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MOLECULAR DETERMINANTS OF THE RESPONSE TO MALARIA THERAPEUTICS

(Tese para a obtenção do grau de doutor no ramo de Bioquímica, especialidade de Biologia Celular e Molecular)

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TÍTULO DA TESE:

"Determinantes moleculares da resposta à terapêutica na malária"

RESUMO

O objectivo geral desta tese foi a análise da variabilidade genética em genes que codificam proteínas importantes no metabolismo de xenobióticos. A análise do metabolismo de antimaláricos, em particular da amodiaquina, e a possível contribuição da biotransformação para a susceptibilidade para a doença foi também avaliada.

Os resultados obtidos na análise de genótipos em populacões da Guiné-Bissau, Portugal, São Tomé e Príncipe, Tailândia e Zanzibar revelaram uma grande variabilidade inter-étnica. Uma grande variabilidade foi também observada entre as diferentes populacões Africanas. O estudo dos genótipos *CYP2C8* em indivíduos tratados com amodiaquina revelou que nenhum dos alelos analisados influencia a eficácia da terapêutica.

Na análise do metabolismo da amodiaquina estudou-se em particular o metabolito desetilamodiaquina. Utilizando microssomas enriquecidos com CYPs recombinantes, foi observada uma redução na concentração de fármaco na presença do CYP1A1. A procura de variabilidade genética no *CYP1A1* identificou vários SNPs, em particular numa população da Papua Nova Guiné. Porém, esta variabilidade não se mostrou associada aos extremos observados nos parâmetros farmacocinéticos.

O estudo da influência do genótipo "null" *GSTT1* na malária não-severa revelou que a delecção do gene poderá constituir um factor protector para a doença.

Palavras-chave: Farmacogenética, Malária, Amodiaquina, Desetilamodiaquina, Metabolismo de xenobióticos

TÍTULO DA TESE EM INGLÊS:

"Molecular determinants of the response to malaria therapeutics"

ABSTRACT

The overall aim of this thesis was the analysis of the genetic variability in genes coding for proteins important in drug metabolism. The analysis of the metabolism of antimalarial drugs, particularly amodiaquine, and the possible contribution of biotransformation for the susceptibility to the disease was also studied.

The results obtained in the genotyping studies in the populations of Guinea-Bissau, Portugal, Sao Tome and Principe, Thailand and Zanzibar showed a high interethnic variability. A high variability in Africans was observed. The study of the effect of *CYP2C8* genotype in the AQ treatment outcome revealed that none of the alleles analysed influence therapeutic efficacy.

In the analysis of amodiaquine metabolism we focused particularly on the metabolite desethylamodiaquine. Using microsomes enriched with recombinant CYPs, a reduction in drug concentration was observed in the presence of CYP1A1. The posterior investigation of the genetic variation in *CYP1A1* identified several SNPs, particularly in the Papua New Guinea population. However, this variability could not be associated with the extreme variability observed in pharmacokinetic parameters.

The study on the influence of the *GSTT1* null genotype in uncomplicated malaria revealed that the lack of this gene seems to be a protective factor in this disease.

Keywords: Pharmacogenetics, Malaria, Amodiaquine, Desethylamodiaquine, Drug metabolism

ABBREVIATIONS

ABC – ATP-Binding Cassette

ACT – Artemisinin based Combination Therapy

AhR – Aryl hydrocarbon receptor

AIDS – Acquired immunodeficiency syndrome

AL – Artemether + Lumefantrine

AQ – Amodiaquine

ARNT – Aryl hydrocarbon receptor nuclear translocator

ART – Artemisinin

AS – Artesunate

ATP – Adenosine 5'- triphosphate

AUC – Area under the plasma concentration-time curve

CAR – Constitutive androstane receptor

CI – Confidence Interval

CQ – Chloroquine

CYP – Cytochrome P450

G6PD - Glucose-6-phosphate dehydrogenase

DBD – DNA-binding domain

DEAQ – Desethylamodiaquine

DOTS - Direct Observed Treatment Strategy

GSH - Glutathione

GST – Glutathione S-Transferase

HIV – Human immunodeficiency virus

HPLC – High Performance Liquid Chromatography

INH – Isoniazid

LBD – Ligand-binding domain

LUM – Lumefantrine

MDR – Multidrug Resistance

MRP – Mutidrug Resistance Protein

NADPH – Nicotinamide-adenine dinucleotide phosphate (reduced)

NAT – *N*-Acetyltransferase

OR – Odds Ratio

ORF – Open Reading Frame

PDB – Protein database

PCR – Polymerase Chain Reaction

PfATP6 – *Plasmodium falciparum* Calcium-dependent ATPase

P-gp – P-glycoprotein

PK - Pharmacokinetic

PRR – Parasite Reduction Ratio

PXR – Pregnane X receptor

RBC – Red Blood Cells

RFLP – Restriction Fragment Length Polymorphism

ROS – Reactive oxygen species

SERCA – Sarco/Endoplasmic Reticulum Calcium-dependent ATPase

SNP – Single Nucleotide Polymorphism

SP – Sulphadoxine-Pyrimethamine

STP – Sao Tome and Principe

TB – Tuberculosis

WHO – World Health Organization

XMEs – Xenobiotic-Metabolizing Enzymes

XRTs – Xenobiotic-related transporters

LIST OF PUBLICATIONS

This thesis is based on the following articles, which are referred to in the text by Roman numerals:

I. **Cavaco, I**, Gil, JP, Gil-Berglund, E, Ribeiro, V. *CYP3A4* and *MDR1* alleles in a Portuguese population. *Clin Chem Lab Med*; 2003; 41: 1345 – 1350.

II. **Cavaco, I**, Reis, R, Gil, JP, Ribeiro, V. Cytochrome P450 3A4 and N-acetyltransferase 2 genetic polymorphism in a native African population. *Clin Chem Lab Med*; 2003; 41: 606 – 609.

III. **Cavaco, I**, Strömberg-Nörklit, J, Kaneko, A, Msellem, MI, Dahoma, M, Ribeiro V, Björkman A, Gil, JP. *CYP2C8* polymorphism frequencies among malaria patients in Zanzibar. *Eur J Clin Pharmacol*; 2005; 6: 15 – 8.

IV. **Cavaco, I**, Piedade, R, Gil, JP, Ribeiro, V. *CYP2C8* polymorphism among the Portuguese. *Clin Chem Lab Med* 2006; 44(2): 168-170.

V. **Cavaco, I**, Piedade, R, Martins, JP, do Rosário, V, Gil, JP, Ribeiro, V. *CYP2C8* SNP profile of four malaria endemic populations. Submitted.

VI. **Cavaco, I**, Martensson, A, Ribeiro, V, Björkman, A, Gil, JP. CYP2C8 polymorphisms and efficacy of amodiaquine therapy. Manuscript.

VII. Piedade, R, **Cavaco, I**, do Rosário, V, Gil, JP, Ribeiro, V. *CYP3A5*3* is associated to polymorphisms in the pregnane X receptor in a degree that depends on ethnicity. Manuscript.

VIII. **Cavaco, I**, Martensson, A, Bhattarai, A, Ribeiro, V, Björkman, A, Gil, JP. Glutathione-S-Transferase M1 and T1 polymorphisms – association with uncomplicated malaria. Manuscript.

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INTRODUCTION

General Background

The first description of individual idiosyncratic reactions to xenobiotics goes back to the year of 510 B.C. when Pythagoras identified potentially fatal reactions in some individuals after the ingestion of fava beans (Nebert, 1999). The cause of this adverse reaction was identified in 1956 when African American US Army soldiers had adverse effects after the intake of the antimalarial drug primaquine and during a high altitude flight. These were attributed to a deficiency in erythrocyte glucose 6-phosphate dehydrogenase (G6PD) activity (Carson *et al.*, 1956).

The concept behind the variability observed was **pharmacogenetics**, defined in 1959 by Friedrich Vogel as the “study of the role of variability in drug response due to heredity” (Vogel, 1959; Nebert, 1999).

Pharmacogenetics related to drug metabolism is an important field of research, but to understand the processes that follow drug administration it is also necessary to know what happens in terms of **pharmacokinetics** and **pharmacodynamics.** Pharmacokinetics describes the process of absorption, distribution, metabolism and excretion of a drug in the body. Pharmacodynamics describes the pharmacological effect of a drug in the body or disease causing pathogen and, subsequently, the effect on the clinical phenotype.

Pharmacokinetic Aspects

Drugs must enter the bloodstream after administration to be distributed to the site of action. **Absorption** is defined as the process by which unchanged drug proceeds from the site of administration to the site of measurement within the body (Rowland and Tozer, 1995). Absorption is not an intervening factor if the drug is given intravenously, but in the case of oral ingestion, for example, the drug needs to be absorbed from the gut. The absorption process mostly happens though passive diffusion where no energy is required for the process, but active transporters can also be involved and this is an energy-dependent process across the membrane. The extent of absorption determines the fraction of a dose that gains access to the circulation (**bioavailability**). For some drugs bioavailability may be influenced by metabolism in the gut and liver before reaching the systemic circulation, since these tissues are sites of elimination. This process reduces the amount of drug reaching the systemic circulation and is called **first-pass effect** (Rowland and Tozer, 1995).

When the drug reaches the systemic circulation, it is distributed throughout the body into different conceptual body compartments (e.g. plasma water, interstitial fluid, intracellular water or fat). The **distribution** is dependent on the lipid solubility of the drug, concentration in plasma and in various tissues, permeability of tissue membranes, blood perfusion and binding of the drug to plasma proteins and/or transport proteins. The binding is a reversible process with the unbound drug usually being the actual pharmacologic action agent (Buxton, 2006).

Elimination is the irreversible loss of drug from the site of measurement and can occur by two processes, excretion and metabolism (Rowland and Tozer, 1995). **Excretion** is the elimination of the drug in the unchanged form. **Metabolism** is the process by which a drug undergoes biotransformation normally with the objective of

reducing lipid solubility and increasing the possibility of further elimination. Metabolism is also normally referred as **biotransformation** and is the major mechanism for elimination of drugs from the body (Rowland and Tozer, 1995).

The biotransformation process occurs mainly in the liver, being performed by **Xenobiotic-Metabolizing Enzymes (XMEs)**, and is generally divided in **Phase I** and **Phase II** (Williams, 1959; Nebert and Dalton, 2006) (see section ***Drug Clearance and Infectious Diseases***). Phase I reactions expose or introduce a functional group (e.g. –OH, -COOH, -SH, -NH_2) by oxidation, reduction or hydrolysis, normally creating more reactive products. Several enzymes are involved in this Phase I process, e.g. alcohol and aldehyde dehydrogenases, carboxyesterases, epoxide hydrolase and monooxygenases, being the Cytochromes P450 (CYP) the most important among them. Phase II reactions involves coupling of the drug or its polar metabolite with endogenous substrates such as glucuronic acid, sulfate, amino acids, acetyl groups, methyl groups and the tripeptide glutathione. Examples of enzymes involved in these reactions are the UDP-glucuronosyl transferases (UGT), sulfotransferases (SULT), catechol-*O*-methyltransferase (COMT), *N*-acetyltransferases (NAT), and glutathione S-transferases (GST).

Biotransformation is generally a detoxification process, but sometimes the metabolic product is more toxic than the initial compound. This enzymatic formation of more reactive metabolites is termed bioactivation. The bioactivation is important in the cases when the administered drug is an inactive prodrug that is converted into a pharmacologically active species.

In addition to metabolizing enzymes, **transport** across biological membranes is important in drug disposition. In recent years, the **xenobiotic-related transporters (XRTs)** have been proposed and/or demonstrated to have a role in regulating the

absorption, distribution and excretion of many compounds (Eichelbaum *et al.*, 2006; Nebert and Dalton, 2006). Nonpolar compounds can pass through the lipid bilayer and cross the membrane unassisted, but a membrane protein for transmembrane movement is usually necessary in the case of polar or charged compounds. The organic anion transporters (OATP, SLC21A) are an example of uptake carriers and the ATP-binding cassette (ABC) superfamily of membrane proteins are an example of efflux transporters. The transport proteins allow the conceptual definition of a **Phase III** of drug elimination (Ishikawa, 1992), where the products, normally of phase II (conjugates), are effluxed out of the cytosol.

All these biotransformation processes are known to display inter-individual variability due to genetic polymorphisms that are associated to variable plasma levels of the drug.

Pharmacodynamic Aspects

Pharmacodynamics describes the effect of the drug – or its active metabolite – in the body though its binding to a receptor that mediate its effect. The pharmacological effect is dependent of different factors such as the drug dose, drug pharmacokinetics, and the receptor number at target organ or the other drugs interacting or competing for similar receptors (Perazella and Parikh, 2005).

Drug target pharmacogenetics aims at the identification of the inherited basis for interindividual variability in drug response and toxicity, particularly when this variability is not explained by differences in drug concentration (variability in the pharmacokinetic process). Pharmacogenetic variants have been described in drug targets (e.g. the β_2-adrenergic receptor, the angiotensin-converting enzyme or the

apolipoprotein E) and in their activation pathways that can influence drug response (Small *et al.*, 2002; Evans and McLeod, 2003).

Although many non-genetic factors influence drug response (Figure 1), genetics can theoretically account for 20–95 percent of variability in drug disposition and effects (Kalow *et al.*, 1999). In the case of CYP3A4 it was estimated that more than 60% of the enzyme activity is under genetic control and that approximately 90% of the interindividual variability in hepatic CYP3A4 activity is genetically determined (Ozdemir *et al.*, 2000).

The study of variability began with a focus on drug metabolizing enzymes, but it has been extended to membrane transporters and also to the genes that code for drug targets (Meyer, 2004; Evans and McLeod, 2003).

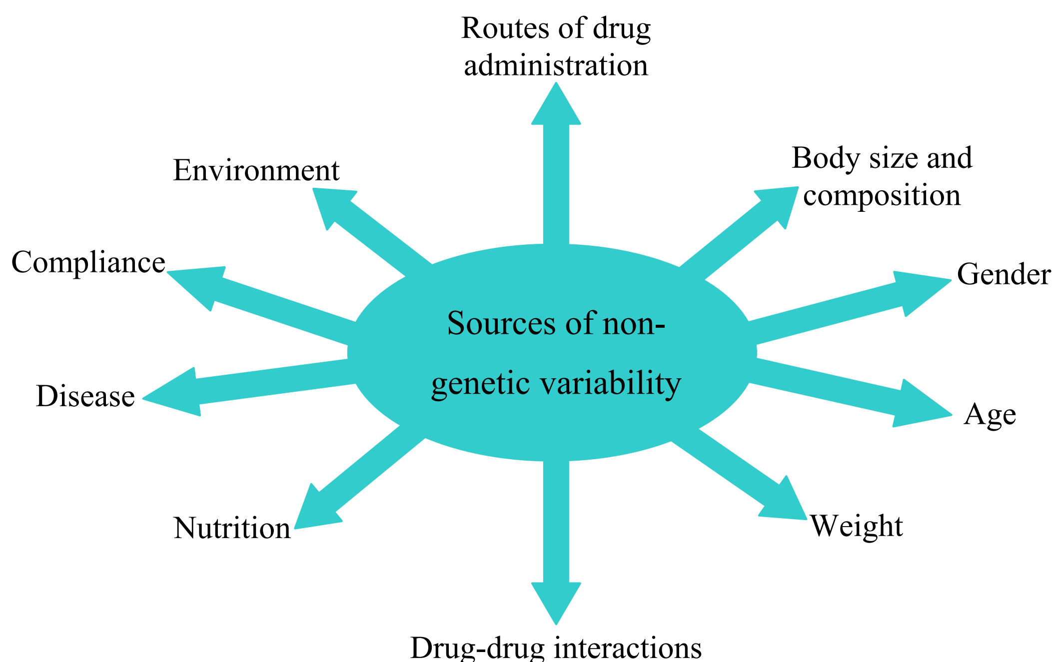


Figure 1 – Non-genetic parameters influencing the variation in drug effect.

Malaria

Malaria is globally the most important parasitic disease, with transmission occurring throughout Africa, Oceania, Asia and Latin America. In 2005 the global incidence of malaria has been estimated at 350-500 million new clinical cases annually, which can cause up to 3 million deaths (Sachs, 2005). More than 90% of the malaria burden occurs in sub-Saharan Africa, where tuberculosis and HIV/AIDS are also important public health problems. In this continent, the children under the age of 5 years and pregnant women are the ones more affected with malaria (WHO and UNICEF, 2005).

The malaria epidemic situation worsened in the last decade with the spread of drug resistance in the parasite against the commonly used antimalarial therapies based on chloroquine (CQ) and sulfadoxine-pyrimethamine (SP). A new hope appeared with the introduction of artemisinins and combination therapy (White *et al.*, 1999).

Malaria parasites are included in the protozoan subkingdom, class sporozoa. Four species of the *Plasmodium genus* are the canonically accepted as responsible for malaria in humans: *P. falciparum*, *P. ovale*, *P. malariae* and *P. vivax*. *P. falciparum* is essentially the sole agent responsible for the high mortality of the disease, a composite result of its virulence and drug resistance. The later is a major motor for the world trend of increased prevalence of this species over the other human infecting Plasmodia parasites.

Plasmodium life cycle

The life cycle in the four species of *Plasmodium* is relatively similar comprising two basic phases (Figure 2): an exogenous sexual phase (sporogony) in the female *Anopheles* mosquito and an endogenous asexual phase (schizogony) in human body. The asexual phase can be divided in a liver stage and an erythrocytic stage. When the mosquito infected with *P. falciparum* bites the human host it inoculates the sporozoites into the blood stream from where they migrate to the liver and invade the hepatocytes, a process that takes around 20 minutes. Inside the hepatocytes the sporozoite passes through different forms, being released back to the bloodstream as a merozoite (5 to 20 days after liver invasion). The liver stage is asymptomatic with only a few liver cells being infected.

The merozoites released invade the red blood cells (RBC), beginning the erythrocytic stage. In the erythrocyte the parasites become mature trophozoites that develop into new merozoites in 48 hours. Then they are released and can invade new RBC. Symptomatic disease begins when the asexual parasite multiplies in RBCs. To close the cycle, subpopulations of parasites develop into gametocytes and are ingested during a bite of a mosquito. The female and male gametocytes form a zygote in the insect's midgut. The zygotes develop into motile sporozoites through asexual division in an oocyst attached to the intestinal wall of the mosquito. These sporozoites migrate to the salivary gland to continue the *Plasmodium* life cycle by infecting the next host during the mosquito feeding.

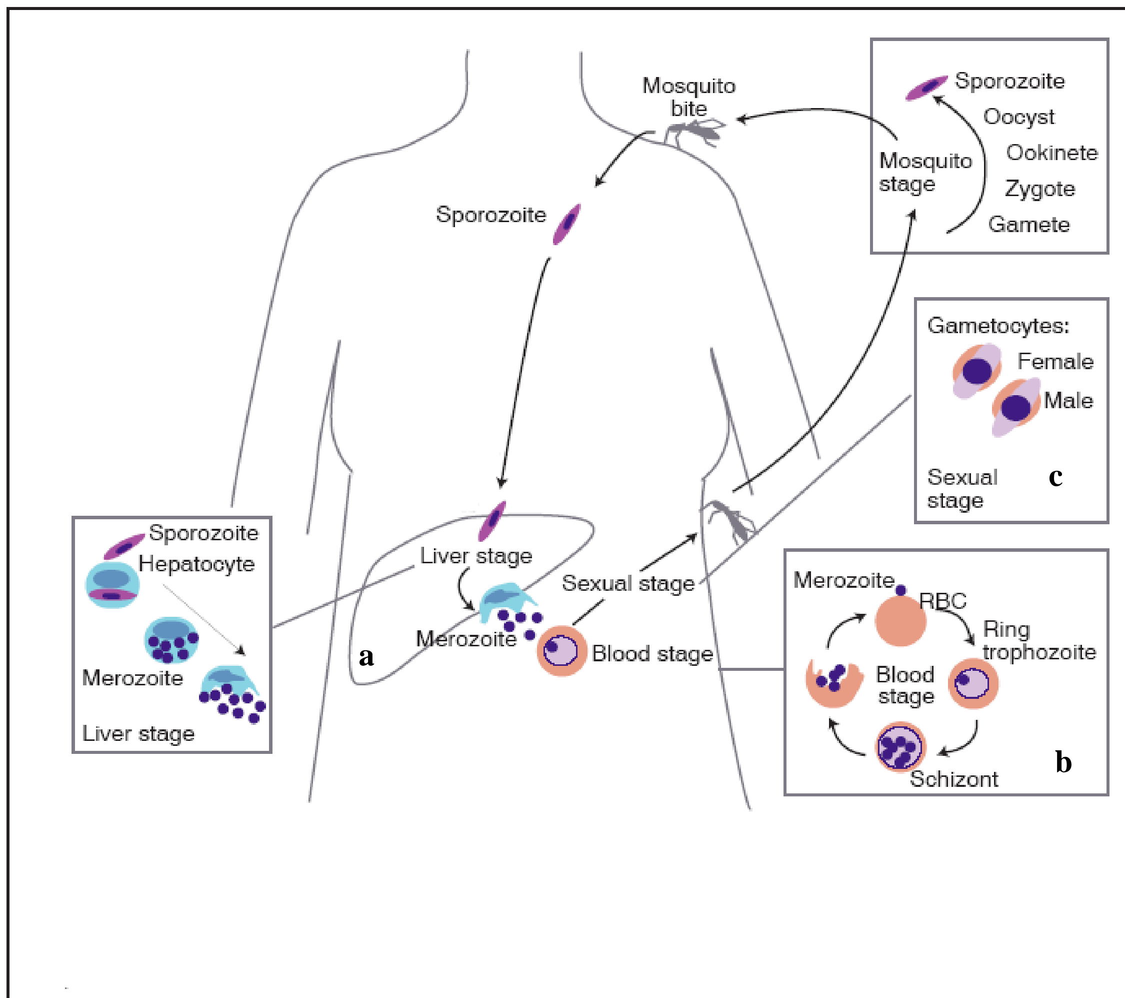


Figure 2 – Life cycle of *P. falciparum* and typical stages of action of antimalarial drugs (adapted from http://www–ermm.cbcu.cam.ac.uk).

a – 8-Aminoquinolines (primaquine), folate inhibitors (proguanil) and atovaquone. **b** – Artemisinin drugs (artemisinin, dihydroartemisinin, artesunate, artemether), 4-aminoquinolines (chloroquine, amodiaquine), quinoline methanols (quinine, mefloquine, halofantrine), folate inhibitors (proguanil, pyrimethamine, dapsone, sulfonamides) and atovaquone. **c** – Artemisinin drugs, Primaquine. (Baird, 2005; Patel and Kain, 2005; O'Neill *et al.*, 2006)

The Human Host – Genetic Resistance

In highly endemic areas the malaria risk is elevated in children and in pregnant women. This risk has been associated with the immune status of an individual. The innate immunity includes not only the cells of the immune system (e.g. natural killer cells, natural-killer-T cells or polymorphonuclear cells) but also host genetic factors. In malaria endemic areas certain diseases of red blood cells have been associated to a malaria protection, as the sickle cell trait, thalassemia, and enzyme deficiency or ABO blood groups.

The hemoglobin S gene geographical distribution is virtually identical to areas in the world in which malaria is (or has been) endemic (Ashley-Koch *et al.*, 2000). Early epidemiological studies suggested that heterozygote carriers of the βs gene could acquire *P. falciparum* malaria but had a reduced relative risk of dying of the infection (Allison, 1954). This trait has been related to a premature removal of infected RBC (higher susceptibility to phagocytosis and lower ability to invade and multiply) and, more recently, it was suggested that the protection might involve immunological components, although the mechanism of protection was not yet fully resolved (Min-Oo and Gros, 2005; Williams, 2006).

The thalassemias are disorders of hemoglobin production and they are divided in α- and β-thalassemia that are characterized by underproduction or deletion of the α- and the β-globin respectively (Weatherall and Clegg, 1982). Both alpha and beta thalassemias have been associated with decreased susceptibility to malaria infection (Flint *et al.*, 1986). The protection afforded by thalassemia against malaria may be related to the inability of the parasite to invade or multiply, or to cytoadhere to endothelial cells (Udomsangpetch *et al.*, 1993).

The blood group antigen polymorphisms can have an important role in malaria since the absence of certain glycophorin molecules on the surface of erythrocytes may affect parasite binding and invasion. *P. falciparum* binds specific sites on erythrocyte glycophorin (Pasvol, 1984), namely glycophorin A. Other red-cell-membrane-associated proteins that have been implicated in malaria protection include complement receptors, knop-blood group and the ABO blood groups. Individuals with blood group O are relatively resistant to severe malaria caused by *P. falciparum*, since in individuals with this blood group the forming of rosettes is not so readily observed (Uneke, 2007).

The enzyme glucose-6-phosphate dehydrogenase (G6PD) is part of the pentose phosphate pathway that is responsible for the production of NADPH that have a key role in supplying reducing equivalents in the infected RBCs. One function of NADPH is to be the reducing agent of GSSG to GSH and without this system perfectly working high levels of oxidative stress will be generated in the red blood cell.

Deficiency of the activity of G6PD is extremely frequent in some regions of the world (e.g., tropical Africa, parts of the Middle East and Southeast Asia), areas where malaria is most prevalent. G6PD variants include G6PD A (present in frequencies of 15 to 40%), G6PD B (60 to 80%) and G6PD A^- (0 to 25%) (Ruwende and Hill, 1998). This last variant is responsible for the the lost of almost all enzyme activity since it is reduced to less than 15% (Ruwende and Hill, 1998). In addition to such epidemiological observations, *in vitro* studies have shown that the growth of *P. falciparum* is reduced in G6PD deficient red cells when compared with normal red cells (Miller *et al.*, 1984; Roth *et al.*, 1983). The mechanism behind this observation is not clear, but there is evidence that G6PD-deficient infected RBCs are phagocytosed more rapidly than those from normal individuals (Cappadoro *et al.*, 1998). The G6PD-deficient cells lack the ability to resist sustained oxidative stress adequately and hence the free-radical-

producing parasite is a challenge to such cells. This situation is thought to make the red cell more susceptible to phagocytosis. Also oxidative stress induced by the parasite, plus the normal red cell oxidative stress particularly unquenched by the enzyme deficiency, results in an environment in which normal parasite growth is limited (Senok *et al.*, 1997).

