 (0.00)	1 (0.03)		**T**	0 (0.00)	2 (0.04)	0 (0.00)	2 (0.03)
p			***p***	–		–			***p***	*0.239*		*1.000*	
c.985+39 G>A (rs28371725)													
GG	*41	Decrease	55	23 (0.82)	23 (0.85)	18 (1.00)	28 (0.76)	110	**G**	46 (0.82)	49 (0.91)	36 (1.00)	59 (0.80)
GA				0 (0.00)	3 (0.11)	0 (0.00)	3 (0.08)						
AA				5 (0.18)	1 (0.04)	0 (0.00)	6 (0.16)		**A**	10 (0.18)	5 (0.09)	0 (0.00)	15 (0.20)
p			***p***	0.059		0.073			***p***	*0.268*		**0.002*	

Legend: n = number of parturients; *p<0.05; **p<0.01.
Note: To *3, *6 and *17 it was not possible calculate the p-value because one row is filled with zeros is in the contingency table. In this situation, chi-square analysis is impossible. At least one of these values must be positive.

4. Comment

In this study, we analyze the *CYP2D6* gene, in a sample of 55 Portuguese Caucasian adult parturients, to whom morphine was given morphine for analgesia after cesarean section.

The definition of a specific haplotype for this gene, through the seven variations studied g.997C>G (rs1135840, c.1457C>G), g.2549DelA (rs35742686, c.775delA), g.1846G>A (rs3892097, c.506-1G>A), g.1707DelT (rs5030655, c.454DelT), g.100C>T (rs1065852, c.100C>T) and g.1023C>T (rs28371706, c.320 C>T) g.2988G>A (rs28371725, c.985+39G>A) and the phenotype approach is very closely compared to the profile of an individual's metabolism.

According to the results, only 3 alleles were statistical significant in this study: *2 (rs1135840, c.1457C>G), **4* (rs3892097, c.506-1G>A) and **10* (rs1065852, c.100C>T).

From the present results, it is possible to observe that the most frequent *CYP2D6* combined allele was the normal and functional allele, *wt* or **1*, followed by allele **4*. We have also confirmed the presence of alleles **10*, **17* and **41* in a Portuguese population, which was expected due to the knowledge of existing Asian and African ancestors, respectively[56,94]. The PM frequency observed in parturients (5%) is in agreement with previous studies that estimate 5-10% of PM in the European Caucasian population[56,94]. Accordingly, it is estimated that, taking into account the number of women estimated by CENSUS 2011 (5,515,578)[95] there are approximately 275,778 PMs in Portugal.

The UM frequency observed in parturients is 0.073 (8%) and is slightly higher than the previous studies which is between 3-5%[49]. According to the Portuguese populations CENSUS, we estimate that there are approximately 441,246 UM in Portugal.

Comparing data of this population of parturients with the women Caucasian in general population, it is possible to conclude that the IMs and EMs are very similar in both groups. However, there are more PMs and UMs in the parturients (9% and 7%, respectively) comparing with women Caucasian, population (5% and 8%, respectively) but differences are not significant (Figure 12).

After analysis of this sampling, it was analyzed the alleles *2, *4 and *10 for genotype, allele and their metabolic profile, according to pain scores and presence or absence of pruritus, were analyzed.

Concerning the results of genotypes and pain scoring, it was verified that all 3 alleles present statistical differences for pain scoring. The pain score is higher in parturients that are heterozygous for *4 and *10 frequency, probably due to an enzyme reduction function.

The comparison between predicted metabolic phenotype and pain does not present any statistical significant results.

To explain the continuation of pain after morphine administration, there are some theories discuted in literature.

According to Timothy S. Tracy *et al.*[96], pregnancy is a dynamic state during which a number of physiologic and biochemical changes occur. Furthermore, the extent of these changes may be affected by the stage of pregnancy. CYP2D6 metabolizes several drugs that are used clinically in the care of parturients and has been reported that the metabolism of these drugs increase significantly during pregnancy, compared with the post-partum period. The increase of CYP2D6 activity throughout the course of pregnancy is considering the generally held notion that CYP2D6 is not inducible, at least by xenobiotics. The most obvious potential inducing substance would be the elevated endogenous hormones (e.g., estrogen and progesterone) that occur during pregnancy. However, there are not data to suggest that these hormones can induce CYP2D6 activity, which suggests that other endogenous compounds may be responsible for the observed induction of CYP2D6.