During last several million years the evolution in malaria lead to the selection of many genetic variants that confere some degree of protection against death from the disease, even sometimes it cost to maintain a highly deleterious allele like HbS.

Antimalarial drugs

The first specific chemotherapies for the treatment of malaria, defined as "fevers" used extracts of the *Chinchona* tree (quinine), in the Andes, and *Artemisia annua* (artemisinin), in China. Quinine was for Centuries the only antimalarial drug available in the Western World. Through pressure of the global conflicts in the XX century, significant efforts for the development of synthetic antimalarials were applied. Chloroquine was one of the drugs that resulted from these efforts. It proved to be a safe drug with few toxic effects and was used by the WHO in the program for global malaria eradication. In the late 1950's resistance to CQ was almost simultaneously reported in Thailand, Colombia and Venezuela (Peters, 1970). From these first *foci* of CQ resistance a growing number of countries had to face this problem and today CQ resistance had spread to all the regions where malaria is endemic (Wongsrichanalai *et al.*, 2002). Only some regions in Central America, North of the Panama Canal and on the island of Hispaniola, chloroquine-resistant *P. falciparum* malaria have not been consistely documented (WHO, 2005).

The major antimalarial drugs in use can be separated in 4-aminoquinolines, 8-aminoquinolines, aryl aminoalcohols (or quinoline methanols), sesquiterpene endoperoxides, antifolates and antibiotics. The 4-aminoquinolines includes chloroquine and amodiaquine and the 8-aminoquinolines the animalarial primaquine. The aryl aminoalcohols includes mefloquine, lumefantrine, halofantrine and quinine, and the sesquiterpene endoperoxides the artemisinin-type compounds (e.g. dihydroartemisinin, artesunate, artemether and arteether). Finally, the antifolates includes the Type-I antifolates (e.g. sulfones and sulfonamides) and the Type-II antifolates (e.g. pyrimethamine and proguanil) and the antibiotics the tetracycline, doxycycline and clindamycin. The different known antimalarials have diverse points of action in the *P. falciparum* life cycle (Figure 2).

Some pharmacological properties of the main antimalarials (Figure 3) in use for the treatment of *P. falciparum* malaria are shown in Table 1.

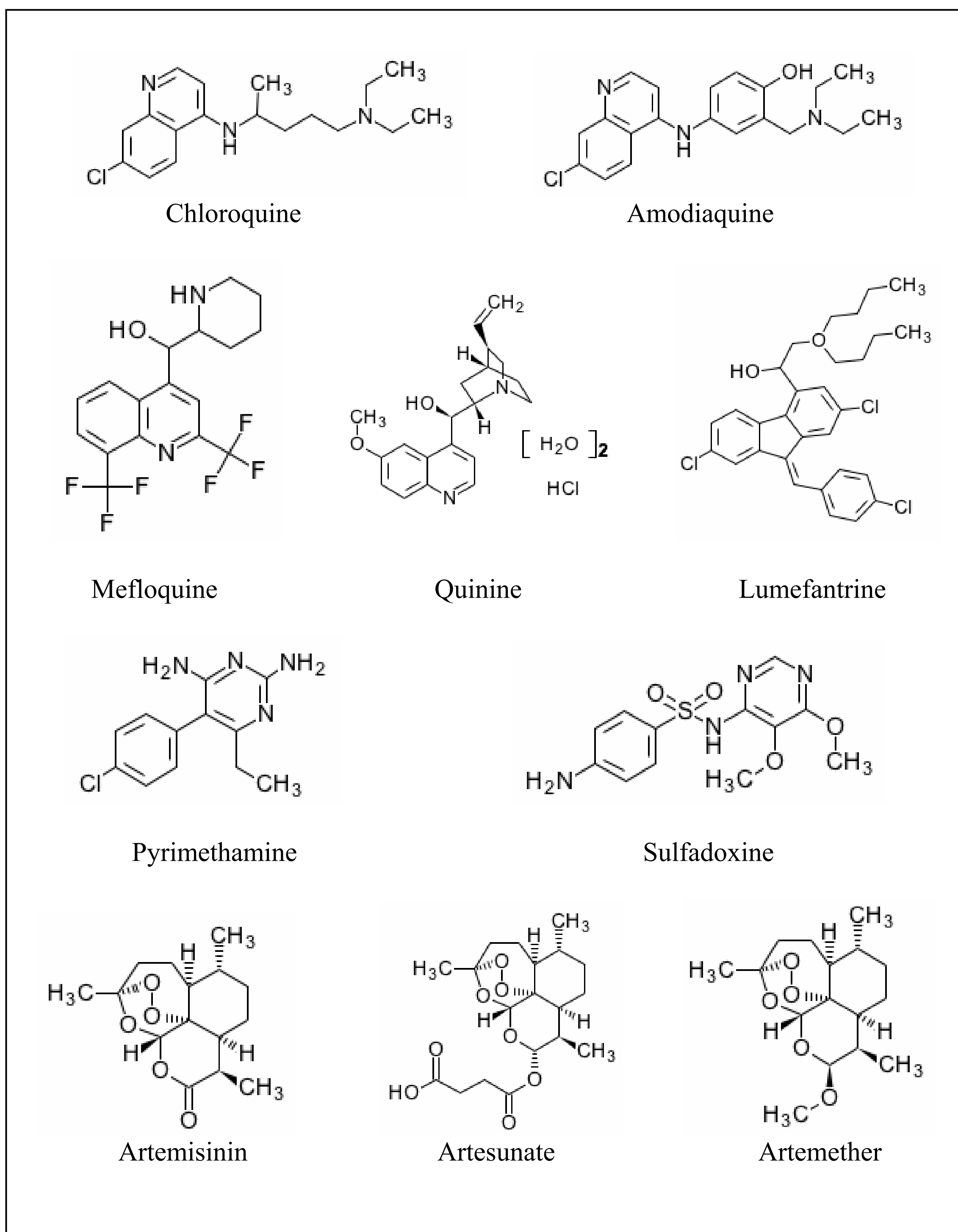


Figure 3 – Structures of the main antimalarial drugs used in first-line treatments (structures from www.genome.jp/kegg/, Kyoto Encyclopedia of Genes and Genomes).

Table 1 – Pharmacological properties of the antimalarials in widespread use[*].

	Chloroquine	*Amodiaquine*	*Quinine*	*Mefloquine*	*Sulfadoxine*	*Pyrimethamine*	*Lumefantrine*	*Artemisinin drugs*
Action	Interferes with parasite haem detoxification	Interferes with parasite haem detoxification (hypothesis)	Inhibits the ingestion of host cell haemoglobin (hypothesis)	Inhibits the ingestion of host cell haemoglobin (hypothesis)	DHPS inhibitor	DHFR inhibitor	Interferes with parasite haem detoxification (hypothesis)	Inhibits SERCA (hypothesis)
t_{max} (hrs)[**]	1 to 3	0.6 to 1.3	1 to 4	6 to 24	2 to 6	2 to 12	10	1 to 3
$t_{1/2\beta}$	8 - 58 days	2 - 10 hrs	11 - 18 hrs	14 to 22 days	4 to 9 days	4 to 6 days	33 hrs - 6 days	45 min to 4 hrs
Protein binding	50 to ~60%	> 90%	80 to 90%	~98%	90 to 95%	80 to 90%	> 99%	~50 to > 90%
Metabolism ***	CYP2C8, CYP3As, CYP2D6[****]	CYP2C8	CYP3As, CYP2C19[****]	CYP3A4	Acetylation	?	CYP3A4	CYP2A6, CYP2B6, CYP3As, Glucuronidation
Toxicity	Skin eruptions, gastrointestinal disturbs, central nervous system toxicity	No evidence for serious toxicity in AQ therapy	Gastrointestinal disturbs, cardiovascular and haematological effects	Nausea, vomiting, anorexia, loss of balance, cardiovascular effects	Allergic reactions, anorexia, Stevens-Johnson syndrome	Skin rashes, gastrointestinal disturbs, haematological effects	Ototoxicity Neurotoxicity	

[*] Data in the table is from Bray *et al.*, 2006; Ezzet *et al.*, 1998; Famin and Ginsburg, 2002; Foley and Tilley, 1997; Giao and de Vries, 2001; Grace *et al.*, 1998; Ilett *et al.*, 2002; Li *et al.*, 2002; Li *et al.*, 2003; Meyer and Zanger, 1997; Projean *et al.*, 2003; Rodriguez-Antona *et al.*, 2005; Schmuck *et al.*, 2002; Svensson and Ashton, 1999; Toovey, 2006a; Wanwimolruk *et al.*, 1991; Winstanley *et al.*, 1987; Winstanley and Ward, 2006; Zhao *et al.*, 1996.

[**] Peak plasma concentrations.

[***] For further information see section ***Drug Clearance and Infectious Diseases***.

[****] These enzymes are involved to a minor extent.

Quinolines

Quinine was the first antimalarial available in the Western World. It is still used today to treat the more severe cases of the disease and also as the second line treatment in some countries (www.who.int/malaria/treatmentpolicies.htm). The 4-aminoquinoline antimalarials (CQ and AQ) and related aryl aminoalcohols quinolines (mefloquine, lumefantrine and halofantrine) are, in general, synthetic derivatives of the quinine structure.

Chloroquine (CQ) was the global mainstay for the treatment of uncomplicated malaria for decades due to its safety, efficacy and low cost. This changed when *P. falciparum* CQ resistance emerged and spread (Rathore *et al.*, 2005).

Amodiaquine (AQ) is an effective antimalarial that emerged as an option for the treatment of CQ resistant malaria in the low-resource settings (Olliaro and Mussano, 2003).

Mefloquine and lumefantrine (previously called benflumetol) are synthetic aryl aminoalcohols quinolines presently in wide use in artemisinin-based combination therapy (ACT). Lumefantrine is only available co-formulated with artemether, under the brand names Coartem® (Developing World) and Riamet® (Industrialised World) (Novartis AG, Basel, Switzerland). Coartem® and AS + AQ represent the main ACTs in use in Africa, while mefloquine in combination with artesunate is mainly used in South East Asia malaria settings (Nosten and Brasseur, 2002). AS + AQ is now available in a fixed combination under the name Artesunate-Amodiaquine Winthrop® (ASAQ) for the public markets, and under the brand name Coarsucam® in the private markets (Sanifi-Aventis, Paris, France and DNDi).

Antifolates

The antifolates are an important group of antimalarials that exploit the ability of malaria parasites to synthesise folate *de novo,* which differs from the human host where the folates come from dietary intake. The antifolate drugs consist of dihydrofolate reductase enzyme (DHFR) inhibitors (such as pyrimethamine) and sulfonamides that inhibit the dihydroopteroate synthetase enzyme (DHPS). The application of antifolates as antimalarial drugs was enhanced by the discovery of synergism in drug combinations involving both classes of inhibitors that block nucleic acids synthesis in the parasite (Wang *et al.*, 2004).

Artemisinin derivatives

Another group of antimalarial drugs includes artemisinin (ART) and its derivatives (Figure 3). This family of drugs is characterized by rapid parasite clearance times and low toxicity. The artemisinin drugs have the highest **Parasite Reduction Ratio** (PRR), defined as the ratio between parasite count before treatment and 48 hours later (one parasite cycle). For ART this value is comprised between 10^3 and 10^5 compared with 10 to 10^2 described for mefloquine or quinine (White, 1997). This higher PRR of ART leads to a rapid clinical and parasitological cure that is highly effective against multidrug resistant parasites.

Artemisinin drugs have the broadest parasite stage specificity, being effective in most intraerythrocytic cycle stages, although with distinctive stage specific activities (Ter Kuile *et al.*, 1993; Skinner *et al.*, 1996). Artemisinin was demonstrated to be rapidly effective against both rings and schizonts (100% growth inhibition within 4 to 6h) but in the case of trophozoites it takes 10h to achive 60% growth inhibition (Skinner

et al., 1996). In the case of artemether the inhibitory effect is more gradual (70% growth inhibition within 8 to 10h), but, in contrast, dihydroartemisinin was described to be highly effective against all stages of the parasites (100% growth inhibition within 24h) (Skinner *et al.*, 1996). The quinghaosu drugs are also effective in reducing gametocyte carrier rates, and as a consequence, transmission of malaria (Chen *et al.*, 1994; Price *et al.*, 1996).

It was shown that after repeated doses of ART, the drug first-pass elimination effect was increased significantly (Ashton *et al.*, 1998). This effect is possibly associated to the fact that ART is an effective ligand of the nuclear receptors PXR (pregnane X receptor) and CAR (constitutive androstane receptor) (Burk *et al.*, 2005; Simonsson *et al.*, 2006) (see section ***Drug Clearance and Infectious Diseases – Pregnane X Receptor***). These nuclear receptors can induce a significant number of detoxification enzymes and transporters, including the enzymes involved in the metabolism of ART and its semi-synthetic derivatives (Table 1). This activation capacity constitutes a potential source of drug-drug interactions, when in the context of large scale combination therapy.

Amodiaquine, and its main metabolite desethylamodiaquine, are two main objects of study in this thesis constituting an important link between most parts of the work herein presented. Hence, more detail will be referred concerning these compounds.

Amodiaquine

Amodiaquine (AQ) is a 4-aminoquinoline, structurally similar to CQ (Figure 3), with a suggested similar mode of action against *P. falciparum*, but more active against most chloroquine-resistant parasites (Foley and Tilley, 1998). This drug was associated with rare but severe events in prophylaxis regimens. These events include agranulocytosis and hepatotoxicity (Meshnick and Alker, 2005). Severe adverse events have not been documented in current treatment therapies (Olliaro and Mussano, 2003).

In line with the recent global strategy for malaria control, the present WHO guidelines recommend the use of AQ as a partner in combination therapy. In ACT the partner of AQ is AS, a combination adopted in different countries as the first line treatment (www.who.int/malaria/treatmentpolicies.htm). In non-ACT AQ is typically combined with SP, a strategy in use by the Malaria Control Programmes of Papua New Guinea and Colombia (www.who.int/malaria/treatmentpolicies.htm; Genton *et al.*, 2005; Blair *et al.*, 2006).

Amodiaquine pharmacokinetics data is limited, particularly in which concerns the paediatric population, the main target group for this antimalarial therapeutic. The studies developed in healthy adults and in children from Papua New Guinea showed that AQ is readily absorbed (t_{max} ranging from 0.6 to 1.3 hours in adult health subjects) from the gastro-intestinal tract, being rapidly and extensively metabolized in the liver to the main metabolite, *N*-desethylamodiaquine (DEAQ) (Winstanley *et al.*, 1987; Hombhanje *et al.*, 2005). Minor metabolites were identified *in vivo* and *in vitro* in 1995 by Jewell and collaborators and include 2-hydroxyl-desethylamodiaquine and *N*-bisdesethylamodiaquine (Jewell *et al.*, 1995). These metabolites were not observed in a posterior *in vitro* study using human liver microsomes and recombinant Cytochromes P450 (Li *et al.*, 2002), but in a *in vivo* study *N*-bisdesethylamodiaquine was identified in

a blood sample (Minzi *et al.*, 2003). A recent study has analysed the *in vitro* metabolism of AQ using recombinant CYP2C8 proteins and also did not identify other metabolites besides DEAQ (Parikh *et al.*, 2007). The differences observed in the different studies may be the result of different *in vitro* or *in vivo* approaches.

DEAQ constitutes the main compound responsible for the long term effect of AQ therapy (Winstanley *et al.*, 1987; Hombhanje *et al.*, 2005), with AQ being rapid and extensive metabolized into DEAQ (Li *et al.*, 2002).

AQ is primarily eliminated through biotransformation and has been suggested to be excreted in bile since the percentage of the drug recovered in urine is very small (Winstanley *et al.*, 1987). The elimination pathways of DEAQ have not been clearly elucidated, but this metabolite was suggested to be eliminated by renal excretion (Giao and de Vries, 2001).

AQ is eliminated in humans via extensive first-pass biotransformation to DEAQ resulting from the hepatic activity of CYP2C8 (Li *et al.*, 2002). Two other enzymes, CYP1A1 and CYP1B1, were shown to metabolise AQ *in vitro*, and may contribute to the elimination of AQ at an extrahepatic level (Li *et al.*, 2002).

Resistance to antimalarial drugs

Antimalarial drug resistance is defined by the WHO as "the ability of a parasite strain to survive and/or multiply despite the proper administration and absorption of a medicine given in doses equal to or higher than those usually recommended" (WHO, 2005).

Resistance has been reported for all antimalarial classes of drugs (for artemisin drugs only *in vitro* resistance was described (Jambou *et al.*, 2005; Afonso *et al.*, 2006)). The molecular basis and mechanisms of action behind these phenotypes are still not totally understood (Krishna *et al.*, 2006; White, 2004), although it might partially include mutations in the SERCA protein coded by the PfATP6 gene (Eckstein-Ludwig *et al.*, 2003).

Numerous factors can be involved in the development of resistance and they can go from socio-economic factors to pharmacokinetics or parasite biology. One of the most important is subtherapeutic drug pressure that occurs when a sufficient number of human hosts carry residual drug levels to which infecting malaria parasites are exposed (Hastings and Watkins, 2005). This exposure to subtherapeutic drug levels result in a higher tolerance in the parasite population.

The quantity of drug that reaches the target (parasite's metabolism and detoxification of haemoglobin, antifolate synthesis, mitochondria or the sarco/endoplasmic reticulum Ca^{2+}-ATPase) in the parasite is determined by the human host pharmacokinetics, which is in turn influenced by the properties of each drug (Daily, 2006). Artemisinin derivatives are eliminated very fast (tipically $t_{1/2}$ < 1h) and are in general never presented to parasites at subtherapeutic drug concentrations for sufficient time to allow the selection process to occur. The same does not happen with

drugs that are slowly eliminated (e.g. mefloquine, chloroquine). In this case the parasites are exposed to low concentrations of drug for enough time to complete several intraerythrocyte cycles and this leads to the selection of resistant parasites. If gametocytes are allowed to be produced this selection can be further transmitted.

Pharmacogenetic diversity can influence individual drug pharmacokinetics through variability in XMEs, drug transporters and the nuclear receptors that control the expression of the former, affecting the metabolism and elimination of antimalarial drugs, and leading to variable drug levels in circulation. The different enzymes involved in the metabolism of antimalarials are possible sources of variability in the treatment outcome.

In summary, different pharmacokinetic characteristics can lead to different periods of sub-therapeutic doses of antimalarials, hypothetically leading to the aforementioned drug resistance parasite selection.

Combination therapy

Monotherapy is no longer an effective option against malaria in endemic regions, a consequence of the development of *P. falciparum* resistance against the mainstay antimalarial drugs. Mirroring the development of chemotherapy in other major infectious diseases (e.g. TB, AIDS) combination therapy represents the present effort to delay the spread of drug resistance and to maintain high efficacy of current drug regimens. Among the several possible and available combinations, artemisinin-based combination therapy (ACT) has been used in South-East Asia for a decade now with considerable success. ACT combines artemisinin compounds that produce a rapid clearance of parasitaemia and fever, with slowly eliminated antimalarial drugs like amodiaquine or lumefantrine (Figure 4).

The success obtained in Asia led WHO to promote the massive introduction of ACTs in most countries affected with malaria, with particularly emphasis in sub-Saharan Africa (Mutabingwa, 2005). However, the long term success of ACT observed in South-East Asia, an area with low transmission of malaria, is not clear to be repeated in most countries in Africa, with high transmission rates. This difference is related to the fact that in low transmission areas the risk of re-infection is low; therefore there is a reduced probability of new parasites being submitted only to the presence of the long life partner (after the fast elimination of ART). But in areas where the transmission is high the patients are submitted to frequent re-infections, with the new parasites facing only the long life partner. This can lead to the selection of parasites with growing drug tolerances and, in the future, to the development of *in vivo* resistance to ACT (Sisowath *et al.*, 2005; Hastings and Ward, 2005).

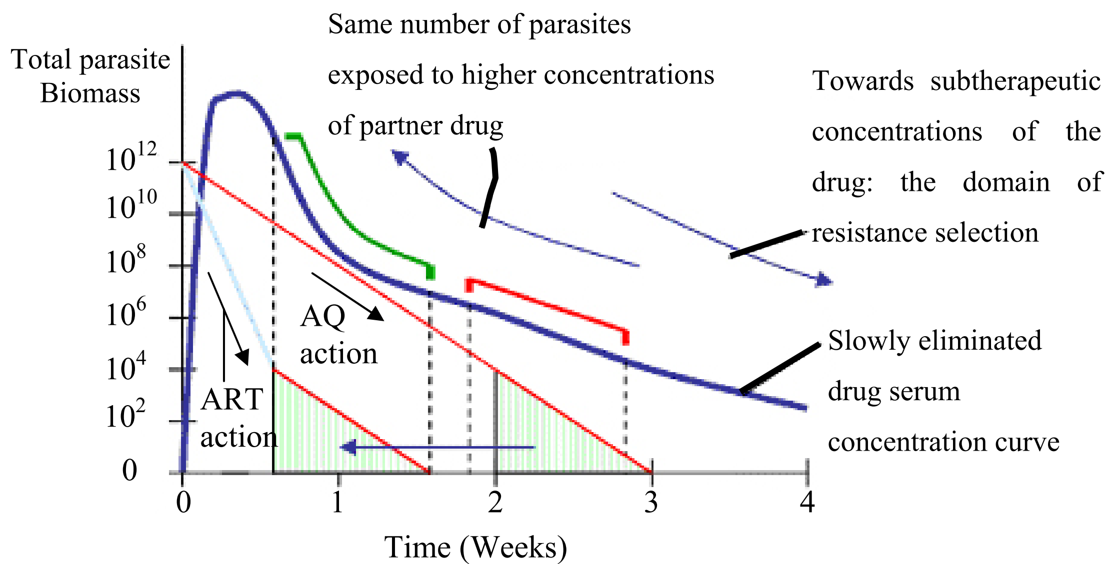


Figure 4 – The pharmacodynamic concept of artemisinin combination therapy (ACT). Reducing the parasite biomass with the high PRR drug (artesunate) protects the partner drug (amodiaquine), only obliged to face with a number of parasites several orders of magnitude smaller (adapted from Gil and Gil-Berglund, 2007).

Presently the use of combination therapies involves some disadvantages: (1) treatment courses with higher costs as compared with common monotherapy regimens (Wiseman *et al.*, 2006); (2) The possibility of inappropriate use of artemisinin derivatives if the ACT is not available in a fixed formulation – ART use as monotherapy can lead to high recrudescence rates and possibly to the rise of resistance (Jambou *et al.*, 2005); (3) the lack of information about safety (e.g. the embryotoxicity and potentially teratogenic effect associated with artemisinin drugs in animals is unknown in humans, but there are some evidence from clinical of neurotoxicity associated with these compounds), associated to a possible increase in the risk of

adverse effects and/or drug-drug interactions (Mutabingwa, 2005; Toovey *et al.*, 2006b).

The combination therapies adopted around the world as first-line treatment against *P. falciparum* malaria are presented in Figure 5.

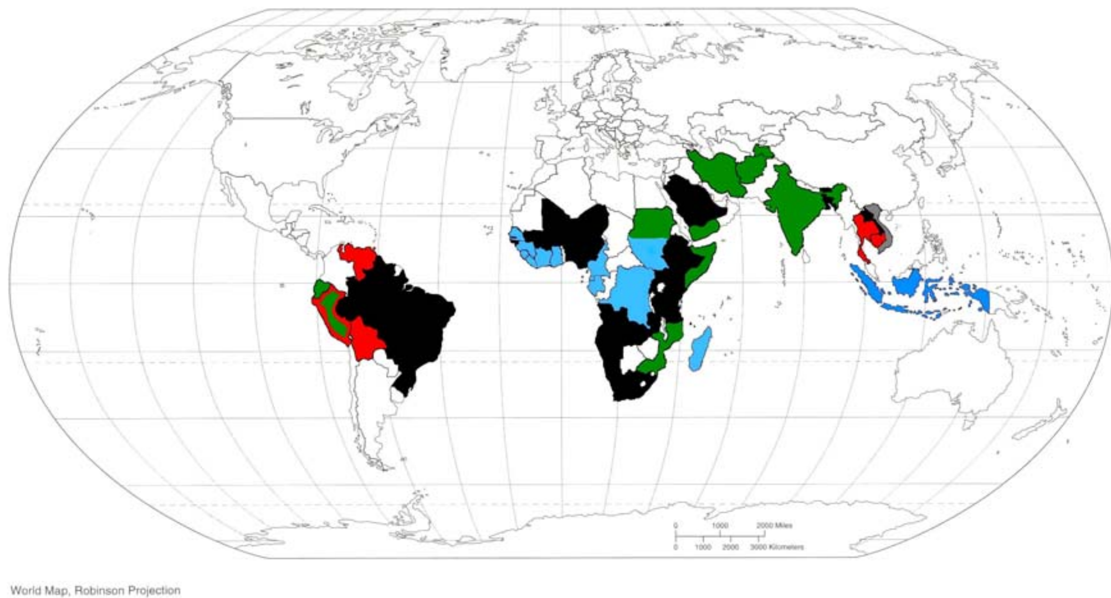


Figure 5 – Distribution of artemisinin combination therapies officially adopted as first-line treatment in malaria endemic countries (data from www.who.int/malaria/treatmentpolicies.htm). Blue – Artesunate + Amodiaquine (Artesunate-Amodiaquine Winthrop® or Coarsucam®); Black – Artemether + Lumefantrine (Coartem®); Green – Artesunate + SP; Red – Artesunate + Mefloquine; Grey - Dihydroartemisinin + piperaquine (Artekin®).

Drug Clearance and Infectious Diseases

In most tropical regions infectious diseases represent the major burden for the national public health systems. The major infectious diseases were responsible for 14.7 million deaths in 2001 (http://www.who.int). Among them malaria, HIV/AIDS and tuberculosis (TB) – the Big Three (Hotez *et al.*, 2006) – account for the biggest burden: an average of two million people with malaria die each year, three million people died from AIDS while four million have active tuberculosis at any time (Fauci, 2005; WHO, 2004).

Differences on ethnicity may have a notable impact on drug clearance, thus affecting the safety and efficacy of the dosing regimens. Drug clearance might be further affected in the context of tropical regions due to drug-drug interactions associated to the multiple drug therapy for concurrent infectious diseases.

Scarce data is available on the pharmacogenetic characteristics of populations concerning the genes involved in the elimination of the drugs currently used for the treatment of the major infectious diseases. The evaluation of pharmacogenetic differences would be a useful tool in the definition of average recommended doses, so that the starting dose in one population would correspond to the mean value in the main affected ethnic group. To note that ethnic differences in drug exposure are related not only to biological factors, but also social, cultural or economical aspects of a region. This includes local medical practice and compliance with prescribed medications, as well as diet, and particularly exposure to xenobiotics e.g. tobacco, alcohol (ethanol), industrial pollutants.

The metabolism of several antimalarial drugs has been related to different cytochrome P450s including CYP2B6 (artemisinin derivatives), CYP2C8 (CQ, AQ), CYP2D6 (CQ), CYP3A4 (CQ, LUM, quinine and artemisinin derivatives) or CYP3A5

(quinine) (Li *et al.*, 2003; Simonsson *et al.*, 2003; Svensson and Ashton, 1999; Grace *et al.*, 1998; Rodriguez-Antona *et al.*, 2005). Drug transporters are associated to antimalarials metabolism and, an example, is the *in vitro* interaction of mefloquine with MRP1 (ABCC1) and MRP4 (ABCC4) (Wu *et al.*, 2005). ABC transporter ABCB1/MDR1 (P-glycoprotein, Pgp) is involved in the transport of different antimalarials and this issue will be developed in section ***Drug Transporters – Multidrug Resistance 1 gene***. Completing this picture, the nuclear receptors PXR and CAR, were recently demonstrated to be activated *in vitro* by artemisinin derivatives (Burk *et al.*, 2005; Simonsson *et al.*, 2006), as this protein is able to influence the transcription activity of all the previously mentioned *loci*.

The metabolism of several anti-HIV/AIDS drugs is also mainly performed by cytochrome P450s, including protease inhibitors (normally CYP3A4; nelfinavir, CYP2C19) and non-nucleoside transcriptase inhibitors (CYP3A4 and CYP2B6) (Khoo *et al.*, 2005). The ABC proteins P-glycoprotein (Kim, 2003) and MRP2 (ABCC2) (Gutmann *et al.*, 1999; Huisman *et al.*, 2002) have also been proposed as transporting HIV protease inhibitor drugs.

This thesis is particularly focused on the pharmacogenetics of the cytochrome P450 superfamily, responsible for the highest contribution for variability among XMEs. Besides that, the pharmacogenetics of two Phase II enzymes, N-acetyltransferase 2 (NAT2) and Glutathione S-Transferase (GST), as well as an ABC transporter (ABCB1/MDR1), and the nuclear receptor pregnane X receptor was also investigated.

Cytochrome P450 (CYP)

The superfamily of CYPs, involved in the phase I metabolism of most drugs, has the highest impact among XMEs. In *Homo sapiens* the CYP superfamily includes 107 members, 57 of them known to code for active enzymes (http://drnelson.utmem.edu/CytochromeP450.html). Most CYP proteins are localized in the liver, contributing for the importance of this organ in xenobiotic metabolism. Extra-hepatic localizations are particularly important concerning the gut and the kidney with respect to orally administered and renally cleared drugs. The CYP enzymes are classified in families (enzymes sharing ≥ 40% amino acid sequences) and subfamilies (enzymes sharing ≥ 55% amino acid sequence). The first Arabic number indicates the family (CYP1), the letter indicates the subfamily (CYP1A) and another Arabic number corresponds to the individual gene (*CYP1A1*). The gene name is written in italic (*CYP1A1*) and the mRNA and protein in regular capital letters (CYP1A1).

The major human CYP enzymes responsible for catalyzing drug biotransformation are members of families CYP1, CYP2 and CYP3. Two subfamilies are particularly abundant in human liver microsomes, CYP2C and CYP3A, accounting for approximately 20% and 30%, respectively, of total CYP content (Shimada *et al.*, 1994). Genetic polymorphisms with functional consequences have been described in most CYP families (http://www.cypalleles.ki.se).

In the studies included in this thesis we have primarily focused in:

a) CYP2C8 because of its importance in the hepatic metabolism of AQ (Li *et al.*, 2002);

b) CYP1A1 and CYP1B1 involved in the *in vitro* biotransformation of AQ and with a possible role in the extrahepatic metabolism (Li *et al.*, 2002);

c) CYP3A4 described to be involved in the metabolism of LUM, quinine and ART derivatives and CYP3A5 for quinine (Li *et al.*, 2002; Lefevre *et al.*, 2000; Grace *et al.*, 1998; Rodriguez-Antona *et al.*, 2005).

CYP1A1

CYP1A subfamily includes two members, CYP1A1 and CYP1A2. The *CYP1A1* and *CYP1A2 loci* are located at chromosome 15q22-qter (*CYP1A1* reading towards the telomere and *CYP1A2* reading towards the centromere) and the two genes are separated by 23 kilobases (Kb) (Corchero *et al.*, 2001). CYP1A1 is an important enzyme in extra-hepatic tissues and is mainly expressed in human heart, lung, prostate, thymus, intestine, placenta, and lymphocytes (Ding and Kaminsky, 2003; Shimada *et al.*, 1994; Choudhary *et al.*, 2005). Its expression is highly inducible by several xenobiotics (e.g. polycyclic aromatic hydrocarbons), via the AhR/ARNT pathway (Ma, 2001). The enzyme's endogenous substrate is unknown but CYP1A1 was described to be involved in the *in vitro* metabolism of many drugs, including drugs important in the context of tropical medicine such as amodiaquine, quinine, and chloroquine, although only as a minor contributor (Li *et al.*, 2002; Li *et al.*, 2003).

CYP1A1 is a polymorphic gene and fourteen allelic variants have been determined (http://www.cypalleles.ki.se) (Figure 6). The protein coding region includes six of seven exons. One of the major SNPs present in this region is the 2455A>G

substitution in exon 7, leading to the amino acid change Ile462Val located near the heme binding region (Hayashi *et al.*, 1991). This alteration is present in both *CYP1A1*2B* and **2C* alleles and its influence over the enzyme activity is still under discussion (Cosma *et al.*, 1993; Crofts *et al.*, 1994; Persson *et al.*, 1997).

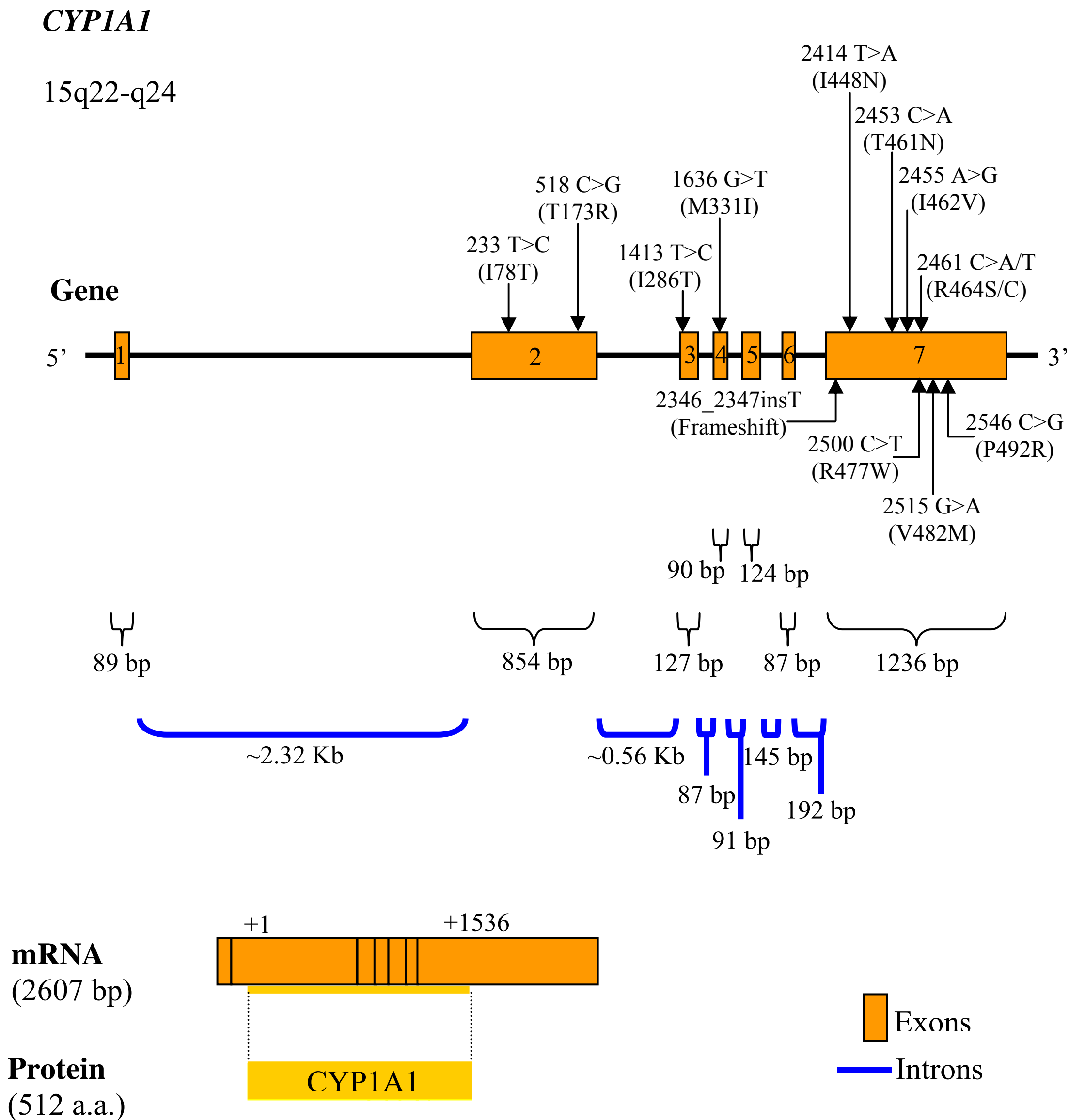


Figure 6 – Structure of the *CYP1A1* gene (data from http://www.ensembl.org/). The ATG start codon is close to the 5' end of exon 2 producing a protein of 512 amino acids. Arrows indicate the location of the nucleotide changes present in the coding region (data from http://www.cypalleles.ki.se).

CYP1B1

The *CYP1B1* gene, located in chromosome 2, is composed of three exons with the open reading frame (ORF) starting on exon two (Figure 7) (Tang *et al.*, 1996). The coded enzyme is mainly of extrahepatic expression, and is present in a large range of tissues, including kidney, prostate, mammary gland, and ovary (Sutter *et al.*, 1994; Shimada *et al.*, 1996; Tang *et al.*, 1999). Like the other members of CYP1 family, the induction of CYP1B1 is regulated by the AhR/ARNT pathway (Sutter *et al.*, 1994). This enzyme is involved in the biotransformation of estradiol, tegafur, docetaxel, mitoxantrone, flutamide and amodiaquine (*in vitro* and to a minor extent) (Rodriguez-Antona and Ingelman-Sundberg, 2006; Michael and Doherty, 2005; Li *et al.*, 2003).

Different physiological roles have been reported for CYP1B1, including the participation in the metabolism of endogenous substrates such as the steroid 17β-estradiol and steroid hormones estrone, testosterone and progesterone (Shimada *et al.*, 1999). Another physiological function of CYP1B1 is in ocular development and differentiation in humans, supported by the identification of rare mutations in the gene in patients with primary congenital glaucoma (Stoilov *et al.*, 1997; Stoilov *et al.*, 1998; Kakiuchi *et al.*, 1999).

Twenty-two alleles were described in *CYP1B1* (http://www.cypalleles.ki.se). Six alleles (*CYP1B1*2* to *CYP1B1*7*) are the result of different combinations of five nonsynonymous SNPs that give origin to the amino acid substitutions R48G, A119S, L432V, A443G and N453S (Stoilov *et al.*, 1998; Bailey *et al.*, 1998). The effect of these alleles is not well established, but *CYP1B1*6* and *CYP1B1*7* seem to be associated with a highly significant decrease in turnover numbers for both 2- and 4-hydroxylation of 17β-estradiol (Aklillu *et al.*, 2002).

CYP1B1

2p22-2p21

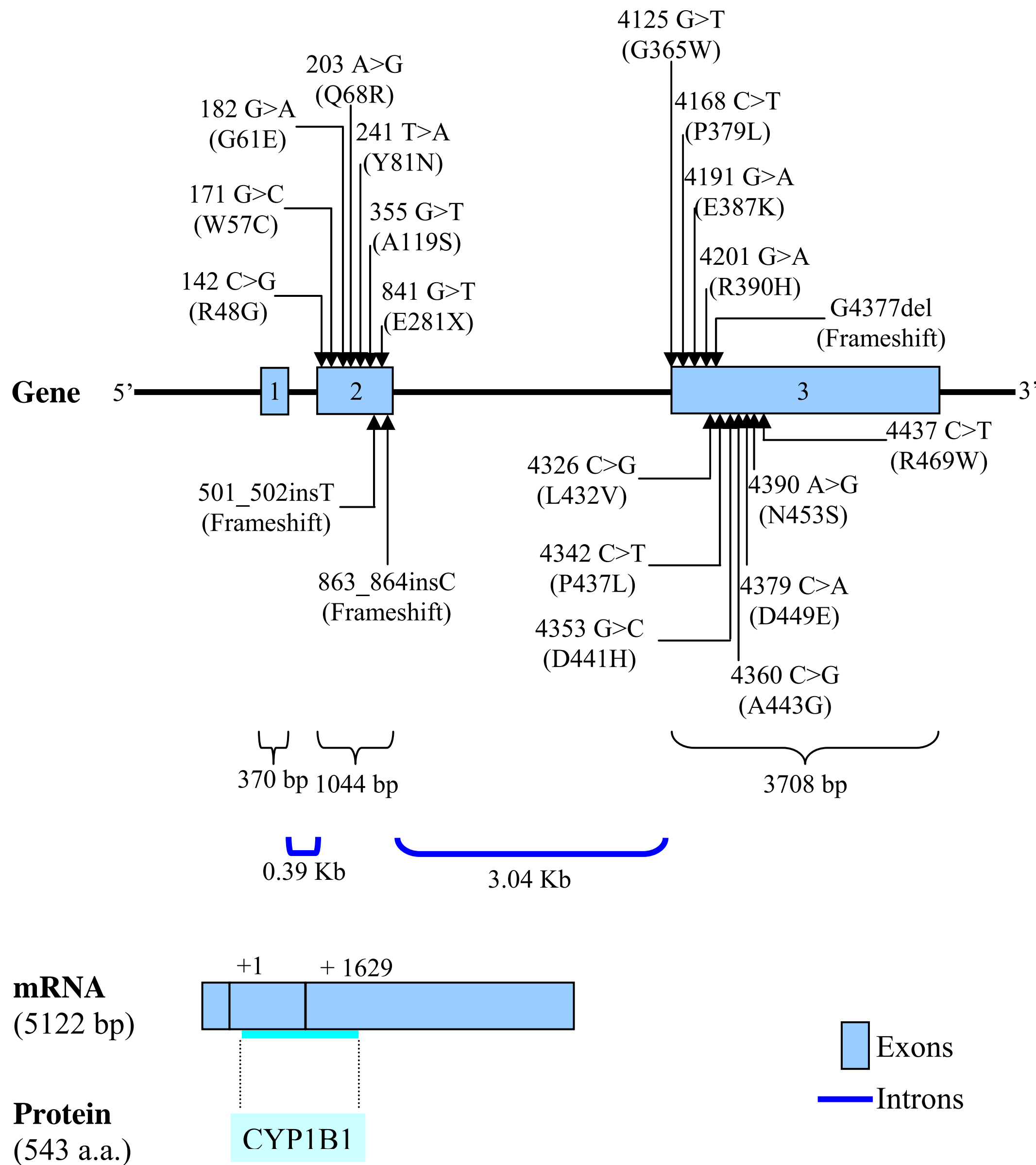


Figure 7 – The *CYP1B1* gene has 12Kb and contains three exons (data from http://www.ensembl.org/). The translation begins close to the 5' end of the second exon producing a protein of 543 amino acids. The nucleotide changes in the coding region are indicated by arrows (data from http://www.cypalleles.ki.se). For simplicity, the large deletions that give origin to the alleles *CYP1B1*16*, *CYP1B1*17*, *CYP1B1*22* and *CYP1B1*26* are not represented.

CYP2C8

The human *CYP2C8* gene (Figure 8) is one of the four members in the *CYP2C* cluster (Cent–RBP4–*CYP2C18*–*CYP2C19*–*CYP2C9*–*CYP2C8*-Tel) at chromosome 10 (Gray *et al.*, 1995). The CYP2C8 enzyme is mainly expressed in the liver, accounting for 7% of the total microsomal CYP content of this organ, but also in various extra-hepatic tissues (Shimada *et al.*, 1994; Klose *et al.*, 1999). It plays an important role in the metabolism of several therapeutic drugs including the anticancer paclitaxel, the anti-arrhythmic amiodarone, the calcium channel blocker verapamil or the antidiabetic troglitazone (Rahman *et al.*, 1994; Borlak *et al.*, 2003; Tracy *et al.*, 1999; Yamazaki *et al.*, 1999). Particularly important in the context of this thesis is the main role of CYP2C8 in the metabolism of the antimalarial amodiaquine (Li *et al.*, 2002).

This enzyme is also involved in the metabolism of several non-steroidal anti-inflammatory drugs (NSAIDs), such as diclofenac, as well in the metabolism of the cholesterol lowering drug cerivastatin (for a more complete list of substrates see Totah and Rettie, 2005). Besides the exogenous substrates, CYP2C8 has an endogenous role since it has been described to be involved in the biosynthesis of endogenous vasoregulating factors from their arachidonic acid precursor (Dai *et al.*, 2001).

The wide range of substrates presented for CYP2C8 may be a consequence of a large active site (1438 $Å^3$), akin to the observed in CYP3A4 and significantly larger than the active site of the other members of CYP2C subfamily (Schoch *et al.*, 2004). Although CYP2C8 shares 70% sequence homology with CYP2C9 and 25% with CYP3A4, this enzyme shares many more substrates with CYP3A4. The structure of CYP2C8 was determined by X-ray diffraction as a symmetric dimmer in the presence of two molecules of palmitate (Schoch *et al.*, 2004). No additional structures of CYP2C8 in a complex with other substrates were determined and substrate docking is the only

available information besides the crystal structure (Li *et al.*, 2002; Lewis, 2002; Melet *et al.*, 2004).

In total only eleven alleles were described for *CYP2C8* (http://www.cypalleles.ki.se) and this reflects a relatively low number of non-synonymous SNPs documented for *CYP2C8* (reviewed by Gil and Gil Berglund, 2007). The main polymorphisms, *CYP2C8*2* (harbouring 805 A>T), *CYP2C8*3* (carrying both 416 G>A and 1196 A>G) and *CYP2C8*4* (792 C>G), lead to the amino acid changes I269F, R139K, K399R and I264M, respectively. These variants have been documented to be associated with altered *in vitro* enzyme activity towards the probe drug paclitaxel (Dai *et al.*, 2001; Bahadur *et al.*, 2002). Specifically, *CYP2C8*2* allele was observed to cause an increase on the K_m for paclitaxel transformation while *CYP2C8*3* was documented to be associated with an enzyme activity significantly lower for paclitaxel hydroxylation and a decrease in the metabolism of arachidonic acid (Dai *et al.*, 2001). Conversely, another study has analysed *CYP2C8*3* and *CYP2C8*4* activities in human liver microsomes having shown small differences in paclitaxel hydroxylation for both alleles (Bahadur *et al.*, 2002). A recent study had determined the kinetic parameters of DEAQ formation by CYP2C8 "wild-type" and main variants (Parikh *et al.*, 2007). The *CYP2C8*2* allele is reported to present a lower V_{max} and intrinsic clearance, and a higher K_m when compared with *CYP2C8*1*. For *CYP2C8*3* allele the authors report that the decrease of AQ desethylase activity is profound, precluding the determination of the kinetic parameters.

The prevalence of the three major alleles has been suggested to differ significantly between ethnic groups (Dai *et al.*, 2001; Bahadur *et al.*, 2002; Nakajima *et al.*, 2003).

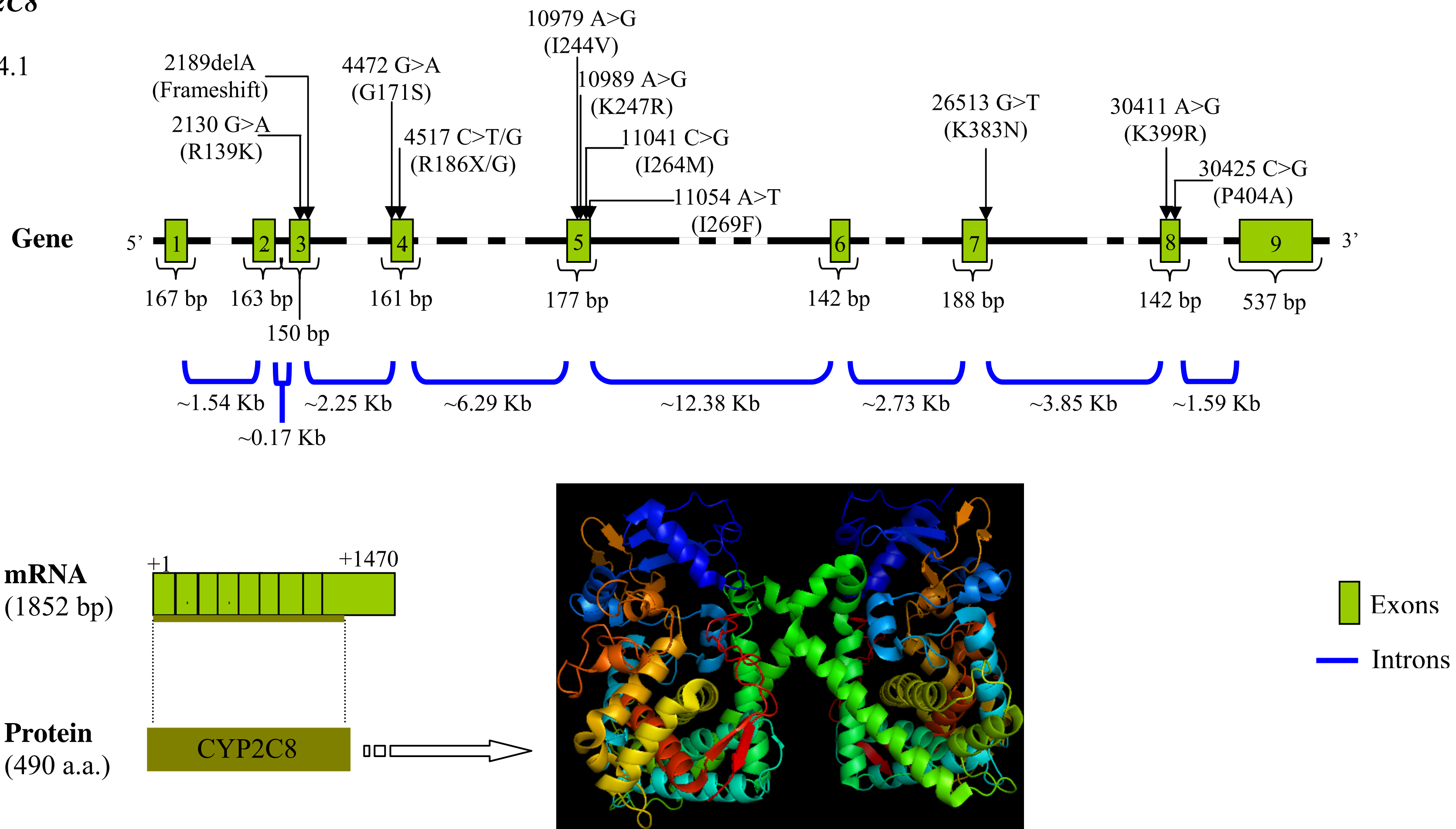


Figure 8 – *CYP2C8* gene structure where the nucleotide changes present in the coding region of the gene are represented (http://www.cypalleles.ki.se/). The 3D structure of CYP2C8 protein was obtained from PDB (PDB access number 1PQ2). CYP2C8 was crystallized as a symmetric dimer (Schoch *et al.*, 2004).

CYP3A4

The CYP3A subfamily includes four genes localized in a cluster at chromosome 7 (Cent–*CYP3A43*–*CYP3A4*–*CYP3A7*–*CYP3A5*–Tel). CYP3A4 is one of the quantitatively most important drug metabolising enzymes involved in both hepatic and intestinal metabolism of drugs. CYP3A4 metabolizes about 50% of all prescribed drugs, including taxanes, vinca-alkaloids, irinotecan, etoposide, antimalarials and antiretroviral drugs (Guengerich, 1999; Li *et al.*, 2003; http://medicine.iupui.edu/flockhart/). The large list of CY3A4 substrates is consistent with a large active site that goes from ~950 $Å^3$ in a ligand-free structure to 1650 $Å^3$ with ketoconazole and 2000 $Å^3$ in the presence of erythromycin (Ekroos and Sjogren, 2006).

The CYP3A4 enzyme was also described to be involved in the metabolism of endogenous compounds, contributing in a minor extent to the bile acid biosynthesis in humans since it is involved in the conversion of cholesterol to 4β-hydroxycholesterol (Bodin *et al.*, 2001). It is also responsible for carrying out the 25-hydroxylation of the bile acid intermediate 5β-cholestane-3α, 7α, 12α-triol (Furster and Wikvall, 1999; Honda *et al.*, 2001) and metabolizes different steroids such as testosterone, progesterone, pregnenolone, cortisol and estradiol (Guengerich *et al.*, 1986; Ged *et al.*, 1989; Niwa *et al.*, 1998; Kerlan *et al.*, 1992; Miller *et al.*, 2004).