CYP2D6 is highly polymorphic and plays an important role in the hepatic metabolism of therapeutic agents including drugs affecting the CNS (Central Nervous System) and other xenobiotics. *CYP2D6* is also expressed in the human brain. Furthermore, recent *in vitro* and *in vivo* animal studies support CYP2D6 involvement in the biotransformation of endogenous substances such as 5-methoxyindolethylamine O-demethylase potentially contributing to regeneration of serotonin from 5-methoxytryptamine (5MT). As dopaminergic neurotransmission is regulated by serotonin, CYP2D6 may exert a nuanced (serotonergic) influence on dopaminergic tone in the pituitary. The 5MT exists in the brain including in the serotonergic raphe

nucleus and the pineal gland. Dopamine, another neuroactive amine, is thought to be formed in part by hydroxylation of tyramine via CYP2D6 intervention. The total amount of CYP2D6 in the brain is much lower than in the liver, and tyramine derived from food does not cross the blood–brain barrier. The concentration of dopamine in the brain is high, since the classic biosynthetic route to dopamine from tyrosine is very active. However, tyramine can be formed in the brain, especially in the basal ganglia, via the aromatic hydroxylation of phenylethylamine or via tyrosine decarboxylation to give tyramine, which is a CYP2D6 substrate for dopamine synthesis[60,61,97].

Accordingly to Lledó *et al*[60], CYP2D6 PMs, who have null enzyme capacity, appear to present a reduction in regeneration of serotonin, which in turn may cause a slightly elevated dopamine tone due to the inverse correlation between the serotonin and dopamine systems. Anatomical and pharmacological evidence suggests that the dorsal raphe serotonin system and the ventral tegmental and substancia nigra dopamine system may act as mutual opponents for some psychological functions.

Pharmacological studies show that dopamine is involved in activating behaviors that serotonin inhibits and vice-versa. As previously described in literature, serotonin is implicated in the maintenance of psychological balance, and malfunction in its production is for example responsible for depression, aggression, impulsivity, obsessionality, analgesia, hallucinations, attention and mood[63].

Dopamine neurotransmission has an important influence on outcome prediction, attention, response inhibition, and motivation, as well as affective symptoms associated with anxiety and depression[24].

It is known that the action of morphine in the CNS promotes the activity of the dopaminergic neurons by the increase of release of dopamine to the synaptic gap to control pain.

So it possible to conclude that the haplotypes resulting in absence on reduction of enzyme function are positively correlated with higher pain scores (Figure 14). This is possibly related to a decrease of dopamine synthesis by tyramine pathway. Consequently, the activation of signal transduction pathway that controls neuronal pain and analgesic effect may be reduced, leading to an increase of pain after cesarean. However, this is only verified if we consider the PM and IM together because the

number of samples is low, so it is necessary to take into account the preliminary nature of this work.

Thus, subjects with partial or null function of CYP2D6 enzyme could not metabolize tyramine effectively, leading to decreased of dopamine content. Consequently, the activation of signal transduction pathway that controls neuronal pain and analgesic effect may be reduced, leading to increased of pain after cesarean section.

The results by genotype show that there are not statistical differences for presence or absence of pruritus for *2, *4* and **10* allele. It was demonstrated that there are an increased number of homozygous of *wt* allele comparing to heterozygous. Analysis by allele, show that there are statistical significant results for alleles *2 (rs1135840, c.1457C>G) and to **10* (rs1065852, c.100C>T). For **4* (rs3892097, c.506-1G>A), there is not significance (p=0.0985). This results show that parturients with pruritus have higher prevalence of variant allele, meaning that allelic variation influence the emerging of ADRs.

Comparison between PM phenotype *vs* others phenotypes and pruritus, shown that there are significant differences (p = 0.0350). This demonstrates that a higher number of PM parturients have pruritus comparing with PM parturients. In figure 15 it is possible to observe that 44% of parturients from the set of PM and IM have pruritus comparing with 46% of PM and IM set parturients with absence of pruritus.

This present study is relevant because there is no data in literature that relates the presence of pruritus with metabolic profiles associated to *CYP2D6* gene. However the scientific knowledge about this side effect is limited. Pruritus is an important symptom of many skin, systemic and autoimmune diseases, a troubling side effect of a number of medications, like morphine[41]. By definition, it is a sensation that provokes the uncontrollable need to scratch and can be aroused by a variety of mechanical, electrical and chemical stimuli. The exact mechanism of neuroaxial opioid-induced pruritus remains unclear. Postulated mechanisms include the presence of an "itch center" in the central nervous system, medullary dorsal horn activation, and antagonism of inhibitory transmitters. Modulation of the serotoninergic pathway and involvement of prostaglandins may also be important in the etiology of neuroaxial opioid-induced pruritus. Prostaglandins (PGE_1 and PGE_2) enhance C fiber transmission

to the CNS and they are also known to release histamine and potentiate pruritus induced by histamine[98,99].