CYP3A4 exhibits an important individual variability, which cannot, at present, be satisfactorily attributed to genetic polymorphism. This is also observed in other CYP enzymes, as is the case of CYP1A2 where a recent *in vivo* study could not found an association between the genotype and the phenotype that explains the interindividual differences (Jiang *et al.*, 2006). Among the SNPs identified to date, some are reported to affect function, but are not present at a high frequency in any ethnic group studied,

being therefore not likely to have major pharmacological consequences. The metabolic variability may rather be due to environmental factors and/or to polymorphisms in factors regulating gene transcription.

More than thirty allelic variants (http://www.cypalleles.ki.se) have been identified in the *CYP3A4* gene, resulting from combinations of SNPs located in the ORF, promoter and intronic regions (Figure 9). Most of them seem not to have significant consequences for gene expression or protein activity. An exception in this context is the relatively common −392A>G (*CYP3A4*1B*) SNP, notably frequent in populations of African origin (Ball *et al.*, 1999; Rodriguez-Antona *et al.*, 2005). Of interest in the frame of this thesis, it was recently demonstrated that *CYP3A4*1B*, previously suggested to be a transcriptionally less active allele (Rebbeck *et al.*, 1998), is associated to a reduced *in vivo* metabolism of quinine in Tanzanians (Rodriguez-Antona *et al.*, 2005).

The initial search for variants with functional significance led to the report of two non-synonimous SNPs in the coding region of CYP3A4, leading to the amino acid changes S222P (*CYP3A4*2*) and M445T (*CYP3A4*3*) (Sata *et al.*, 2000). *CYP3A4*2* was associated with lower intrinsic clearance for nifedipine when compared with the reference allele (Sata *et al.*, 2000).

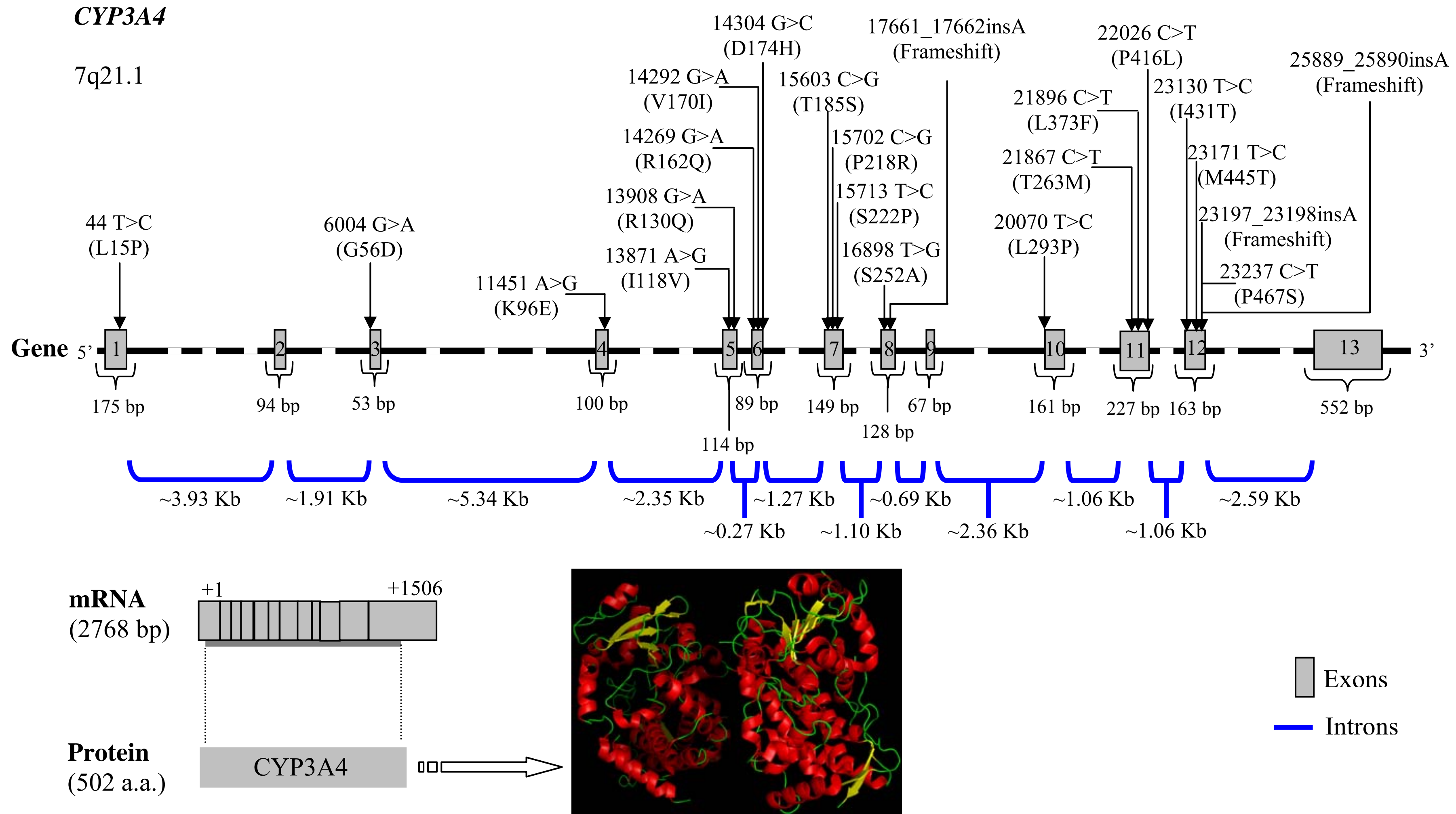


Figure 9 – The *CYP3A4* gene is constituted by 13 exons in 26.5 Kb. The data about the size of the introns and exons is from Gellner *et al.*, 2001. Only the SNPs present in the coding region of the gene are represented (http://www.cypalleles.ki.se/). The 3D structure of CYP3A4 protein was obtained from PDB (PDB access number 1TQN).

CYP3A5

The *CYP3A5* gene is composed of 13 exons and an ORF that originates a protein with 502 amino acids (Figure 10).

CYP3A5 substrates are less well documented as compared with CYP3A4. However, it has been shown that CYP3A5 plays a major role in the metabolism of a wide variety of substrates (Kuehl *et al.*, 2001), such as HIV-1 protease inhibitors (Koudriakova *et al.*, 1998), and the antimalarial quinine (Rodriguez-Antona *et al.*, 2005). The CYP3A5 protein is expressed in liver but also abundantly in different extra-hepatic locations, such as lung (Kivisto *et al.*, 1996), kidney (Schuetz *et al.*, 1992; Haehner *et al.*, 1996), breast (Huang *et al.*, 1996) and leukocytes (Janardan *et al.*, 1996).

The contribution of CYP3A5 in hepatic CYP3A drug metabolism displays a high variation - from 6 to 99%. However, this enzyme can represent at least 50% of the total CYP3A hepatic content in subjects where it is expressed (Wrighton *et al.*, 2000; Kuehl *et al.*, 2001). Although several variants have been described for *CYP3A5* (http://www.cypalleles.ki.se), the documented polymorphic expression of CYP3A5 is mainly due to a polymorphism (6986 A>G) within intron 3 (*CYP3A5*3*), present in all ethnic groups, in variable frequencies (Figure 10). As a result, individuals homozygous for the *CYP3A5*3* allele appear to be defective in CYP3A5, as this SNP generates a cryptic splice site resulting in the incorporation of the intronic sequence in the mature mRNA. This ultimately leads to the production of a truncated protein due to a premature termination of translation (Kuehl *et al.*, 2001). The activity of CYP3A5 may be important for the metabolism of specific drugs. This was shown for the case of the immunosuppressive drug tacrolimus where the plasma concentration and dose requirement was clearly demonstrated to be correlated to the presence of the *CYP3A5*3* allele (Hesselink *et al.*, 2003; Thervet *et al.*, 2003).

CYP3A5

7q21.3-q22.1

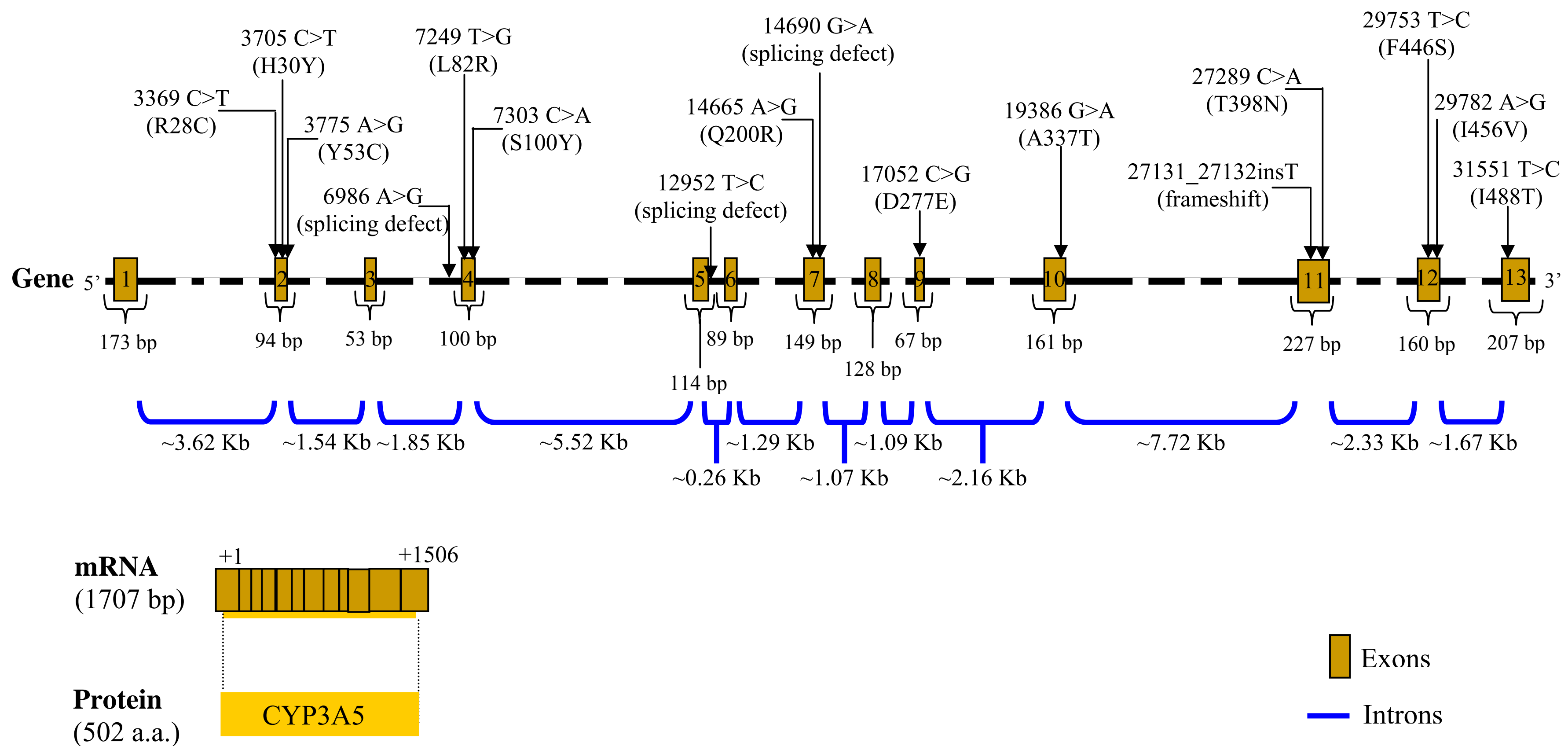


Figure 10 – The *CYP3A5* gene is constituted by 13 exons (Gellner *et al.*, 2001) that give origin to a protein with 57.1 kDa. CYP3A5 variation is mainly due to a SNP present in the intron 3 (6986 A>G) (http://www.cypalleles.ki.se/).

The Phase II of metabolism

Phase II reactions are also called conjugation reactions, whereby the drug molecule or its metabolite(s) is coupled with an endogenous hydrophilic substrate. These include glutathione, glucuronic acid, sulphate, acetate, bile acid, or an amino acid. Major enzymes in Phase II are the UDP glucuronosyltransferase (UGT), sulfotransferases (SULT), arylamine *N*-acetyltransferases (NAT), glutathione *S*-transferases (GST), thiopurine *S*-methyltransferases (TPMT) or the catecholamine-*O*-methyltransferases (COMT). In this thesis we focused on the analysis of polymorphisms in N-acetyltransferase 2 (*NAT2*) and Glutathione S-transferase M1 and T1 (*GSTM1* and *GSTT1*) genes.

N-Acetyltransferase 2 (*NAT2*)

N-acetylation is the major route of biotransformation for xenobiotics containing an aromatic amine (R-NH2) or a hydrazine group (R-NH-NH2). This reaction is performed by two cytosolic enzymes, NAT1 and NAT2. The corresponding genes were mapped to chromosome 8p21.3-23.1 and each of the two respective genes has a single, intronless protein-coding exon with an ORF of 870 bp (Figure 11) (Grant *et al.*, 1989).

The *N*-acetyltransferase 2 enzyme encoded by the *NAT2* gene plays a primary role in the activation and/or inactivation of a diverse number of aromatic amines (e.g., procainamide) and hydrazine drugs (e.g., isoniazid) used in therapeutics. NAT2 is expressed predominantly in the human liver, being found also in the intestine, placenta, bladder or prostate (Boukouvala and Fakis, 2005). The slow NAT2 acetylator, characterized by a decreased enzyme activity, is expected to be at higher risk for drug side effects (isoniazid and sulfasalazine, Hiratsuka *et al.*, 2002 and Tanaka *et al.*, 2002,

respectively), while therapeutic failure may be expected in rapid acetylators after standard doses (Sabbagh and Darlu, 2006).

The *NAT2* allelic variation has been extensively studied, due to its long known impact on the acetylator phenotype (Weber, 1987). The "wild-type" *NAT2*4* and 35 *NAT2* allelic variants have been identified in human populations, as the result of the different combinations of the known SNPs (http://www.louisville.edu/medschool/pharmacology/NAT.htm) (Figure 11).

Of the alleles described, *NAT2*4* is responsible for the fast acetylator phenotype, together with *NAT2*12A*, **12B*, **12C* and **13* alleles, since these contain polymorphisms which are either silent or lead to conservative amino acid changes (Cascorbi *et al.*, 1996; Hein *et al.*, 1994; Lin *et al.*, 1993). The remaining *NAT2* alleles have been demonstrated to code for enzymes with reduced activity and/or stability and, therefore, conferring slow acetylator phenotypes (Ferguson *et al.*, 1994; Hein *et al.*, 1995; Leff *et al.*, 1999). The mutation 191 G>A, previously reported as "African-specific", defines the *NAT2*14* alleles and is known to have a drastic effect on NAT2 enzymatic activity, being associated with the slow acetylator phenotype (Fretland *et al.*, 2001).

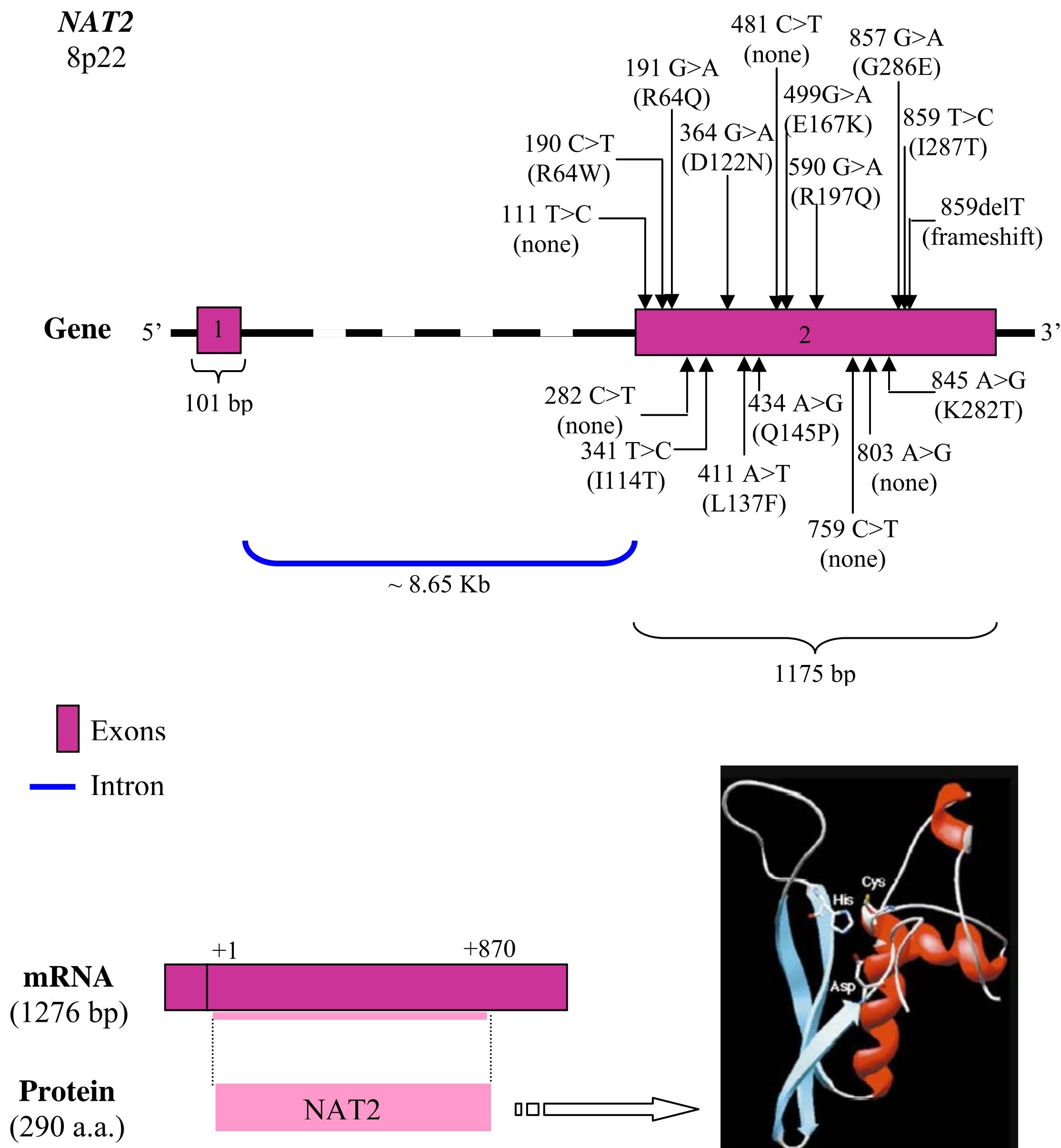


Figure 11 - The *NAT2* gene codes for a 290 amino acids protein (33.5 kDa). The conjugation of the 17 nucleotide changes represented in the figure gives origin to different allelic variants that are compiled in http://www.louisville.edu/medschool/pharmacology/NAT.htm. The structure of the catalytic N-terminal domain of NAT2 was determined by homology models with the crystallographic structure of *Salmonella typhimurium* NAT (Rodrigues-Lima and Dupret, 2002).

Glutathione S-transferases – GSTM1 and GSTT1

The Glutathione S-transferase (GST) superfamily of enzymes comprises a range of cytosolic, mitochondrial, and microsomal proteins which are capable of multiple reactions with a multitude of substrates, both endogenous and exogenous. The GST enzymes facilitate the detoxification of various carcinogens, therapeutic drugs, environmental toxins and products of oxidative stress (Hayes *et al.*, 2005). These enzymes have been described to be involved in the detoxification of anticancer agents (e.g. adriamycin, *cis*-platin or busulfan), environmental chemicals and metabolites (e.g. DDT, inorganic arsenic or methyl parathion) and epoxides (e.g. the antibiotic fosfomycin, aflatoxin B1, polycyclic aromatic hydrocarbons (PAHs) or styrene) (Hayes *et al.*, 2005).

Different physiological roles have been reported for GSTs besides the involvement in the degradation of products resultant from oxidative stress. GSTs are also involved in the degradation of aromatic amino acids, synthesis of steroid hormones, biosynthesis of important metabolites of arachidonic acid and in the modulation of signalling pathways (for a review see Hayes *et al.*, 2005).

Soluble GSTs are biologically active as like dimeric proteins and at least seven distinct classes of soluble GST highly expressed in the mammalian liver have been identified: alpha (A), mu (M), pi (P), sigma (S), theta (T), kappa (K), and zeta (Z) (Hayes *et al.,* 2005). Although the classes share some overlapping properties, each GST isoform possesses unique substrate specificities. These allow the GSTs to provide a broad spectrum defence against the potentially damaging effects of a range of xenobiotics and endogenously produced toxic compounds.

The genes encoding the mu class of enzymes are organized in a gene cluster on chromosome 1p13.3 (5' - *GSTM4* - *GSTM2* - *GSTM1* - *GSTM5* - 3'). *GSTM1* is one of the genes in this cluster. It is organized in eight exons, coding for a protein with 217 aminoacids (Figure 12). GSTM1 is expressed is the liver and also in the kidney (Rowe *et al.*, 1997), lung, colon or leukocytes (Anttila *et al.*, 1993; Szarka *et al.*, 1995; Wang *et al.*, 2000).

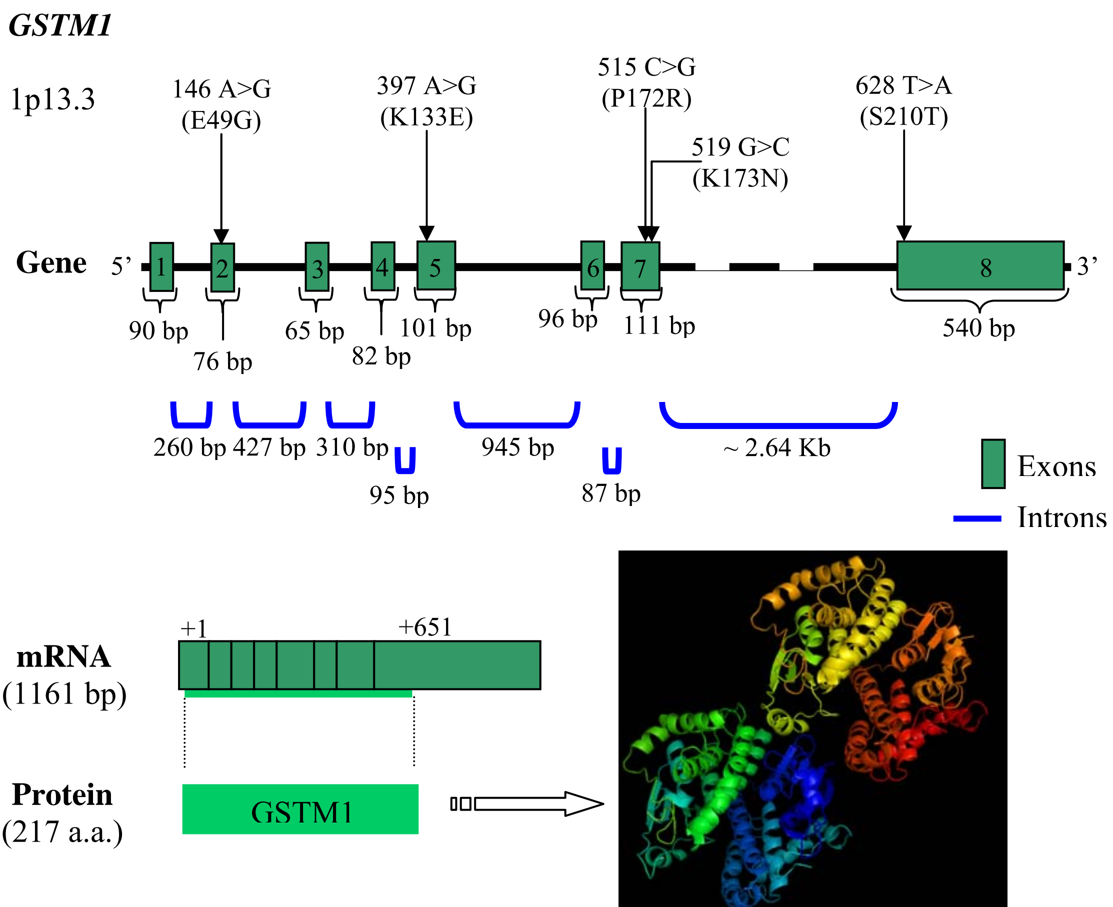


Figure 12 – The *GSTM1* gene is constituted by eight exons where few variations were described (data from Tetlow *et al.*, 2004 and http://pgeni.unc.edu/). GSTM1 3D structure was obtained from PDB and is presented as homodimer (PDB access number 1XW6).

The theta class includes two members, GSTT1 and GSTT2, and the genes are localized in chromosome 22. The *GSTT1* gene is located approximately 50kb upstream from the *GSTT2* gene. Both GSTT1 and GSTT2 were described to be expressed in liver, erythrocytes, lung, kidney, brain, skeletal muscles, heart, small intestine, and spleen (Juronen *et al.*, 1996). The *GSTT1* is composed by five exons and an ORF coding for a protein with 239 amino acids (Figure 13).

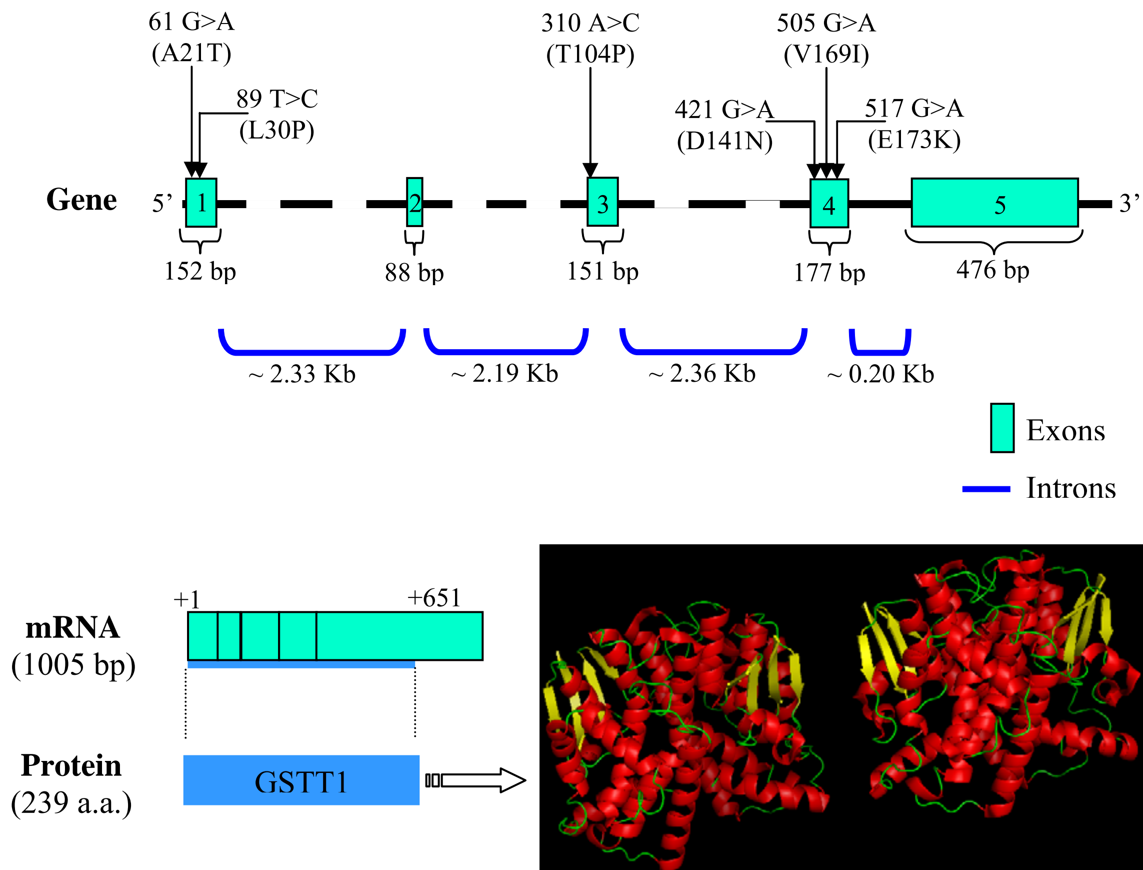


Figure 13 – The *GSTT1* gene was mapped in chromosome 22 and different variations were reported (data from http://pgeni.unc.edu/). The 3D structure of GSTT1 protein was obtained from PDB and is presented as homodimer (PDB access number 2C3N).

Human cytosolic GSTs display polymorphisms likely to contribute to interindividual differences in responses to xenobiotics. The main polymorphism in two important isoforms, GSTT1 and GSTM1, is the total deletion of the gene, resulting in the complete lack of enzyme activity. Studies in this area addressed the question of whether individuals lacking GSTM1 and/or GSTT1 showed a higher incidence of common neoplasias, due to decreased protection against xenobiotic carcinogens (Parl, 2005). Since glutathione-conjugation represents a detoxification pathway, the total absence of GSTM1/GSTT1 activity, due to homozygous gene deletion (genotype *GSTM1*0/*0* and/or *GSTT1*0/*0*) may be linked to increased drug toxicity or cancer susceptibility (Parl, 2005).

Gluthatione S-Transferases and the Oxidative stress in Malaria

The level of oxidative stress is critical for survival, depending on the balance between the production and clearance of reactive oxygen species (ROS). These species are generated not only after pathological stress conditions but as the result of normal metabolism (aerobic respiration leads to the production of the superoxide anion, hydrogen peroxide and the hydroxyl radical). The mammalian cells have developed different systems (non-enzymatic and enzymatic) in order to control the levels of ROS. These species are scavenged by antioxidants, such as reduced glutathione (GSH), ascorbate (vitamin C), and carotenoids and by the catalytic activities of superoxide dismutase (SOD) and catalase (CAT) and glutathione peroxidase (GPX). In addition to these antioxidants systems we find the GST enzymes. GSTs play an important role in the conjugation of GSH to the products of endogenous lipid peroxidation and in the inactivation of organic hydroperoxides via selenium-dependent glutathione peroxidase

activity (Sharma *et al.*, 2004). These actions protect the cell from the deleterious effects of oxidative stress.

Oxidative stress plays an important role in malaria pathology. When infected by *P. falciparum* the host erythrocyte suffers oxidative aggressions by the parasite metabolism and by its own immune system (see section ***The Human Host – Genetic Resistance***). The host immune defence system uses the phagocytosis and the production of nitric oxide and oxygen radicals to fight the infection, contributing this defence to the pathology of the disease (Becker *et al.*, 2004). The oxidative aggressions that come from the parasite are the result of the haemoglobin degradation. The antioxidant system of the parasite is very efficient and is based in an enzymatic antioxidant defence system that includes glutathione- and thioredoxin-dependent proteins. This system is not completely efficient and the toxic compounds generated in haemoglobin degradation can cause redox damage to the host proteins and membranes, leading to the lysis of the erythrocytes or making the RBCs more susceptible to phagocytosis (Becker *et al.*, 2004). Antimalarials like chloroquine or amodiaquine seem to be associated with an increase in the levels of ROS.

In malaria infection increased levels of ROS were described, as well as a decrease in the antioxidants, such as catalase, glutathione peroxidase, glutathione, ascorbate or albumin (Pabón *et al.*, 2003; Das and Nanda, 1999). The oxidative stress inside the erythrocyte is determinant to the progression of the disease since it is involved in the clearance of the parasites (Clark and Hunt, 1983; Greve *et al.*, 1999).

Drug Transporters - Multidrug Resistance 1 gene (*MDR1*, *ABCB1*)

The xenobiotic-related transporters (XRTs) consist of uptake and efflux transporters, depending of the intracellular or extracellular transport directions. An example of uptake carrier systems is the organic anion transporters (OATP, SLC21A).

Most drug efflux transporters belong to the ATP-binding cassette (ABC) superfamily of membrane proteins, which may influence the intracellular concentration of numerous compounds. Efflux drug transporters act as barriers, preventing exogenous compounds from being absorbed, but when expressed in the hepatocytes or in the kidney, they can contribute to excretion (Szakács *et al.*, 2006). In *H. sapiens* the ABC superfamily comprises approximately fifty members, classified in seven subfamilies (ABCA to ABCG), a proportion of them being located in the plasma membrane and able to extrude a variety of structurally diverse drugs and metabolites (Human ABC Proteins Database - http://nutrigene.4t.com/humanabc.htm). Export of these compounds occurs in an active, ATP-dependent manner, and can take place against considerable concentration gradients. The first member of this superfamily, P-glycoprotein (P-gp), was discovered in 1976 and is still the most intensively studied (Juliano and Ling, 1976; Couture *et al.*, 2006).

P-gp protein is coded by the multidrug resistance gene (*MDR1*, *ABCB1*), and displays a wide substrate specificity including molecules of significant difference in structural and chemical characteristics (e.g. anticancer agents, cardiac drugs, HIV protease inhibitors, immunosuppressants, antibiotics, steroids or calcium channel blockers) (for a list of substrates see Fromm, 2002). P-gp is assumed to represent a protective mechanism against potentially toxic xenobiotics, a function that is reflected in its most extreme form in the pivotal role of this transporter in anti-cancer drug resistance. Its typical normal physiological expression in the apical membrane of

normal tissues such as intestine, kidney, liver, and the blood-brain barrier reinforces its significant excretory and xenobiotic barrier role (Thiebaut *et al.*, 1987; Cordon-Cardo *et al.*, 1990). By contributing for the disposition of a large range of drugs, drug-drug interactions are expected to occur when its activity is altered by one drug resulting in a change of the clearance of other substrates.

P-gp was documented to be involved in the disposition of several antimalarials. The antimalarials chloroquine, quinine and mefloquine were reported to be weak P-gp substrates *in vitro* (Pereira *et al.*, 1995; Pham *et al.*, 2000; Riffkin *et al.*, 1996; Crowe *et al.*, 2006). Recently Hayeshi and collaborators analysed a large number of compounds, including quinine, amodiaquine (and DEAQ) and chloroquine and demonstrate *in vitro* that they are P-gp inhibitors but only quinine is a substrate (Hayeshi *et al.*, 2006). Another study, this time performed in healthy White travellers, showed that *MDR1* polymorphisms were associated with neuropsychiatric adverse effects of mefloquine, particularly in female travellers (Aarnoudse *et al.*, 2006).

The *MDR1* gene is located in the long arm of chromosome 7 (7p21.1) and consists in 29 exons (Bodor *et al.*, 2005) that give origin to a protein with 1280 amino acids. The protein is phosphorylated and glycosylated and is composed by two homologous halves, each one containing six transmembrane segments and an intracellular ATP-binding site (Figure 14-A).

Genetic variants can alter P-gp expression and function. To date more than 40 SNPs have been found in *MDR1* gene distributed by the ORF, promoter and intron regions (Kroetz *et al.*, 2003). Among the different SNPs (Figure 14) presently known in the *MDR1* gene, the -129 T>C, 3435 C>T and 2677 G>T/A polymorphisms have been particularly studied and associated to altered P-gp expression and function (Hoffmeyer *et al.*, 2000; Kim *et al.*, 2001; Tanabe *et al.*, 2001).

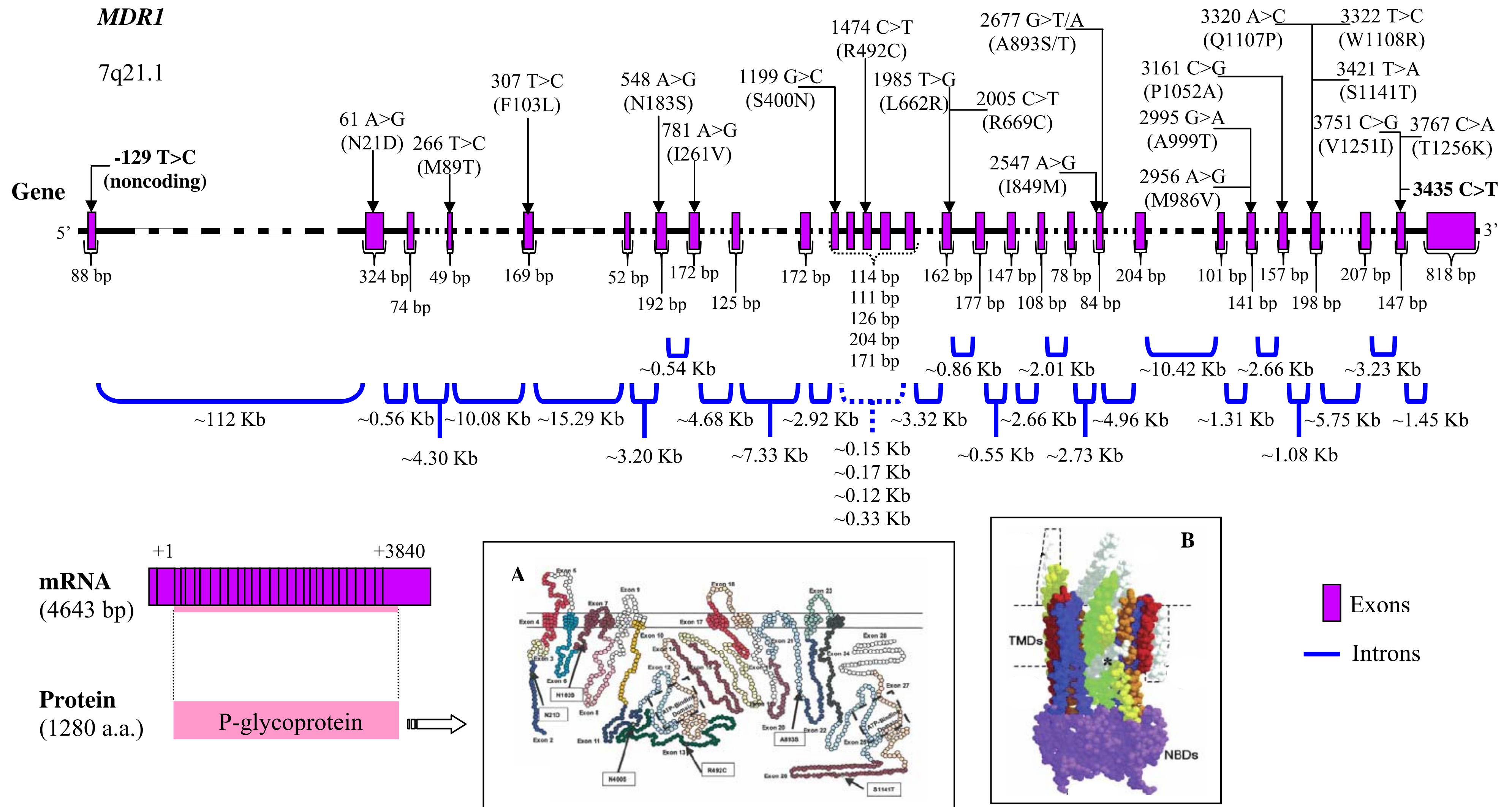


Figure 14 – The *MDR1* gene with 29 exons extends for over more than 200 Kb. The coding region nonsynonymous SNPs are represented in the figure and also the SNP in the promoter -129 T>C and the SNP 3435 C>T in exon 28 (Kroetz *et al.*, 2003; Sakaeda, 2005). A) Schematic drawing of human P-glycoprotein transporter (Kim *et al.*, 2001). B) Space-filling model of human P-gp protein in the nucleotide bound form (from Sarkadi *et al.*, 2006).

The -129 T>C alteration, located in the promoter region, has been associated with significantly lower levels of P-gp in placentas of individuals carrying the T/C genotype when compared with the T/T genotype (Tanabe *et al.*, 2001).

Regarding the 3435 C>T, the TT homozygotes were described to have a lower level of intestinal P-gp resulting frequently (but not always) in an increase of the probe drug digoxin plasma levels, as compared to the CC genotype group (Hoffmeyer *et al.*, 2000; Kim *et al.*, 2001; Morita *et al.*, 2003; Owen *et al.*, 2005; Sakaeda *et al.*, 2001). But, considering that 3435 C>T is a silent mutation, research focused on the reasons that could explain these differences. The first hypothesis to solve this question had pointed to the existence of a linkage between this SNP and others functional alterations. The 2677 G>T/A SNP was described to be in linkage disequilibrium with 3435 C>T and proposed to be the main responsible for the phenotype associations with the later polymorphism (Kim *et al.*, 2001). However, the linkage is normally not observed in 100% of the cases. Another hypothesis appeared in 2005 when a quantitative analysis of allelic 3435 C>T P-gp expression in liver samples and cell lines revealed that livers harbouring TT express significantly less mRNA than the ones containing 3435CC and that the SNP was associated with a decrease in the mRNA stability (Wang *et al.*, 2005). A recent study performed by Kimchi-Sarfaty and collaborators at the National Institute of Health, Bethesda, may have found the main answer since they have shown that the 3435 C>T silent mutation alters the coded protein substrate specificity (Kimchi-Sarfaty *et al.*, 2007). It was suggested that this happens because of the presence of a less commom codon (considering the typical *H.* sapiens codon usage – http://www.kazusa.or.jp/codon/) that affects the timing of co-translational folding and further insertion of P-gp into the membrane (Kimchi-Sarfaty *et al.*, 2007). The

additional time spend in this process can alter the structure of substrate and inhibitor sites (Kimchi-Sarfaty *et al.*, 2007).

Pregnane X receptor (*PXR*, *NR1I2*)

Nuclear receptors (NRs) such as the pregnane X receptor (PXR, NR1I2) and the constitutive androstane receptor (CAR, NR1I3) play an important role in protecting the body against toxic xenobiotics. NRs are a large class of ligand activated transcription factors that function to regulate a wide array of cellular processes (Alarid, 2006).

PXR is a xenobiotic-induced nuclear receptor identified as a transcriptional regulator controlling the expression of numerous proteins which are collectively involved in the metabolism and excretion of lipophilic substances from the body. Among them are phase I enzymes such as CYP1A1, 1A2, 2A6, 2B6, 2C8, 3A4 and 3A5, phase II enzymes like GSTs, UGTs and SULTs, and membrane transporters such as P-gp and MRP2 (Maglich *et al.*, 2002; Tirona and Kim, 2005).

This nuclear receptor was identified independently in 1998 by three different groups as a protein involved in the induction of the cytochromes P450, in particularly CYP3As, and was named PAR, SXR and PXR, depending of the publication (Bertilsson *et al.*, 1998; Blumberg *et al.*, 1998; Kliewer *et al.*, 1998). In 2001 the human *PXR* gene with 38 Kb was located at the chromosome 3q13-q21 (Zhang *et al.*, 2001). It comprises 9 exons and the translation start site is located in exon 2 encoding a protein with 434 amino acids. The analysis of human PXR has shown a multiplicity of transcripts and 10 protein isoforms have been documented (Figure 15) (Lamba *et al.*, 2004; Kurose *et al.*, 2005; Fukuen *et al.*, 2002a). Studies that show the tissue distribution of the variants or their importance in the induction of the target genes are still lacking.

The structure of the PXR protein is similar to other nuclear receptors with a highly conserved DNA binding domain (DBD) linked via a flexible hinge region to the ligand-binding domain (LBD) (Carnahan and Redinbo, 2005). Contrary to other nuclear receptors, PXR is very promiscuous capable of biding a variety of structurally diverse ligands. It has a ligand-binding pocket unusually large (> 1100 Å^3) and flexible, spherical in shape and extremely hydrophobic (twenty of the twenty eight residues in the ligand-biding pocket are hydrophobic) (Watkins *et al.*, 2001; Orans *et al.*, 2005; for a review in structure and function of PXR see Carnahan and Redinbo, 2005).

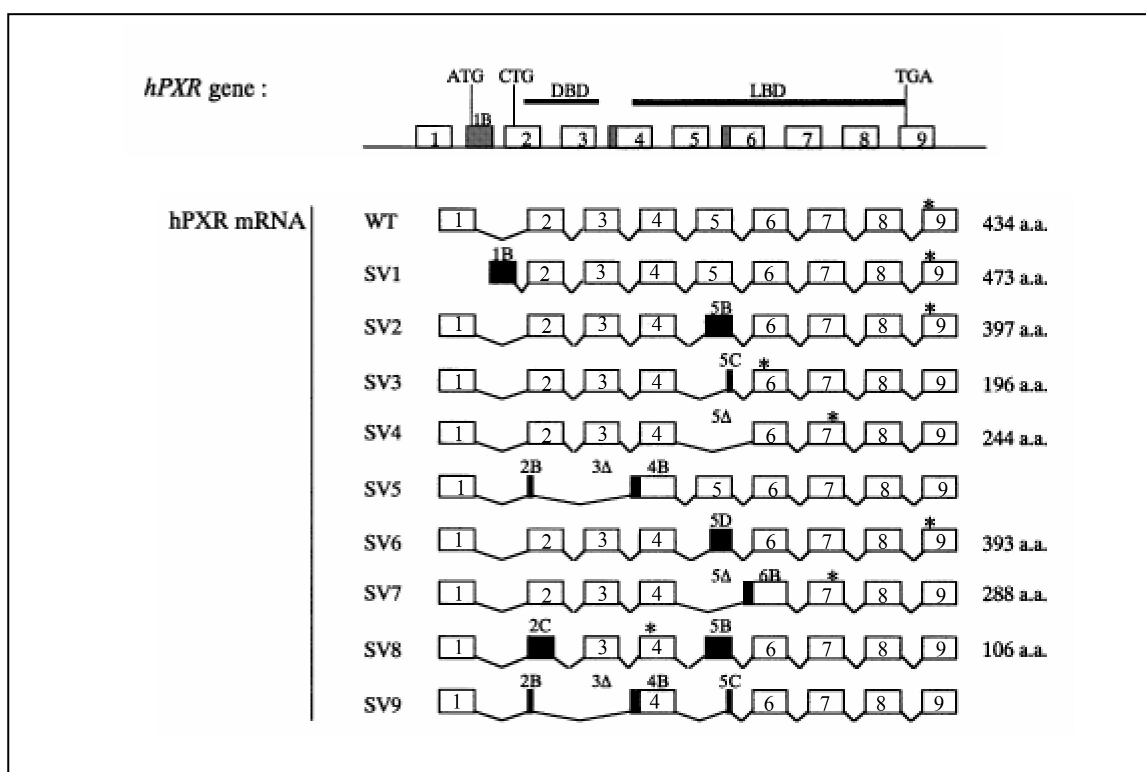


Figure 15 – Schematic representation of the hPXR alternative mRNAs (adapted from Fukuen *et al.*, 2002a). The exons are numbered and boxed and shaded in dark are the insertions. For complete skipping the exon number is missing. The termination codons of open reading frame are depicted as asterisks.

In 2001 Zhang and collaborators, as well as Hustert and collaborators, simultaneously described several SNPs both in the coding and non-coding regions of the *PXR* gene (Figure 16), associated with individual phenotype variations in basal and rifampin inducible expression of CYP3A and P-gp (Zhang *et al.*, 2001; Hustert *et al.*, 2001a). In addition to this original description of SNPs in *PXR*, additional polymorphisms have been described since then (Koyano *et al.*, 2002; Koyano *et al.*, 2004; Lim *et al.*, 2005; for a review in the genetic variation of PXR see Lamba *et al.*, 2005; http://www.hapmap.org).

From the different variants described in 2001, the 7635 A>G and 8055 C>T SNPs localized in intron 5 and 6, respectively were associated with a higher induction activity of PXR, with the consequent increase in erythromycin metabolism after rifampicin treatment. Two SNPs localized in the 3’ untranslated region, the 11156 A>C and 11193 T>C SNPs were correlated with decreased P-gp levels in the gut (Zhang *et al.*, 2001). The different variants identified in *PXR* gene can lead to altered induction transcription rates of target genes. These may affect the disposition of many currently administrated drugs, metabolised and transported by PXR induced proteins. Ultimately, polymorphisms in *PXR* leading to functional changes in this receptor may play a key role in the regulation of drug response.

The antimalarial chemotherapy includes many drugs that are substrates of XMEs and/or XRTs regulated by PXR. A new connection to this receptor was established with the discovery that artemisinin and the derivatives artemether and artesunate activate and act as ligands of human PXR (and also CAR). This leads to the induction of the expression of different CYPs and the transporter P-gp (Burk *et al.*, 2005). By *in vitro* methods the authors showed that artemisinin drugs induce the expression of *CYP2B6*, *CYP3A4* and *MDR1*.

PXR / NR1I2

3q13-q21

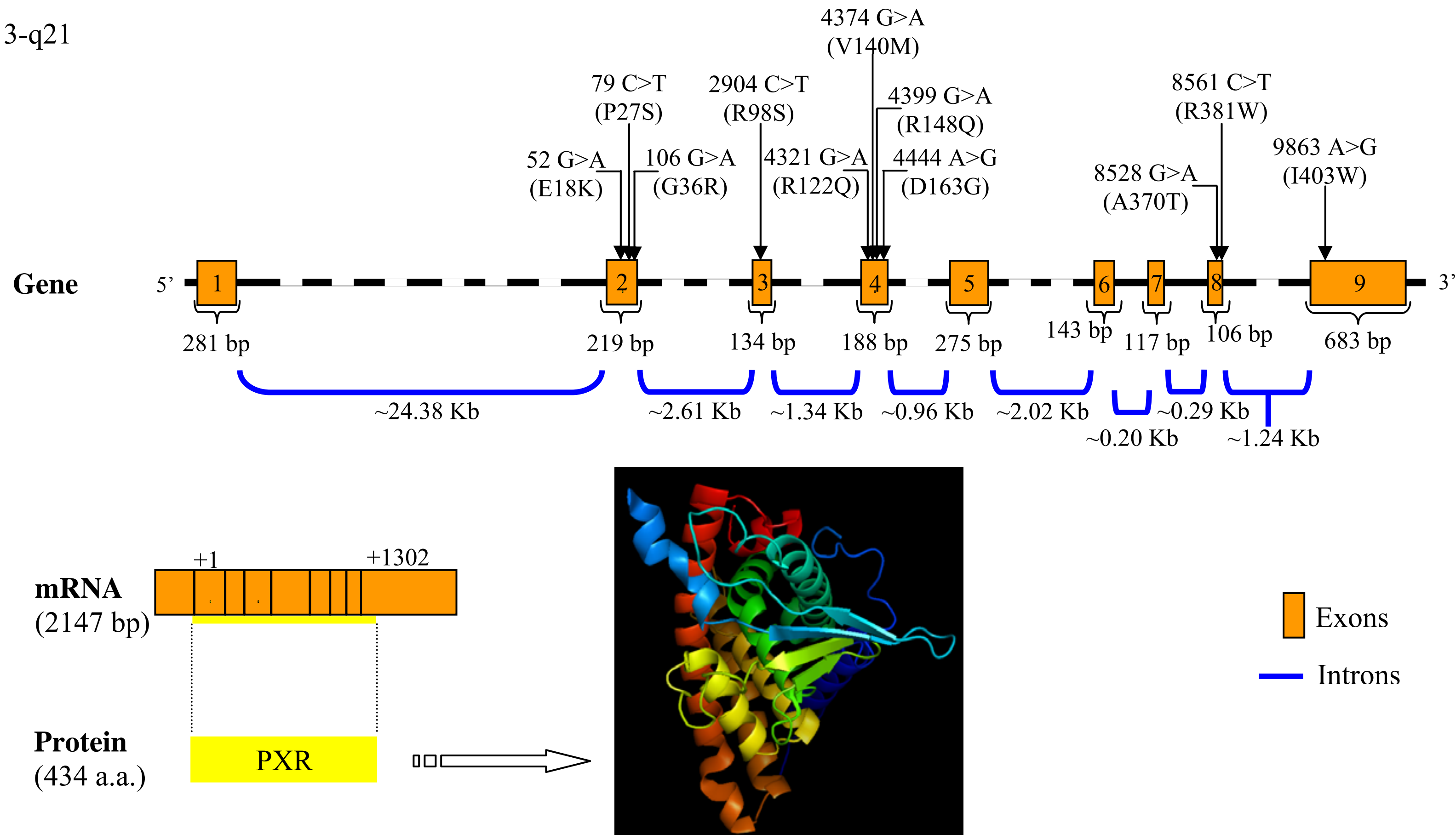


Figure 16 – The genomic structure of *PXR* gene was characterized in 2001 (Zhang *et al.*, 2001) and different nucleotide variants were identified since then. The coding SNPs are represented (data from Lamba *et al.*, 2005). The structure of PXR LBD was determined in 2001 (Watkins *et al.*, 2001) and the 3D from PDB is shown (PDB access number 1ILG).