Histamine, and compounds which release peripheral histamine from mast cells, are the primary experimental itch mediators in humans[41].

The reported incidence of pruritus varies between 30% and 100%. Parturients of the present work appear to be more susceptible, with an incidence between 60% and 100%. This increased incidence may be due to an interaction of estrogen with opioid receptor. The treatment of intrathecal and epidural opioid-induced pruritus remains a challenge. However, many pharmacological therapies, including antihistamines, 5-HT3-receptor antagonists, opiate-antagonists (ex. intravenous naloxone), propofol, nonsteroid antiinflammatory drugs, and droperidol, have been studied. In the present study, when women had severe pruritus, it was administrated naloxone that can reduced or reverse morphine-induced pruritus in 80%, according to Jeon *et al*[40].

Morphine produces part of its analgesic effect through the release of serotonin. There are dense concentrations of serotonin receptors in the dorsal part of spinal cord and the nucleus of the spinal tract of the trigeminal nerve in the medulla opioid density is also high. These observations suggest that the 5-HT3 receptor may be implicated in the development of the pruritus associated with administration of neuroaxial opioids[41,67].

However, according to Green *et al*[41], serotonin is a powerful pruritic agent in mice, but it appears that there are important differences between rodents and humans in the relative involvement of histamine and serotonin in the mediation of itch.

Comparing pruritus and pain by phenotype, it was concluded that there are not statistically significant results, suggesting that there is not a correlation between both parameters.

5. Conclusions

This study allowed the setting the *CYP2D6* pharmacogenetic analysis in a population of Portuguese Caucasian adult parturients and it relation with pain scores and the presence or absence of pruritus.

According to the results, it is possible to conclude that the haplotypes that result in absence and reduction of enzyme function are positively correlated with higher pain scores. This is possibly related to a decrease of dopamine synthesis by tyramine pathway. Consequently, the activation of signal transduction pathway that controls neuronal pain and analgesic effect may be reduced, leading to an increase of pain after cesarean. However, the exact mechanism of neuroaxial opioid-induced pruritus remains unclear.

It was also analyzed the homogeneity of frequencies in the Portuguese Caucasian women, with the same range of ages. Interindividual differences in drug disposition are important causes for ADRs and lack of drug response. However, in future, it is necessary to increase the number of samples in order to obtain a better confidence level of analysis.

There is no doubt that genetic variants affect drug responses to an extent that can have relevant implications beyond just the efficacy of a prescribed drug. For the clinician, and in particular for the anesthesiologist providing anesthesia and post-operative pain management, there are to date no guidelines or recommendations that suggest any pharmacogenetic testing prior to administering any anesthesia-related drug.

Pharmacogenetics is a rapidly developing field. Faster and cheaper methods of genotyping will be available in the near future and this will enable cost-effective preoperative testing of parturients[88] in order to improve drug safety, dosage recommendations, decreased of pain and pharmacovigilance programs which would benefit millions of parturients worldwide.

This work contributes to a better understanding of how the CYP2D6 allelic variants may affect pain (and pruritus) and bring light to the treatment of labor analgesia. However, further studies with a higher number of parturients will be necessary to confirm the present results.

6. Future directions

The scientific developments in pharmacogenomics, and the potential clinical and economic impact of this area, will eventually make genotyping a clinical practice routine. Before widespread clinical use becomes a reality, a host of pharmacogenomics-based clinical trials will be carried out[13].

The future impact of PGx is likely to be considerable both in the selection of the right drug at the proper 'individual' dose and in the prevention of adverse effects. By translating the increasing knowledge of human genetic diversity into better drug treatment, improved health through personalized therapy remains a realistic future scenario in many fields of medicine[100].

The goal of individualized medicine is to prescribe an appropriate medication to the right target of the disease, at the right dose, to achieve maximal therapeutic benefit with minimal, tolerable ADRs. However, good clinical data to support the use of genetic testing for drug treatment for most diseases are still not available[10].

On the other hand, pharmacogenomic polymorphisms are definitely important in interindividual variability in the analgesic effects and occurrence of ADRs of commonly used medications, but genetic factors will provide only a partial answer to this interindividual variability. Other factors, including biological variations (ethnicity, age and gender), environmental factors (smoking status), co-morbidity and co-medications (potential for drug-drug interactions) must be considered, because is the synergestic effect that also influence the pharmacokinetics and pharmacodynamics of medications used for pain management[32].

In the near future, pharmacogenomic approaches in pain management could lead to individualized therapy to best select the appropriate analgesic from the onset to provide sustained efficacy with the lowest side effect profile[32].

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"It's far more important to know what person the disease has than what disease the person has."

Hippocrates