AIMS OF THE STUDY

The overall aim of this thesis was:

A. The study of the genetic variability in enzymes involved in drug metabolism, especially in populations from regions where malaria represents a major public health issue. A particular emphasys was given to the antimalarial drug amodiaquine.

B. The possible contribution of XMEs associated to the management of REDOX stress in the susceptibility to the disease.

Specific aims of the study were:

- To develop new genotyping methods to study the variation in genes that code for enzymes involved in drug metabolism, membrane transporters and transcription factors. Development and optimisation of methodology was performed in the Portuguese population. This population was also the comparator Caucasian population.
- To study the variability of *CYP2C8* in the Zanzibar islands and its possible influence in the efficacy of amodiaquine. This was followed by the characterization of *CYP2C8* in different countries from Africa, Southeast Asia and South America, namely Sao Tome and Principe, Guinea-Bissau, Thailand and Colombia.
- To characterize the variability of *CYP3A5* and *PXR* in Sao Tome and Principe, Guinea-Bissau and Thailand.
- To investigate the enzymes responsible for the metabolism of desethylamodiaquine and posterior study of the variability of the enzymes involved.
- To analyse the influence of the deletion of *GSTM1* and *GSTT1* genes in the pathology of uncomplicated malaria.

METHODS

Genotyping Studies

Studied populations

In **Studies I**, **IV** and **VII**, healthy Caucasian Portuguese subjects from local medical check-ups were included in genotyping studies. Blood samples obtained from individuals of the Balanta ethnic group from Guinea-Bissau (Nhacra sector, a rural area between the rivers Mansoa and Geba, 26 to 51 Km from the capital) were included in **Study II**. Concerning **Study III** and the **Study** of *CYP1A1* and *CYP1B1* polymorphisms, the studied population comprised unrelated Zanzibari children with uncomplicated malaria from Unguja (Uzini Health Care Unit) and Pemba (Konde Health Care Unit). In **Study V** and **Study VII** unrelated subjects from Sao Tome and Principe, Guinea-Bissau and Thailand were analysed. A randomely selected population from Colombia was also genotyped in **Study V**.

Genotyping Methods

Genotyping is a procedure that allows the determination of frequencies of allelic variants in a certain gene. This is accomplished by the analysis of DNA extracted from different origins (e.g. a blood sample or a buccal swab). Different methods are available for genotyping - polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP), single stranded conformation polymorphism (SSCP), sequencing, real-time PCR using TaqMan probes and microarrays, to mention the most common.

The genotyping methods used in **Study I** to **V, VII** and in the **Study** of *CYP1A1* and *CYP1B1* polymorphisms were based on PCR-RFLP analysis and analysed SNPs in the following genes: *CYP1A1* (2455 A>G), *CYP1B1* (4326 C>G; 4360 C>G; 4390 A>G), *CYP2C8* (416 G>A; 1196 A>G; 792 C>G; 805 A>T), *CYP3A4* (-392 A>G; 673 T>C), *CYP3A5* (6986 A>G), *NAT2* (191 A>G), *MDR1* (-129 T>C; 3435 C>T; 2677 G>T) and *PXR* (7635 A>G; 8055 C>T; 11156 A>C; 11193 T>C).

In the analysis of *CYP1B1* polymorphisms, sequencing was used for the study of *CYP1B1* SNPs 142 C>G and 355 G>T. The sequencing was performed in Macrogen (www.macrogen.com, Seoul National University, Seoul, Korea) and the analysis of the results was made using the Chromas© software (McCarthy, Queensland, Australia)

The primers and enzymes used in the different studies are presented in Table 2. Specific assays were designed or were adopted from previous reports.

Influence of CYP2C8 polymorphism in AQ therapy

Experimental design

In **Study VI** the studied population comprised a control group of 110 children (patients with a positive outcome) and a case group of 63 children with recurrent infection, both groups with uncomplicated malaria. The population analyzed is from the two Zanzibar islands, Pemba (controls n = 44; cases n = 53) and Unguja (controls n = 66; cases n = 10). These children were treated with the first line treatment based on three daily doses of amodiaquine-artesunate (4 mg/Kg artesunate + 10 mg/Kg amodiaquine). As a comparator, another group of 96 controls (Pemba n = 34; Unguja n = 62) and 56 cases (Pemba n = 49; Unguja n = 7) were also included in the study corresponding to treatments with the second line treatment of six doses of a lumefantrine-artemether fixed combination (Coartem®, Novartis AG, Basel, Switzerland) (1 to 4 tablet twice daily, 20 mg of artemether and 120 mg of lumefantrine per tablet).

CYP2C8 SNPs 416 G>A, 792 C>G and 805 A>T were analysed using the same PCR-RFLP methods applied in the **Study III** (Table 2). To confirm the presence of heterozygous patterns in the case of 416 G>A (R139K) the samples were genotyped using Real Time PCR 5'-exonuclease based Taqman® assays (Applied Biosystems, Fresno, CA, USA) with the recommended protocol, in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Fresno, CA, USA).

Table 2 – PCR oligonucleotide primers used for RFLP analysis and sequencing of the different genes (lower case denotes the introduction of nucleotide mismatches for the generation of restriction enzyme cleaving sequences).

Detected Polymorphism	**PCR-RFLP Conditions**		**Reference**
	Primers 5' – 3'	**Analysis of the SNP**	
NAT2			
191 G>A	Fw1 - GAT CAC ATT GTA AGA AGA AAC CG Rv1 - GGA TGA AAG TAT TTG ATG TTT AGG Fw2 - GAT CAC ATT GTA AGA AGA AAC CG Rv2 - TTG GGT GAT ACA TAC ACA AGG G	*Msp* I	Study II
CYP3A4			
-392 A>G	Fw - AAT GAG GAC AGC CAT AGA GAC AAG GcC Rv - CAA TCA ATG TTA CTG GGG AGT CCA AGG G	*Bst* NI	Study I and II
673 T>C	Fw - AGA TTT GAT TTT TTG GAT CCA TTC TTT gTC Rv - CAA ATC ACT GAA CTG TAT ATT TTA AGT GG	*Alw*26 I	Study I

MDR1			
-129 T>C	Fw - TCT CGA GGA ATC AGC ATT CAG TCA ATC C Rv - CTA AAG GAA ACG AAG AGC GGC CTC TG	*MspA*1 I	Study I
3435 C>T	Fw - ATG GGC TCC GAG CAC ACC TG Rv - AGG CAG TGA CTC GAT GAA GGC	*Sau*3A I	Study I
2677 G>T	Fw - GTA CCC ATC ATT GCA ATA GCA Rv - TTT AGT TTG ACT CAC CTT CCg AG	*Alw*21 I	Study I
CYP2C8			
416 G>A	Fw - AGG CAA TTC CCC AAT ATC TC Rv - ACT CCT CCA CAA GGC AGT GA	*Bse*R I	Dai *et al.*, 2001
1196 A>G	Fw - CTT CCG TGC TAC ATG ATG ACG Rv - CTG CTG AGA AAG GCA TGA AG	*Bcl* I	Study III - V
792 C>G, 805 A>T	Fw - ATG TTG CTC TTA CAC GAA GTT ACA Rv - ATC TTA CCT GCT CCA TTT TGA	*Taq* I / *Bcl* I	Bahadur *et al.,* 2002

CYP3A5			
6986 A>G	Fw - TGA GCA CTT GAT GAT TTA CC RV - GGT CCA AAC AGG GAA GAG gT	*Rsa* I	Study VII
PXR			
7635 A>G	Fw - GCACTAGCTGTAGGTCAGGA Rv - CAGCAGCCATCCCATCATCAA	*Hph* I	Study VII
8055 C>T	Fw - TTGCTGAGAAGCTGCCCCTC**g**AT Rv - AGGAGCAAGGCCATAGACTGG	*Hinf* I	Study VII
11156 A>C 11193 T>C	Fw - CACACCGGAGAAGAACCATT Rv – TCTTACGCCGGAGTCTTCA	*Dde* I / *Bsi* YI	Study VII
CYP1A1			
2455 A>G	Fw - CGG TTT CTC ACC CCT GAT GGT GCT A Rv – CTC AAG CAC CTA AGA GCG CA	*Bse M*I	-

CYP1B1			
4326 C>G	Fw – GTC AAC CAG TGG TCT GTG AAT CAT GA Rv – CTG CAT CTT AGA AAG TTC TTC GCC A	*Bse N*I	-
4360 C>G 4390 A>G	Fw – ACC TGC CCT ATG TCC TGG CCT TCC T Rv – CTG CAT CTT AGA AAG TTC TTC GCC A	*Alu* I *Mwo* I	-
142 C>G 355 G>T	Fw- CACGCTCCTGCTACTCCTGTCG Rev- AGAAGTTGCGCATCATGCTG SEQ- AGTAGTGGCCGAAAGC	Amplification Sequencing primer	-

Metabolic pathways of DEAQ – The role of CYP1A1

Microsomal incubations

Human liver microsomes (HLM) are fractionated from other subcellular organelles by differential ultracentrifugation. *In vivo* clearances of several substrates can be accurately predicted using the substrate loss method with incubations using human liver microsomes (Mohutsky *et al.*, 2006).

Microsomes from human livers previously prepared and characterized (Westlind *et al.*, 1999) were used in this study. The first experiment was performed using 100 µM of DEAQ. In the other incubation a linear range regarding the concentration of drug (0 mM to 2 mM) was used. NADPH was used as co-enzyme in a final concentration of 1 mM. The reactions were allowed to proceed at +37ºC in a water bath for 20 minutes and were ended with the addition of ice-cold acetonitrile in a proportion of 50:50. Samples were immediately put on ice and stored at -80ºC until further analysis.

In all incubations duplicate samples were run, in parallel with controls without the presence of NADPH.

*InVitro*Somes™

Incubations using the *InVitro*Somes™ sample kit (InVitro Technologies, Inc., Baltimore, Maryland, USA) were performed following the instructions of the manufacturer. The kit consists in human cytochrome P450 enzymes and CYP-reductase co-expressed in *Saccharomyces cerevisiae*. The kit includes ten human recombinant CYPs: CYP1A1, CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2D6, CYP2E1, CYP3A4 and CYP3A5.

In the experiment a concentration of 100 µM of DEAQ was tested. After an incubation of 30 minutes or 1 hour at +37ºC, reactions were terminated with the addition of ice-cold acetonitrile in a proportion of 50:50. Samples were immediately put on ice and stored at -80ºC until further analysis. In the case of the 30 minutes incubation, duplicate samples were run. NADPH was used as co-enzyme and in both runs a control sample was included.

Chemical assay

Drug concentration determinations were conducted at Gothenburg University, Gothenburg, Sweden. Samples were thawed at room temperature. Following vortex mixing and a brief centrifugation at 3400 rpm for 5 minutes, 50µL was transferred to a new tube and 180 mL of phosphate buffer pH 2.0 was added. The concentration of DEAQ was determined using a previously described HPLC method with a Zorbax SB-CN column (Chromtech AB, Hägersten, Sweden) and UV detection at 242 nm (Lindegardh *et al.*, 2002). Linear calibration curves were generated by log-transformation of both axes. The coefficient of variation (CV) in quality control samples for DEAQ was 15 %, 4 %, and 4 % at 10, 75 and 1000 µM respectively. The lower limit of quantification (LLOQ) was set at 3 nM (CV was 13 %).

CYP1A1 sequencing

The microsomal incubations indicated that CYP1A1 was the enzyme involved in the metabolism of DEAQ. To evaluate the role of *CYP1A1* gene variability in the treatment outcome a search for new mutations in the gene was performed. DNA from malaria patients from Papua New Guinea (n= 19) and Zanzibar (n= 12) was used to sequence *CYP1A1* exons and part of the introns and promoter region. These patients were characterized in terms of AQ and DEAQ pharmacokinetics and were genotyped for the main *CYP2C8* SNPs (Hombhanje *et al.*, 2005; Friberg Hietala *et al.*, 2007).

Specific primers were designed for the amplification and sequencing of CYP1A1 using the sequence NC_000015 (Table 3). A long PCR was performed to amplify a fragment encompassing all the exons and introns after the initiation codon. This fragment of 3023 bp was used to amplify four smaller sequences analysed by sequencing in Macrogen (www.macrogen.com, Seoul National University, Seoul, Korea). Sequencing analysis was performed using the Chromas© (McCarthy, Queensland, Australia) and the Sequencher™ software (Gene Codes Corporation, Ann Arbor Michigan, USA).

Table 3 – Sequence of the primers used for *CYP1A1* amplification and sequencing.

	Primers 5' – 3'	**Fragment length (bp)**
1st Amplification (Long PCR)	GTC TGC ATC CAA CAC TTT CTG CAG GCT GAA CCT TAG ACC ACA	3023
Fragment 1	AGT AAG CAG TTC TGG TGG AAA G CAG TTC CTC TTA CCT TTG ACC T	1045
Fragment 2	TCA TCC CTA TTC TTC GCT ACC CTT AGG TAG GGA AAG TCC ACA	674
Fragment 3	TGT GCT AGG AAT AGT GAA GGA C CTT TCC TCT GCA TCT CTG AAC	865
Fragment 4	CCA CTG CTG TCT GTT ACT GAT C CAG GCT GAA CCT TAG ACC ACA	788

Pyrosequencing analysis of *CYP1A1*

New sequence variation in *CYP1A1* promoter was observed by the analysis of the sequencing results. To confirm three of these variations a pyrosequencing assay was developed, since their proximity in the gene did not allow the use of other methods. The protocol for analysis was designed with the aid of the Pyrosequencing™ Assay Design Software, version 1.0 (Biotage AB, Uppsala, Sweden). The primers used for the amplification of the fragment were FW 5'- GTT TCC CCT TTC CCT GAC A – 3' and RV 5' – GAG AGA AGG TGC AGG AAG AAA AAA – 3' and the primer for the sequencing reaction was 5' – CCT TTC CCT GAC ACT CTA – 3'. Samples were analyzed on a PSQTM 96MA instrument using pyrosequencing software PSQTM96MA (Biotage, Uppsala, Sweden).

The role of GSTM1 and T1 in uncomplicated malaria

In **Study VIII** a group of 1885 patients from Unguja (Uzini Health Care Unit) and Pemba (Konde Health Care Unit) were analysed. Among these, 1515 patients corresponded to selected children with uncomplicated malaria. A group of 370 patients enrolled in this study were verified to be negative for the presence of *P. falciparum* and were used as controls.

The analysis of *GSTM1* and *GSTT1* deletion polymorphism was performed adapting a PCR mutiplex method (amplification of *GSTM1*, *GSTT1* and a control gene (*CYP1A1*)) described by Abdel-Rahman and co-workers (Abdel-Rahman *et al.*, 1996).

Statistical analysis

Allelic frequencies and confidence intervals were assessed using the program CIA (Confidence Interval Analysis) (Gardner and Altman, 1989). Chi-square (χ^2) testing was performed with Microstat® software (Ecosoft Inc, Indianapolis, IN, USA) and GraphPad software (www.graphpad.com). For the evaluation of Hardy-Weinberg equilibrium on the analyzed SNPs the GenePop software pack was applied (http://wbiomed.curtin.edu.au/genepop/). The PXR haplotypes and frequencies were obtained by the PHASE Version 2 Software (Stephens *et al.*, 2001; Stephens and Donnelly, 2003; http://stephenslab.uchicago.edu/software.html).

RESULTS AND DISCUSSION

Details of the results are found in the papers I-VIII.

Genotyping Studies

The use of genotyping determination and phenotyping measurements of drugs and/or drug metabolites aims to evaluate the clinical significance of the genotype for the variability in response and side effects. As such, the connection is made between the SNPs in a candidate gene and alterations in pharmacokinetics and/or the clinical outcome.

The common use of phenotyping measurements (e.g. drug pharmacokinetics) in a Developing World typical malaria scenario is far way from becoming a reality, due to several logistic reasons, further aggravated by the characteristic young age of the majority of the malaria patients in those settings. At this stage, pharmacogenetic information about the region's most representative population of malaria patients can be relevant. A better knowledge of the genetic variability in populations from these settings could be an important background for the future pharmaco-vigilance initiatives. In this thesis genotyping studies evaluated the interethnic variability in populations from regions where this information is very limited or even absent.

Study I - *CYP3A4* and *MDR1* alleles in a Portuguese population

The pharmacogenetic characteristics of the Portuguese were assumed as a Caucasian comparator for further discussion with the several non-European populations herein analysed.

In a sample of one hundred unrelated subjects representative of the Caucasian Portuguese population two genes coding for proteins with a major role in drug disposition were analysed: *CYP3A4* and *MDR1*. The frequencies of *CYP3A4*1B* (-392 A>G) and *CYP3A4*2* (673 T>C) were found to be 4.0% and 4.5%, respectively, with no homozygous individuals observed for both alleles (Table 4). Concerning the *MDR1* gene, two of the three variant alleles analysed were present in high frequencies, 3435T with 64.5% and 2677T with 47.5% (Table 4); in the case of the -129 T>C SNP, the presence of the C allele was observed in a frequency of 5.0%. Linkage between the 3435 C>T/ 2677 G>T SNPs was observed, with an association of 3435 C/C with 2677 G/G genotypes in a frequency of 25.8%. We did not find any individual carrying the 3435 C/C associated with 2677 T/T and no significant linkage was observed between the -129 T>C SNP and the other *MDR1* polymorphic positions here analysed.

The description of CYP3A4 and MDR1 SNPs frequencies was the first in the Portuguese population, except for the case of 3435 C>T in *MDR1* previously included as one of several populations described by Ameyaw and collaborators (Ameyaw *et al.*, 2001). The frequency observed in our study for 3435 C>T (64.5%) does not differ significantly from the previously reported in that study conducted in the Centre-North regions of Portugal (57.0%).

The existence of linkage disequilibrium between the synonymous 3435 C>T and 2677 G>T/A (and 1236 C>T) was proposed in 2001 in an attempt to explain the

phenotype associated with 3435 C>T (Kim *et al.*, 2001; Tanabe *et al.*, 2001).The linkage described in the initial reports was around 94% in Japanese, 62% in Caucasian Americans but only 13% in African-Americans, showing a high degree of variability in different populations. In our study the degree of linkage was found to be only of 25%, a value markedely below the registered before. This variability may indicate that the existence of a linkage is not the only explanation for the phenotype observations associated with 3435 C>T. Also, the studies where the influence of *MDR1* SNPs on the disposition of P-gp substrates or treatment outcome was evaluated do not show a conclusive influence of 2677 G>T/A and 3435C>T (Drescher *et al.*, 2002; Owen *et al.*, 2005).

In the absence of results that could fully explain the effect of 3435 C>T in terms of phenotype, the research in the field continued and recently it was shown that 3435 C>T affects the mRNA stability (Wang *et al.*, 2005) and/or alters the substrate specificity through the disturbance of the native folding of the protein (Kimchi-Sarfaty *et al.*, 2007). In particular, this last report challenges the current paradigm by showing that is possible for a synonymous SNP to markedly influence the functionality of the coded protein. The study revealed that the changes observed *in vitro* in the phenotype were not due to the presence of 2677 G>T, that the haplotype 1236C>T – 2677G>T – 3435C>T was associated with similar mRNA or protein levels and that this haplotype is associated with slight alterations in the tertiary structure. More, the authors show that the *MDR1* haplotype can have an important role in the cases where higher levels of mRNA are present, and by consequence more P-gp is being translated. The authors hypothesise that with a higher production of P-gp, the role of codon usage may become critical when certain tRNA species become depleted. They show the presence of lower percentages of codon usage when different *MDR1* variants are present. With these

results the authors presents the hypothesis that when frequent codons are changed and replaced by rare codons it may result in altered function, by alterations in the co-translational folding (Kimchi-Sarfaty *et al.*, 2007).

Table 4 - *CYP3A4* and *MDR1* Single Nucleotide Polymorphism frequencies in the studied Portuguese population (n= 100).

	Genotype	Frequency (95% CI)	Allele	Frequency (95% CI)
CYP3A4				
-392 A>G	A/A	0.920 (0.848 – 0.965)	A	0.960 (0.923 – 0.983)
	A/G	0.080 (0.035 – 0.152)	G	0.040 (0.017 – 0.077)
	G/G	0 (0.000 – 0.0362)		
673 T>C	T/T	0.910 (0.836 – 0.958)	T	0.955 (0.916 – 0.979)
	T/C	0.090 (0.042 – 0.164)	C	0.045 (0.021 – 0.084)
	C/C	0 (0.000 – 0.0362)		
MDR1				
-129 T>C	T/T	0.910 (0.836 – 0.958)	T	0.950 (0.910 – 0.976)
	T/C	0.080 (0.035 – 0.152)	C	0.050 (0.024 – 0.900)
	C/C	0.010 (0.0003 – 0.055)		
3435 C>T	C/C	0.120 (0.064 – 0.200)	C	0.355 (0.289 – 0.421)
	C/T	0.470 (0.369 – 0.572)	T	0.645 (0.579 – 0.711)
	T/T	0.410 (0.313 – 0.513)		
2677 G>T*	G/G	0.310 (0.221 – 0.410)	G	0.525 (0.456 – 0.594)
	G/T	0.430 (0.331 – 0.533)	T	0.475 (0.406 – 0.544)
	T/T	0.260 (0.177 – 0.357)		

*The rare G2677A variant was not analysed in this study. Due to its low frequency among Caucasians, we believe that its discrimination would not change significantly the results and conclusions of this work.

These new data show how a non-synonymous mutation can be important to a final phenotype and have to be considered as being the basis for some current diseases, as well as for variable drug efficacy.

Study II - Cytochrome P450 3A4 and N-acetyltransferase 2 genetic polymorphism in a native African population

In **Study II** we determined the frequency of *CYP3A4*1B* in native subjects from Guinea-Bissau, using the method previously developed for the analysis of the Portuguese population. The same population was also analysed for one of the major function-altering SNPs in the *NAT2* gene, 191 G>A. This study included patients from the Balanta ethnic group, a rural population from Guinea-Bissau, where another study had previously shown that there is a high prevalence of TB, in strong association with HIV-2 infection (Antunes *et al.*, 2002). Guinea-Bissau is a country with poor health infrastructures, while facing the burden of several major infectious diseases, such as TB, typhoid fever, bacillary dysentery, the aforementioned HIV/AIDS and malaria. Malaria is endemic with stable transmission. Guinea-Bissau has recently officially adopted the ACT as first line treatment (AL) in the control of the disease (Figure 5). Full implementation of ACT in this country is several years away, with CQ still being the main drug present and applied in public health centers throughout the territory, since it is very inexpensive and still effective (Kofoed *et al.*, 2007; Ursing *et al.*, 2007a) and readily available.

At the time of the study, the *CYP3A4*1B* polymorphism was chosen for two reasons: it was the polymorphism most extensively documented for its functional effect, and it had been described as frequent in African-Americans.

After our study was performed, new data appeared relating CYP3A4 and the metabolism of antimalarial drugs. Adding to the long known role of CYP3A4 in the metabolism of quinine, this enzyme has also been recently reported to play a role in the metabolism of CQ (Projean *et al.*, 2003) and *CYP3A4*1B* has been specifically associated with a protein that presents a reduced *in vivo* metabolism for quinine (Rodriguez-Antona *et al.*, 2005).

In the 50 Guinea-Bissau patients analysed we determined a frequency of 72.0% for *CYP3A4*1B* (-392 A>G) allele, which corresponds to the presence of 18 heterozygotes and 27 homozygotes with the altered sequence. This suggests that a high percentage of the population can have in circulation higher doses of CQ compared with individuals bearing *CYP3A4*1* alleles (if we assume that CQ follows the same pattern of quinine, however we have to take in account that the PK characteristics of the drugs are very different). These doses are expected to be even higher if we take into account that the use of CQ in Guinea-Bissau does not normally follow the WHO recommendations of 25 mg/kg (10+10+5, 3 days), being used in higher concentrations, up to 75 mg/Kg in a different schedule (Kofoed *et al.*, 2002; Ursing *et al.*, 2007b). The existence of acute toxicity with the higher dosage was, however, not reported (Kofoed *et al.*, 2002).

Besides malaria, tuberculosis (TB) is also a public health problem in Guinea-Bissau, which, as other countries in the world, has implemented the DOTS policy (Direct Observed Treatment Strategy, the internationally recommended control strategy for TB). In a WHO report from 2007 the coverage of DOTS in Guinea-Bissau was reported to be 85% (WHO, 2007). The standard treatment regimen implemented for previously untreated patients consists of 2 months of streptomycin, isoniazid, rifampin and pyrazinamide, followed by 6 months of isoniazid and ethambutol (the 2SHRZ/6HE

regimen). Isoniazid (INH) is an important component of this anti-TB chemotherapy and it is extensively metabolised in the liver by the polymorphic NAT2.

The 191 G>A SNP in the *NAT2* gene, associated with slow acetylation, was suggested to be of African origin by Bell and co-workers, in their early descriptions in African-Americans (Bell *et al.*, 1993). Although studies of the effect of NAT2 genotypes on the plasma concentration of INH had been performed in different populations, the effect of 191 G>A SNP was not evaluated since this allele was not present in the previously studied populations.

We determined the frequency of this variant in 125 Balanta subjects and found a prevalence of 19.2%. As such, an important percentage of the population is expected to display a slow acetylator phenotype, which means that they may accumulate higher circulating levels of INH. This drug is, long known, to be associated to serious adverse effects such as hepatotoxicity, peripheral neuropathy and/or agranulocytosis (Forget and Menzies, 2006). A high frequency of slow acetylator individuals (>15%) may also be the basis for an increased incidence of INH driven drug-drug interactions, as this drug is documented to inhibit several drug metabolizing CYPs (e.g., CYP2A6, CYP2C9, CYP2C19 and CYP3A4) (Wen *et al.*, 2002; Nishimura *et al.*, 2004). When this effect of INH in the inhibition of different CYPs was tested, only standard drugs like coumarin (CYP2A6), warfarin (CYP2C9), mephenytoin (CYP2C19) or midazolam (CYP3A4) were used.

In a country like Guinea-Bissau where beside the chemotherapy against TB, the same individual can be also subjected to antimalarial and/or anti-HIV/AIDS chemotherapy, the problem of drug-drug interactions can take important proportions that normally are not evaluated. A recent study, however, had analysed the interaction between amodiaquine plus artesunate and efavirenz and showed the existence of

hepatotoxicity due to a drug interation (German *et al.*, 2007). They show that the administration of efavirenz togheter with AS+AQ lead to an increase in AQ exposure and decrease in DEAQ exposure, and to increased levels of liver-associated enzymes after the administration of both regimens of drugs (alanine aminotransferases and aspartate aminotransferase) (German *et al.*, 2007). With this first report showing that interations between antimalarial and anti-HIV/AIDS chemotherapy can lead to toxic events it is important try to evaluate the real consequences of the co-administration of drugs.

The full sequencing of the *NAT2* gene was futher performed in a population of Vietnam (side study not formally included in the frame of this thesis – see ***Other publications***). The results obtained revealed a high frequency (90.8%) of at least one *NAT2* fast allele, particularly associated to *NAT2*13*. While this allele is commonly observed at frequencies ≤ 5%, in this study it was detected at a prevalence of 34.3%, significantly different ($P < 0.0001$) from any other population previously analyzed in the region, including studies in Thailand (< 5.1%; χ^2=64.39) (Kukongviriyapan *et al.*, 2003) and Cambodia (**13* not detected; χ^2 = 31.64) (Bechtel *et al.*, 2001). Different SNPs are absent in this population, including the 191 G>A SNP common in the Guinea-Bissau analysed population. The SNPs of the *NAT2*7* allele are among those that were not observed in the analysis of this Vietnamese population, which is in a clear contrast with the results reported for *NAT2*7* in Thailand (20.4%) and Cambodia (6.3%).

The differences in the percentage of slow acetylators in the populations might lead to differences in the incidence of adverse effects caused by over-exposure to INH and also in the incidence of INH driven drug-drug interactions (both higher in populations with high frequencies of slow acetylators) (the effect of acetylator status on drug response and toxicity is summarized in Butcher *et al.*, 2002).

Study III, IV and V – CYP2C8 polymorphism in different populations

As previously mentioned, CYP2C8 is an important enzyme in the metabolism of CQ and the main responsible for the metabolism of AQ. PCR-RFLP methods for the detection of *CYP2C8* major alleles were developed and optimized. The results obtained in the Portuguese population (**Study IV**) show a distribution of 1.2% for *CYP2C8*2*, 19.8% for *2C8*3* and 6.4% for *2C8*4* (Table 5). *CYP2C8*3* was the more frequent allele in line with the other reports in Caucasian populations (e.g. United Kingdom, Western Germany or Central Spain) (Bahadur *et al.*, 2002; Weise *et al.*, 2004; Garcia-Martin *et al.*, 2004). The same trend is not observed in native African populations (Table 5), where *CYP2C8*2* allele is the more frequent instead of *CYP2C8*3*.

CYP2C8 has been described as playing a role in the endogenous metabolism in biosynthesis of vasoactive factors (Fleming, 2004). *CYP2C8*3* allele has been associated to a higher risk of acute myocardial infarction (Yasar *et al.*, 2003). The high frequency of this allele among the Portuguese and in other Caucasian populations lead to a hypothesis that the *CYP2C8*3* may be a possible factor in individual elevated risk for the development of cardiovascular diseases. It would be of value to study a possible association between specific CYP2C8 alleles and the risk of cardiovascular diseases in the Portuguese population.

The characterization of CYP2C8 polymorphism in populations of different ethnic origins from tropical areas where 4-aminoquinoline antimalarial therapy is used was performed in **Study III** and **V**. Alleles that lead to CYP2C8 enzymes with altered activities show different prevalences in the studied populations (Table 5). *CYP2C8*2* is the most frequent variant allele in the African populations analyzed, with frequencies

ranging from 26.6% in Guinea-Bissau to 9.0% in Sao Tome and Principe, in contrast with the 3.8% observed in Colombia (population predominantly of hybrid descendence – “Mestizo”). For *CYP2C8*3* and *CYP2C8*4* a different profile was observed. In the case of *CYP2C8*3* the highest frequency was observed for the Colombians (7.1%), followed by subjects from Sao Tome and Principe (4.0%) and Zanzibar (2.1%). Finally, *CYP2C8*4* allele is present at low allelic frequencies in the studied populations, ranging from 1.6% in Colombia to 0.6% in Zanzibar. In Guinea-Bissau neither *CYP2C8*3* nor *CYP2C8*4* were found. In Thailand all subjects showed the wild type genotype for the polymorphic positions analysed.

Concerning the data obtained for Guinea-Bissau only one of the alleles was observed – *CYP2C8*2* (Table 5). The protein encoded by *CYP2C8*2* has been documented to be associated to a higher K_m than *CYP2C8*1* (but a similar V_{max}) for paclitaxel, which leads to a lower enzymatic activity of CYP2C8 in paclitaxel hydroxylation (Dai *et al.*, 2001). A recent *in vitro* study using AQ as a substrate showed that *CYP2C8*2* allele also present a higher K_m, but a lower V_{max} was observed (Parikh *et al.*, 2007).

In Guinea-Bissau’s population the *CYP2C8*2* allele is present in the homozygote form in 10.4% of the individuals, so most part of the population (~ 90%) have a CYP2C8 enzyme that can eventually compensate the existence of a high frequency of -392 A>G SNP in *CYP3A4*. In the fraction of the population where the allele is present, particularly if combined with a defective CYP3A4 enzyme, the patients are potentially subjected to a longer exposure to drugs metabolized by these enzymes, which can lead to a higher risk of adverse effects.

Table 5 – Genotypic Frequencies observed for *CYP2C8* SNPs in the different studied populations.

Genotype *CYP2C8*	**792 C>G** **(I264M)**	**805 A>T** **(I269F)**	**1196 A>G** **(K399R)**	**Zanzibar** (n = 165)	**Portugal** (n = 164)	**STP** (n = 50)	**Guinea-Bissau** (n = 96)	**Colombia** (n = 91)	**Thailand** (n = 116)
**1/*1*	-/-	-/-	-/-	0.704	0.567	0.740	0.573	0.769	1.00
**1/*2*	-/-	+/-	-/-	0.218	0.018	0.140	0.323	0.077	0
**2/*2*	-/-	+/+	-/-	0.030	0	0.020	0.104	0	0
**1/*3*	-/-	-/-	+/-	0.030	0.262	0.080	0	0.099	0
**3/*3*	-/-	-/-	+/+	0.006	0.043	0	0	0.022	0
**1/*4*	+/-	-/-	-/-	0.012	0.043	0.020	0	0.033	0
**4/*4*	+/+	-/-	-/-	0	0.018	0	0	0	0
**1/*3*4*	+/-	-/-	+/-	0	0.037	0	0	0	0
**1/*2*3*	-/-	+/-	+/-	0	0.006	0	0	0	0
**3/*4*	+/+	-/-	+/-	0	0.006	0	0	0	0

In Sao Tome and Principe malaria is also the main public health problem, accounting to almost 50% of the hospital deaths (http://www.theglobalfund.org/; Project Proposal for Malaria Control in São Tomé and Príncipe). The national antimalarial chemotherapy policy has recently adopted AS+AQ combination as the officially first-line therapeutic, replacing CQ and SP. The change in the drug policy adopted is a consequence of the increase of drug resistance in the islands – CQ has reached a clinical treatment failure of 67% and SP of 20% (http://www.theglobalfund.org/; Project Proposal for Malaria Control in São Tomé and Príncipe). Altought the frequency of SP failure is low when compared with CQ, recrudescence of SP-resistance parasites were also associated with 6% of failure in the test of AS+SP combination. Concerning the data obtained for *CYP2C8* in Sao Tome and Principe we observed that the alleles studied are all present in low frequencies (Table 5). The percentage of individuals that have the homozygous form of the SNPs analysed is *circa* 2%, and if we consider the population that can be exposed to antimalarial chemotherapy (CQ or AQ) it is not a negligible frequency that should be taken in account.

If we compare the results of *CYP2C8* genotyping from Sao Tome and Principe and Guinea-Bissau with the available data we can observe that *CYP2C8*2* is found in Guinea-Bissau at high frequency (26.6%). This result contrast with the results reported for other African populations (Zanzibar - 13.9%, $P = 0.0005$; Burkina Faso - 11.5%, $P = 0.0002$; Ghana - 16.8%; $P < 0.0001$) (Parikh *et al.*, 2007; Röwer *et al.*, 2005). The studied subjects from the islands of Sao Tome and Principe show a significantly lower prevalence of *CYP2C8*2* allele (9.0%; $P = 0.0072$) and in contrast to the observed in Guinea-Bissau, the *CYP2C8*3* and *CYP2C8*4* alleles were detected in Sao Tome and Principe. The frequencies observed for *CYP2C8*2* in Sao Tome and Principe are statistically different from the results in Zanzibar ($P = 0.0111$) and statistically significantly different from the frequencies in Ghana and Burkina Faso ($P = 0.0019$, $P =$

0.0071, respectively). These differences can be a reflection of a richer diversity in Africa compared with, for example, Europe where the diversity is not so evident.

Countries from Central and South America also have to face the problem of malaria and Colombia is not an exception. *P. falciparum* resistance to different antimalarials in Colombia has been reported, namely to CQ (47% to 97%), AQ (3%–7%) and to SP (9%–13%) (Blair *et al.*, 2001; Osorio *et al.*, 1999). The antimalarial policy in Colombia is in constant change. Anyway, the main national first line treatment is still in most cases the combination of the use of AQ (3 days), SP and PQ (www.who.int/malaria/treatmentpolicies.htm). The Colombian population consists in an admixture between local Indian, European (mainly from the Iberian regions) and Africans (Bravo *et al.*, 2001; Salas *et al.*, 2005). This fact is translated also in the frequencies observed for *CYP2C8* alleles, since the pattern does not follow the Caucasian pattern, where *CYP2C8*3* is present at high frequency, neither African populations where *CYP2C8*2* is the predominant allele (Table 5). The *CYP2C8* allelic frequencies observed in the Colombian setting revealed a 2% frequency of predicted slow metabolizers (Table 5). Although the percentage of the population at risk of a longer exposure to the drug seems to be low, this is relative and depending of the total population submitted to antimalarial therapy.

In Thailand none of the studied *CYP2C8* alleles were observed, in line with what was previously observed in Asian populations (reviewed in Gil and Gil Berglund, 2007). Although the population has a different origin, in Papua New Guinea the polymorphism in *CYP2C8* was also not observed (Hombhanje *et al.*, 2005). As such, populations from Asian origin are expected to be all efficient metabolizers for CYP2C8

substrates. This typical profile can contribute to a low frequency of severe adverse effects associated with drugs metabolized by this enzyme, including amodiaquine.

The population affected by malaria from the Zanzibar islands was the most thoroughly characterized from all the studied populations. Besides the aforementioned *CYP2C8* (**Study III**), the main *CYP1A1* and *CYP1B1* polymorphisms were also investigated.

The islands of Zanzibar are a setting where malaria represents a major public health issue. Here the first line treatment adopted is based on three doses of AS+AQ in a three day course (4 mg/Kg artesunate + 10 mg/Kg amodiaquine per day). Concerning the analysis of *CYP2C8*, the population studied follows the same trend subsequently observed in the other African populations studied, a high incidence of *CYP2C8*2* (13.9%) (Table 5). The other variants (**3* and **4*) are present in lower frequencies of 2.1% and 0.6%, respectively. The variant alleles are present in 16.6% of the analysed population, normally in the heterozygous form, where 3.6% corresponds to homozygous form. This study in the Zanzibar islands in the only one presently available in East African populations, being a comparison reference to studies performed in West Africa (previously discussed).

The predicted number of amodiaquine slow metabolizers found in ours and other published works is not negligible, taking in account the incidence of malaria in the respective studied settings. This should be taken in account in the future pharmaco-vigilance initiatives, in particular in frame of a possible trend of increased use of AQ following the recent introduction of an AS + AQ fixed combination by Sanofi-Aventis/DNDi (http://dndi.org/pdf_files/press_release_march_1-eng.pdf).

In an attempt to evaluate the influence of CYP2C8 polymorphism in AQ efficacy we have determined the frequencies of *CYP2C8* major alleles in a Zanzibar population where the treatment outcome was analysed after the antimalarial therapy with AS + AQ (**Study VI -** ***Influence of CYP2C8 polymorphism in AQ therapy***). From the analysis of *CYP2C8* genotype we observed that the genotype had no significant influence in the outcome (Table 6), when comparing a control group where the treatment was successful and another where the patients experience a recurrence of the infection. This was observed in AS+AQ treatment group but also in a group treated with AL (data not shown), the later being a negative control for the study, as CYP2C8 is not involved in the metabolism of these drugs.

The results obtained with the populations of the Zanzibar islands treated with AS+AQ are in line with recently published data from an AQ monotherapy efficacy trial performed in Burkina Faso (Parikh *et al.*, 2007). In that study the analysis is presented only for the case of *CYP2C8*2* (the main allele present in native sub-Saharan African populations) but for the three different groups: (1) adequate clinical and parasitological response, (2) recrudescence and (3) new infection. Even with this stratification, variations in the efficacy outcome were not observed, dependent on CYP2C8 status.

Considering the fact that presently AQ is mainly administred in combination with AS (with a high PRR) and it is rapidly biotransformed in DEAQ, the contribution of AQ in the therapy is, in fact, expected to be minimal. This can explain why in the population analyzed a protective effect by the different alleles was not observed. The present observations might change in long term, with the development of a scenario where the parasite has developed resistance to DEAQ. Then, an expected larger AQ AUC associated to subjects carrying low activity alleles will become significantly more influencial for the success of the therapy.

Table 6 - Frequencies for *CYP2C8* genotypes in the two groups of Zanzibar population of *P. falciparum* malaria patients treated with AS+AQ.

Genotype	Patients with positive outcome (n=110)		Patients with recurrences (n= 63)		*p-value*
	n	Frequency (95% CI)	n	Frequency (95% CI)	
*1/*1	66	0.600 (0.508 – 0.692)	38	0.603 (0.472 – 724)	0.9837
**1/*2*	36	0.327 (0.240 – 0.415)	20	0.317 (0.206 – 0.447)	0.9243
**2/*2*	3	0.027 (0.006 – 0.078)	1	0.016 (0.0004 – 0.085)	0.6385
**1/*3*	4	0.036 (0.010 – 0.090)	4	0.064 (0.018 – 0.155)	0.6866
**1/*4*	1	0.009 (0.0002 – 0.050)	0	0 (0.000 – 0.057)	0.4499

The new data concerning the biotransformation of AQ show that the metabolism is impaired by the presence of *CYP2C8*2* and *CYP2C8*3* (Parikh *et al.*, 2007). These observations had led to the prediction by the authors that in the case of both variants the concentration-time profiles of AQ and DEAQ could be altered. In the case of DEAQ however, and contrary to the hypothesised by the authors, the significant interindividual diversity in PK parameters observed in a study conducted in Papua New Guinea, was not explained by the *CYP2C8* polymorphism, as all subjects were confirmed as only carrying the wild type allele (Hombhanje *et al.*, 2005).

The alterations in the concentration-time profiles of AQ and DEAQ can be important in the risk developing drug induced adverse events. AQ toxicity has been associated to the formation of a quinoneimine reactive product and DEAQ shows significantly reduced capacity to generate this toxic metabolite, when compared with AQ (Clarke *et al.*, 1991; Jewell *et al.*, 1995; Tingle *et al.*, 1995). This suggests that the formation of DEAQ can be a pathway associated to a decreased risk for the advent of

quinoneimine related cytotoxic effects. In the study from Parikh and collaborators this issue was also evaluated and no major adverse events were reported in the population analyzed (Parikh *et al.*, 2007). However, an interesting significant increase in mild side effects (self reported rate of abdominal pain: 52% vs 30%, $P < 0.01$) was detected among the carriers of the *CYP2C8*2*. To note that this allele, although with decreased AQ metabolism activity (significantly increased K_m and decreased V_{max}), is still able to biotransform this drug, while the *CYP2C8*3* associated SNPs seems to severely affect the coded enzyme. In fact, the kinetic parameters of the enzyme were not possible to be determined, as no AQ metabolism was detected (Parikh *et al.*, 2007). These results mirror the previously observed with the probe drug paclitaxel, where a decrease to 15% of the metabolism capacity of the "wild type" allele (*CYP2C8*1*) was documented (Dai *et al.*, 2001). Faced with this new data, we are left to speculate if the *CYP2C8*3* allele – more frequent among Caucasians, as we determined among the Portuguese population – had playing a role in AQ driven serious adverse events observed on the past among Caucasian travellers.

The new information concerning the effect of CYP2C8 variants in the AQ metabolism and possible effect in efficacy and adverse events should be put in context to recently documented drug-drug interctions data. The inhibitory effect of different anti-HIV/AIDS drugs (e.g. efavirenz, nevirapine, saquinavir, lopinavir, *etc*) and also the antimalarial pyrimethamine on CYP2C8 activity has been evaluated (Parikh *et al.*, 2007). The antimalarial pyrimethamine was shown to inhibit CYP2C8 although in a range of concentrations that are not achieved in the standard dosing. More important for the scenario where AQ has been implemented, is the strong inhibition observed in the case of clinically relevant concentrations of anti-HIV/AIDS drugs efavirenz, saquinavir, lopinavir and tipranavir. The concomitant use of these therapeutic can lead to higher

concentrations of AQ and hypothetical sub-sequent increase in the toxicity. Indeed, if we take into account the study performed by German and collaborators we observe that this hypothetical increase in the toxicity was already observed *in vivo* (German *et al.*, 2007). The results in an African American men and in a White woman show that the administration of efavirenz togheter with AS+AQ lead to increased levels of liver-associated enzymes (alanine aminotransferases and aspartate aminotransferase). The hepatotoxicity is due the drug interaction, since it is only observed when to the antimalarial therapeuthic AS+AQ is added the anti-HIV/AIDS drug efavirenz (German *et al.*, 2007).

CYP1A1 and *CYP1B1* polymorphism in Zanzibar malaria patients

The Cytochromes P450 have been suggested to have a role in the extrahepatic metabolism of amodiaquine, namely CYP1A1 and CYP1B1 (Li *et al.*, 2002). In accordance, a study on the variants in the genes coding for these enzymes was performed in patients from the Zanzibar islands.

Concerning *CYP1A1* the 2455 A>G SNP was analysed, which is representative of both *CYP1A1*2B* and **2C* alleles. The analysis of *CYP1A1* 2455 A>G SNP shows that it is present in the Zanzibar population at low frequency (2.4%). This variation is only present in the heterozygote form (5 in 103) and was found to be in Hardy-Weinberg equilibrium. Our studies confirm a previous study in African native populations showing this as a low frequency variant, with a frequency of 1.3% in Tanzanians, and being absent in South African Venda and Zimbabweans (Dandara *et al.*, 2002). The presence of this SNP in mainland Tanzanians and in the Zanzibari population, but not in the other two countries might be related to the fact that in this Eastern African coastal regions gene mixing may have existed between the local Black

population and the Orientals, where 2455 A>G was described to be more frequent (~ 20%) (Dandara *et al.*, 2002; Chowbay *et al.*, 2005).

The analysis of *CYP1B1* genetic variability has involved the genotyping of five SNPs by PCR-RFLP (4326 C>G, 4360 C>G and 4390 A>G) and sequencing (142 C>G and 355 G>T). Due to results in the sequencing that did not allow the unequivocal identification of the variants, some samples had to be excluded and the initial group having been consequently reduced to 82 individuals.

The previoully referred SNPs define different alleles, *CYP1B1*1*, **2*, **3*, **4*, **5*, **6*, and **7*, all present in the studied population (Table 7), except for the *CYP1B1*5*. From the twelve different combinations that ocurr, the *CYP1B1*2/*6* and *CYP1B1*6/*6* are the more frequent (Table 7). The high frequency of *CYP1B1*2*, **3* and **6* alleles is the result of a high frequency of the 142 C>G (70.1%), 355 G>T (70.1%) and 4326 C>G SNPs (65.2%).

From the SNPs analysed, three were found to be in Hardy-Weinberg equilibrium, 4326 C>G, 4360 C>G and 4390 A>G, but the other two did not follow the equilibrium. Both 142 C>G and 355 G>T are deficient in heterozygotes (15 observed vs 34.36 expected). Since the other three SNPs are in equilibrium in the same population is difficult to explain these two cases, unless the two SNPs had been selected in the Zanzibar islands during evolution by some unknown reason. If we take into account the sequencing results we can see that they are very clear in the cases of homozygotes, so a bad interpretation of the not so clear heterozygotes only would lead to a higher number of the two genotypes already in "excess" (Figure 17).

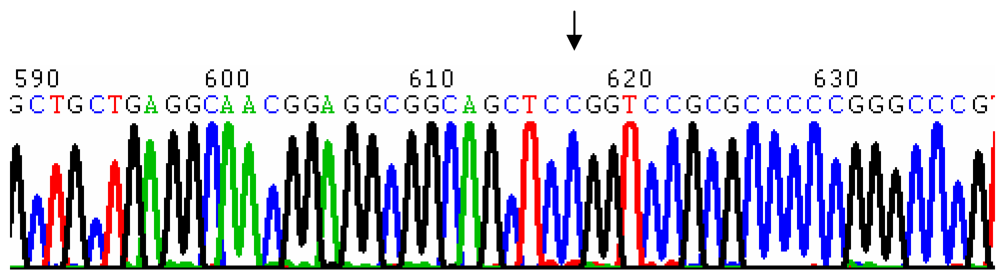
Table 7 – Genotype and allelic frequencies of *CYP1B1*.

CYP1B1 Genotypes (n = 82)			Allele Frequencies (n = 164)		
Genotypes	n	Frequency (95% CI)	Allele	n	Frequency (95% CI)
**1/*1*	4	0.049 (0.014 – 0.120)	*CYP1B1*1*	20	0.122 (0.072 – 0.172)
**1/*2*	2	0.024 (0.003 – 0.085)	*CYP1B1*2*	36	0.220 (0.156 – 0.283)
**1/*3*	5	0.061 (0.020 – 0.137)	*CYP1B1*3*	28	0.171 (0.113 – 0.228)
**1/*4*	1	0.012 (0.0003 – 0.066)	*CYP1B1*4*	1	0.006 (0.0002 – 0.034)
**1/*6*	4	0.049 (0.014 – 0.120)	*CYP1B1*6*	75	0.457 (0.381 – 0.534)
**2/*2*	7	0.085 (0.035 – 0.168)	*CYP1B1*7*	4	0.024 (0.007 – 0.061)
**2/*6*	20	0.244 (0.156 – 0.351)			
**3/*3*	7	0.085 (0.035 – 0.168)			
**3/*6*	7	0.085 (0.035 – 0.168)			
**3/*7*	2	0.024 (0.003 – 0.085)			
**6/*6*	21	0.256 (0.166 – 0.364)			
**6/*7*	2	0.024 (0.003 – 0.085)			

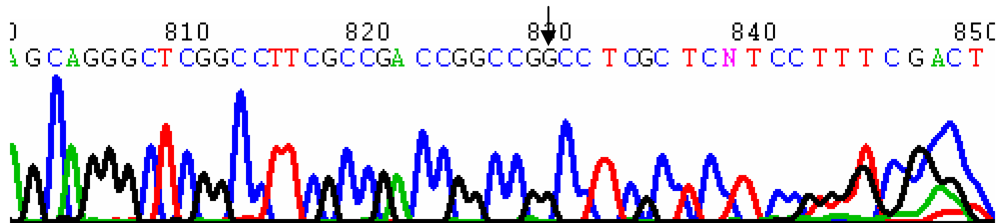
The studies in African origin populations are scarce concerning the *CYP1B1* gene, being an Ethiopian population the only African native population analysed (Aklillu *et al.*, 2002). When we compare the genotypes observed in Zanzibar with the reported by Aklillu and collaborators we can see that Zanzibar does not present many different genotypes (12 vs 18 in Ethiopia). The genotypes described for each population are arranged differently and with different frequencies. In Zanzibar the most frequent genotype (Table 7) is *CYP1B1*6/*6* (25.6%) followed by *CYP1B1*2/*6* (24.4%), frequencies statistically different from the 0.7% and 5.3%, respectively, observed in Ethiopia ($P < 0.0001$). The most frequent genotype among Ethiopians, *CYP1B1*2/*3* (29.3%), was not found in our study in Zanzibar. In terms of allelic frequency, differences are also evident, since in the case of Zanzibar the more frequent allele is *CYP1B1*6* with 45.7% and *CYP1B1*2* (22.0%), while in Ethiopia the highest frequency was observed for *CYP1B1*3* (39.0%), followed by *CYP1B1*2* with 36.7% (Aklillu *et al.*, 2002). These differences may be due to differences in the origins of the studied population, since Zanzibar islands should have more gene mixing than the population in Ethiopia (http://www.zanzinet.org/).

Considering the hypothetical role of CYP1A1 and CYP1B1 in the extrahepatic metabolism of AQ, the results here presented show a high variability that could have an influence in the metabolism of this drug. Unfortunally, the controversy behind the effect of the SNPs, namely in the case of *CYP1A1*, and the lack of a complete analysis of the *in vivo* functional consequences of the *CYP1B1* haplotype, does not allow a theoretical prediction of the effect of polymorphisms in *CYP1A1* and *CYP1B1* in AQ therapeutics.

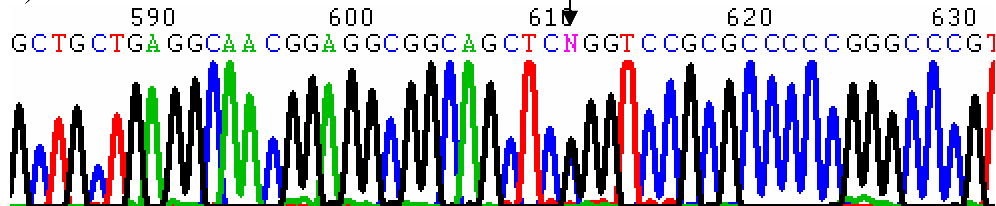
a) 142 CC



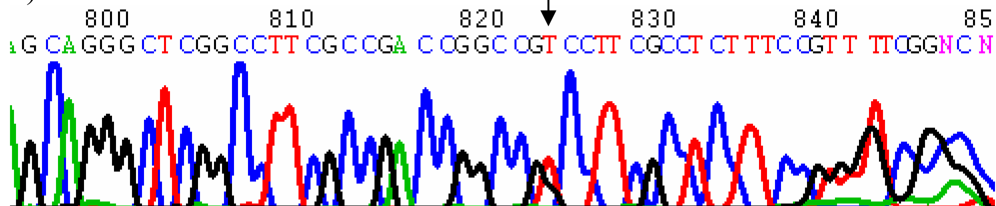
b) 355 GG



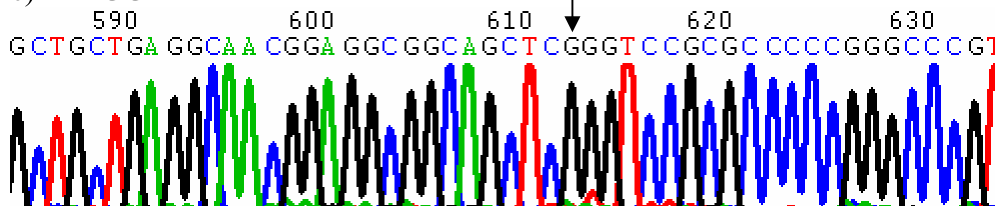
c) 142 CG



d) 355 GT



e) 142 GG



f) 355 TT

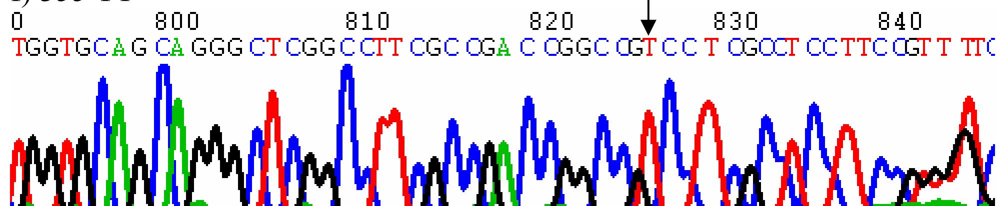


Figure 17 – Example of the results obtained in the sequencing of CYP1B1 142 C>G and 355 G>T SNPs. The arrows indicate the position of the SNPs.

The pharmacogenetic characterization of the population of Zanzibar concerning other important proteins in the metabolism of antimalarials was performed in a parallel study not included in this thesis (Ferreira *et al.*, submitted, ***Other Publications***). In this study the DNA from the children previously analysed for *CYP2C8* was studied for the main functionally relevant SNPs present in genes potentially involved in the elimination of other central antimalarial drugs in current use in Zanzibar. The genes included in this study were the cytochrome P450s *CYP3A4, CYP3A5*, *CYP2B6*, the *MDR1* transporter and the nuclear receptor *PXR*. From all the variants analysed, *CYP3A4*1B* allele was the more frequent being present in 49.5% of the patients in the homozygous form. The other alleles analysed coding for low activity proteins were found in the homozygous form in frequencies ranging from 2.9% in *MDR1* to 14.6% in *CYP3A5*. An important result in the analysis was the fact that ten subjects were found to be predicted low metabolizers simultaneously for CYP3A4 and CYP3A5, which can be a major problem in the case of antimalarial drugs where the metabolism is mainly performed by these enzymes (e.g. quinine, mefloquine and LUM). In this study, regions of *MDR1* and *CYP3A4* promoters associated to the transcriptional control of these genes were sequenced. In *PXR* the sequencing was performed in the exons 2 and 5, coding part of the functionally important DNA binding domain (DBD) and Ligand Binding Domain (LBD) (Lamba *et al.*, 2005). From this analysis only one SNP, previously described (Zhang *et al.*, 2001), was observed (79 C>T, P27S) in a frequency of 11.2%, showing that these two exons are highly conserved. In *MDR1* a new SNP was found, –158 T>C (tagtcatgT/Cactcaaaa) with a prevalence of 7.3%.

This study completes – for the moment – the picture of the main pharmacogenetic factors that can influence potential toxicity of the antimalarial drugs on the islands of Zanzibar. The Zanzibar population is now one of the best characterized African native populations in terms of pharmacogenetic determinants.

Study VII - Ethnic distribution of *CYP3A5* and *PXR* polymorphism

The CYP3A5 enzyme was recently described has having a role in the metabolism of quinine (Rodriguez-Antona *et al.*, 2005), but at the time this study started, the *CYP3A5* gene, in particular the 6986 A>G SNP was choosen because the carriers of this mutation appear to be defective in CYP3A5 enzyme. The high frequency of this variant in Caucasian when compared with the African-Americans was also taken in account to choose this SNP.

In order to evaluate the distribution of *CYP3A5* 6986 A>G SNP, which defines *CYP3A5*3* and the minor alleles **10* and **11*, we analysed the same groups of subjects previously analysed for *CYP2C8* from the populations of Guinea-Bissau, Sao Tome and Principe, Thailand and Portugal. The *CYP3A5* 6986 A>G SNP is present in all the populations analysed with the Portuguese population being the carrier of the highest frequency of the variant (86.9% in the homozygous form). The other populations studied presented lower frequencies (Table 8), particularly the African populations with frequencies that reach as low as 5.3% of subjects homozygous for the G allele in the case of Guinea-Bissau.

In Figure 18 the world distribution of the 6986 A>G SNP is presented. We can see that the frequencies obtained for the Portuguese and Thai population are similar to the ones reported for other Caucasians and Asian populations. The same does not happen in Africa where there are significant differences between the populations analysed up to date.

Table 8 – Genotypic frequencies for the SNPs analysed in *CYP3A5* and *PXR* genes in the studied populations.

	CYP3A5 6986			**PXR 7635**			**PXR 8055**			**PXR 11156**			**PXR 11193**		
	A/A	A/G	G/G	A/A	A/G	G/G	C/C	C/T	T/T	A/A	A/C	C/C	T/T	T/C	T/T
Portugal (n= 61)	0.164	0.115	0.869	0.328	0.443	0.229	0.590	0.328	0.082	0.590	0.328	0.082	0.590	0.328	0.082
Thailand (n= 116)	0.190	0.483	0.328	0.052	0.517	0.431	0.164	0.664	0.172	0.164	0.664	0.172	0.164	0.664	0.172
STP (n= 50)	0.380	0.400	0.220	0.020	0.260	0.720	0.420	0.440	0.140	0.220	0.600	0.180	0.220	0.600	0.180
Guinea-Bissau (n= 95)	0.610	0.337	0.053	0	0.011	0.989	0.316	0.537	0.147	0.200	0.558	0.242	0.200	0.558	0.242

The percentages of 6986GG carriers reflect the population where the CYP3A5 enzyme is not present. From the results in the Portuguese population it is clearly evident that CYP3A mediated metabolism should be particularly mediated by CYP3A4 with a minor contribution of CYP3A5. The same happens, to a lesser extent in the Thai population. However a completely different scenario is observed in Guinea-Bissau and Sao Tome and Principe (and Zanzibar) where a variable degree of CYP3A5-mediated metabolism should be present.

The low frequency of individuals carrying the 6986 A>G SNP in Guinea-Bissau indicates that probably the majority of the persons express the CYP3A5 enzyme. The role of CYP3A5 enzyme in the metabolism of CQ has not been clearly elucidated (Li *et al.*, 2003), so it is conceivable that CYP3A5 could share this substrate with CYP3A4. Although the inexistence of acute toxicity with the higher dosage of CQ used in Guinea-Bissau cannot be related directly with a genetic cause (Kofoed *et al.*, 2007; Ursing *et al.*, 2007b), it should be of interest to evaluate the relative contribution of different expression levels of the CYP3A enzymes.

On the other phenotypic extreme, if we analyse the results of *CYP2C8* and *CYP3A5* together, we find that seven individuals have defective SNPs in both genes. This means that 7.4% of the analysed population is hypothetical low metabolizers (or intermediate, depending of the alleles) concerning these enzymes. We have also to take into account the fact that a higher percentage of the population bears the defective *CYP3A4*1B*.

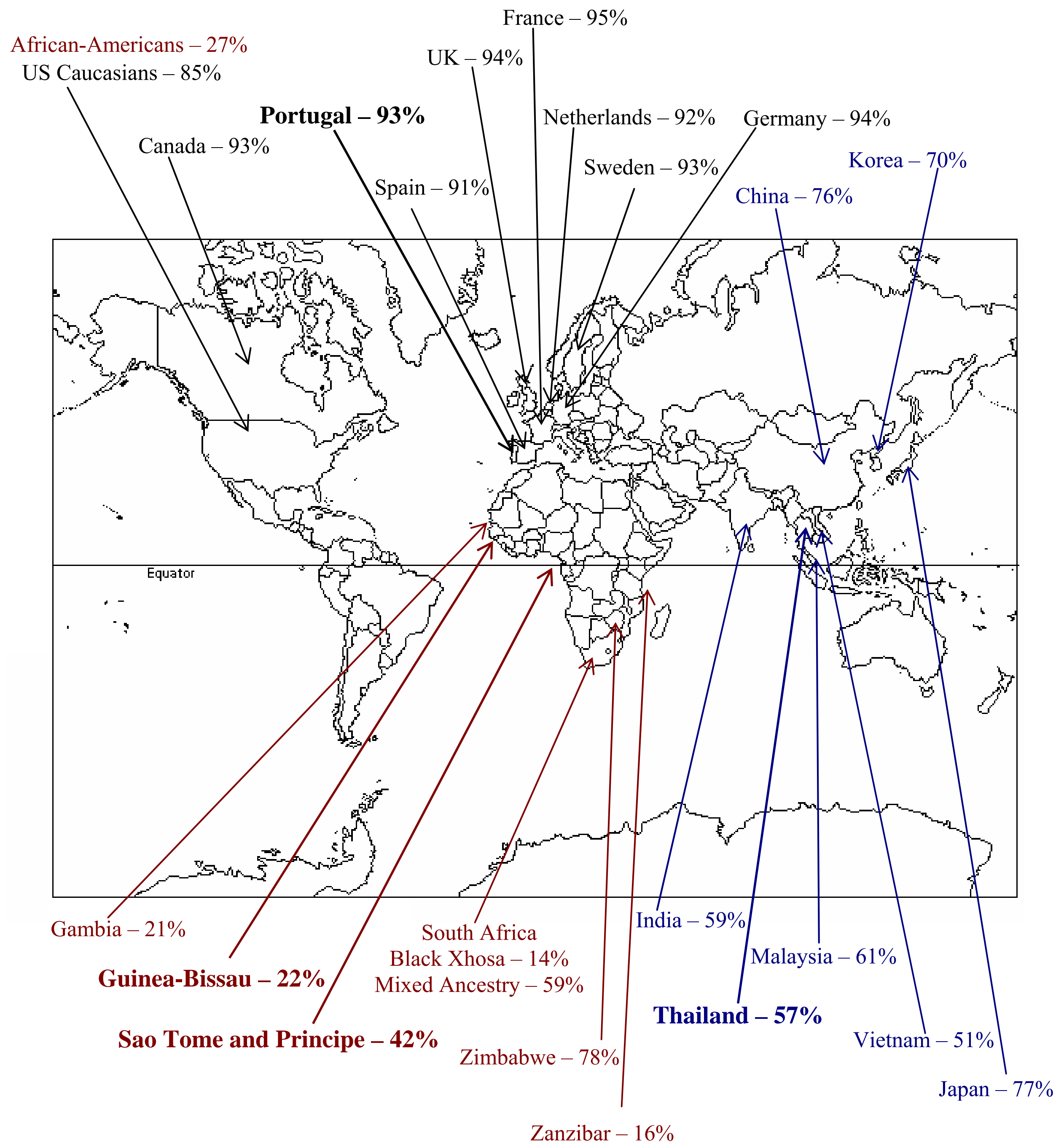


Figure 18 – *CYP3A5* 6986 A>G SNP world distribution (map adapted from http://www.colby.edu/geology/gifs/worldmap.gif) (data from Kuehl *et al.*, 2001; Hustert *et al.*, 2001b; Roy *et al.*, 2005; Gervasini *et al.*, 2005; King *et al.*, 2003; Koch *et al.*, 2002; Wadelius *et al.*, 2004; Thervet *et al.*, 2003; van Schaik *et al.*, 2002; Balram *et al.*, 2003; Fukuen *et al.*, 2002b; Dandara *et al.*, 2005; Wojnowski *et al.*, 2004; Ferreira *et al.*, in press, ***Other Publications***; Veiga *et al.*, submitted, ***Other Publications***).

In Sao Tome and Principe the frequency of individuals homozygous for *CYP3A5* 6986 A>G SNP was 22.0% (Table 8), significantly higher than the observed for Guinea-Bissau (*P* = 0.0006). This difference might reflect the percentage of native Africans present in the two countries - in Guinea-Bissau around 99% but less in Sao Tome and Principe, since the "Mestizo" is a significant part of the population.

The word "Mestizo" is normally connected to a person of mixed race, which means, by example, in Mexico and Central and South America, a person of European (Spanish or Portuguese) and indigenous descent (Columbia Encyclopedia, Columbia University Press). In the case of Sao Tome and Principe the islands were uninhabited before the Portuguese explorations and the majority of the current population is descendent from the mixing of the Portuguese that initially settled the islands from the 15th Century onwards and the slaves brought from Africa mainland to work in agriculture (Tomas *et al.*, 2002).

When we analyse the presence of *CYP2C8* and *CYP3A5* defective alleles in the same individuals we observe that 3 in 50 carry mutations in both genes, and may therefore present an impaired metabolism.

In Thailand the allelic frequency for *CYP3A5* 6986 A>G SNP was determined to be 56.8% and a high percentage is present in the homozygous form (32.8%) (Table 8). The allelic frequency observed is very similar to the frequencies reported in Malaysia and Vietnam, nearby countries (Figure 17). Contrary to CYP2C8 where the Asian populations seem to be normal metabolizers, for CYP3A5 a high fraction of the populations do not have the enzyme and another important fraction has lower levels of this enzyme. The frequencies described in these Asian countries for *CYP3A5* 6986 A>G SNP are between the two extremes observed in populations from African and Caucasian

origin. Like in Caucasians, in the Asian populations the clearance of drugs which are CYP3A substrates should be mediated mainly by CYP3A4.

The variation that is observed around the world in terms of *CYP3A5* 6986 A>G SNP frequency will be translated in a high degree of variation in the expression of the enzyme. This variation in expression may be a genetic contributor to taking into account in interindividual and interracial differences that have been reported in CYP3A-dependent drug clearance.

All the genes previously discussed (except *NAT2*) are regulated (directly or not – CYP1 family is regulated indirectly *via* the receptor AhR) by the nuclear receptor PXR. Several highly prevalent variants in *PXR* gene were described in 2001 by Zhang and collaborators and among them we choose to study four, 7635 A>G, 8055 C>T, 11156 A>C and 11193 T>C, all associated with a phenotypic alteration and non-negligible frequencies (Zhang *et al.*, 2001). The SNPs analysed were found to be present in all the populations and normally in high frequencies, particularly in African populations (Table 8). We observed that two of the studied SNPs are in complete linkage (11156 A>C and 11193 T>C) in all the populations (Table 8). In Portuguese and Thai populations another SNP, 8055 C>T, appears in linkage with these two.

Regarding the results obtained in Guinea-Bissau all the PXR SNPs studied are present in frequencies above 50% (Table 8). The more frequent is 7635 A>G present in 98.9% of the studied population as G/G homozygote. This SNP (and also 8055 C>T) were described as being associated with a higher induction activity of PXR (Zhang *et al.*, 2001). As such, they may lead to higher levels of expression of the genes involved in the metabolism of CQ (*CYP2C8* and *CYP3A4*).

Concerning the two SNPs localized in the 3' untranslated region, 11156 A>C and 11193 T>C, these were observed in a complete linkage at high frequencies. These two SNPs were correlated with decreased P-gp levels in the gut (Zhang *et al.*, 2001) which potentially can alter the bioavailability and pharmacokinetic profile of substrates. Decreased P-gp levels might contribute to an increase in the absorption of orally taken antimalarial drugs.

We performed a haplotype analysis for the analysed SNPs of PXR using the PHASE Version 2 software (Stephens *et al.*, 2001; Stephens and Donnelly, 2003; http://stephenslab.uchicago.edu/software.html). This analysis led to the identification of eight possible different haplotypes for PXR (Table 9). In Guinea-Bissau all the possible haplotypes were present, although four of them are predicted to be present in low frequency. This is in contrast with Portugal where only three of the haplotypes were estimated to be present and all in high frequencies (Table 9). These differences may be a consequence of the complete linkage that is observed between two of the SNPs for Guinea-Bissau and three SNPs for Portugal. The diversity observed in Guinea-Bissau in terms of haplotypes is a somewhat expected phenomenon, in the light of the fact that the modern humans are known to have evolved from African ancestors (Forster, 2004). It has been proposed that ancestral alleles are "African", in the sense that these are expected to reach their highest diversity and frequency in natives of that continent (Satta and Takahata, 2002; Takahata *et al.*, 2001).

Table 9 - Haplotypes for PXR and their estimated frequencies in the studied populations.

Haplotype	Nucleotide Position				Portuguese	Thai	STP	Guinea-Bissau
	7635	8050	11156	11193				
1	A	C	A	T	0.549	0.310	0.149	0.001
2	A	C	C	C	-	-	0.001	0.001
3	A	T	A	T	-	-	-	0.001
4	A	T	C	C	-	-	-	0.002
5	G	C	A	T	0.205	0.186	0.335	0.344
6	G	C	C	C	-	0.004	0.156	0.238
7	G	T	A	T	-	-	0.036	0.133
8	G	T	C	C	0.246	0.500	0.323	0.280

A very interesting observation was the strong association of *CYP3A5* 6986 A>G SNP with PXR variants, with 67% of Portuguese bearing at least one G in conjunction with one PXR variant. The same phenomenon was observed in 76% of subjects from Thailand, 60% in Sao Tome and Principe and 39% in Guinea-Bissau. It is tempting to speculate that carriers of a low CYP3A5 expression will, in a significant number of cases, harbour an increase in CYP3A4 expression, due to the linked PXR polymorphism reflecting a possible selected compensatory effect.

By contrast we have detected subjects bearing the “wild-type” *CYP3A5* (individuals that express CYP3A5) in conjunction with one or more PXR variants associated to increased PXR transcriptional activity. These individuals are expected to possess a higher CYP3A-mediated metabolic activity, either basal or after exposure to known PXR activators. In the populations analyzed the association was found with a

frequency of 19% in subjects from Thailand, of 38% in Sao Tome and Principe and of 61% in Guinea-Bissau. None of the Portuguese Caucasians were found to bear these characteristics. These different allele combinations probably implicate significant interindividual differences in drug clearance, an hypothesis yet to be tested.

The description that artemisinin drugs are activators of PXR and CAR revealed that a vast list of genes may be affected by exposure to this drug. In the report of Burk and collaborators it was shown that ART activates human PXR and can induce *CYP3A4*, *CYP2B6* and *MDR1* mRNA expression (Burk *et al.*, 2005). The activity of P-gp was demonstrated to be partially inhibited by ART but in the case of CYP3A4 this is only suggested (Burk *et al.*, 2005). A strong induction effect of ART was observed *in vivo* in which concerns the mouse Cyp2b10, both at mRNA and enzyme activity levels (the same was unable to be shown for Cyp3a) (Simonsson *et al.*, 2006), pointing to a regulator role only of CAR (since it was not observed *in vitro* activation of PXR). More recently, however, it was demonstrated that artemisinin antimalarials are inducers of CYP3A (Asimus *et al.*, 2007). These two reports are, in some way, contradictory since only Burk and collaborators see an activation of PXR after the treatment with ART (Burk *et al.*, 2005), which may be the result of different experimental approaches.

However, considering the regulatory role of PXR and the fact that it is activated by ART, the massive introduction of ACT may bring serious problems concerning drug-drug interactions, efficacy and adverse effects. CYP3A4, as an example, is the enzyme responsible for the metabolism of different antimalarial drugs (see ***Malaria – Antimalarial drugs*** for details). If we look to the implementation of ACT, one of the most used combinations is artemether + lumefantrine (see Figure 5), both drugs

metabolized by CYP3A4. Since artemether was demonstrated to activate PXR and to induce PXR and CYP3A (Burk *et al.*, 2005; Asimus *et al.*, 2007), the use of a combination where the two drugs are substrates for CYP3A4 enzyme can be a problem. This situation can lead to a decrease in the efficacy of the therapeutic and, hypothetically, to the development of resistance since subtherapeutic levels of the drugs can be reached. However, CYP3A4 is also responsible for the metabolism of many other drugs, including many anti-HIV drugs and in the scenario already mentioned of co-infections (malaria and HIV/AIDS) this can result in important drug-drug interactions that have to be evaluated. With the recent report of hepatotoxicity resulting from drug interaction because of administration of AS + AQ and efavirenz (German *et al.*, 2007) the evaluation of resulting adverse effects is even urgent. In these cases it should be interesting to analyse also the role of the different PXR polymorphisms and see if they lead to a reduction (or increase) in the induction effect, and consequently of the drug-drug interactions. The effect of PXR activation by ART in the expression of other CYPs involved in the metabolism of antimalarials, as is the case of CYP2C8, was not determined yet.

Taken together, our results show a very high prevalence of PXR SNPs and a strong association between CYP3A5 and PXR polymorphic *loci* in African populations, strengthening the idea that ethnic differences may be important, and must be documented in order to better understand and eventually optimize therapeutic outcome.

Generally, the results obtained in the genotyping studies show the variability that exists between different continents, a fact already observed in the many genetic studies performed during the last decades. A relatively new observation is the variability that exists in the same ethnic group, which is particularly true in the case of the Africans.

Factors like the concomitant use of anti-HIV drugs, anti-TB, or many other drugs and herbal compounds (the reality in Africa) are normally not taken into account during the clinical trials and many of the adeverse effects, or even deaths, can be the result of drug-drug interactions and not from the disease. The problem of drug-drug interactions has also to be considered because of the possibility of diminishing the therapeutic efficacy of chemotherapy.

Metabolic pathways of DEAQ – The role of CYP1A1

In the *in vitro* assay for DEAQ metabolism in human liver microsomes, no variations were observed in concentrations of DEAQ, pointing at one of several outcomes: (1) the drug is not metabolized *in vivo*; (2) it is metabolized so slowly that the incubation times of this type of *in vitro* system are not efficient enough to detect it; (3) it is metabolized by a non-hepatic expression enzymes. The incubation with microsomes enriched with CYP1A1 (*InVitro*Somes™, InVitro Technologies, Inc., Baltimore, Maryland, USA) revealed a decrease in the concentration of DEAQ. This was not observed with other recombinant enriched microsomes.

The CYP1A1 enzyme had been previously suggested to mediate the *in vitro* biotransformation of DEAQ (Li *et al.*, 2002). In order to evaluate the *in vivo* contribution of *CYP1A1* genetic variation as a basis for the interindividual variability in pharmacokinetic (PK) parameters observed previously in patients from Papua New Guinea (Hombhanje *et al.*, 2005) and Zanzibar (Friberg Hietala *et al.*, 2007) we sequenced the full coding region of *CYP1A1* gene and a small region of the proximal promoter from these subjects.

Significant sequence variation was detected among the Papua New Guinea subjects, although not associated to the documented PK data (Table 10). The high diversity of SNPs found among the Papua New Guinea subjects led us to confirm a subset of them through another robust technique. Pyrosequencing of the promoter located SNPs -92 A>T, -86 C>G and -80 T>G revealed them as probable sequencing artifacts. All the other nucleotide changes founded during sequencing should be also verified to confirm the results obtained.

The Zanzibar samples only harboured two SNPs, 233 T>C (already described before) and 3309 A>G (intronic), both not related to the available pharmacokinetic data (Sofia Friberg Hietala, Göteborg University, manuscript in preparation). Interestingly, this low variation is concomitant with preliminary data indicating low variation on the PK parameters of DEAQ among the studied Zanzibari population (e.g. interindividual AUC variantions not larger that 30% (Sofia Friberg Hietala, Göteborg University, pers. commun). The hypothesis stays open that the higher diversity detected among the Papua New Guinea patients might be related to not detected, but functionally important linked SNPs (e.g. in distant regulatory regions of the gene).

Table 10 – Nucleotide changes detected by the analysis of the results of the sequencing of CYP1A1 in the samples from Papua New Guinea. The position considers the ATG has the +1 nucleotide.

	-63 G>A	-57 Ins G-56	134 G>A	167 G>A	354 T>A	3303 G>A *	3335 G>A *	3486 G>A *	3534 G>A *	4508 A>G	4588 G>A *	4597 C>T *	4633 T>C *	$t_{1/2}$ (days)[a]
1	-	x	-	-	-	?	?	-	-	-	-	-	-	3.5
2	x	x	-	-	-	x	x	-	-	-	-	-	-	3.3
3	x	x		-	-	x	x	-	-	-	-	-	-	6.7
4	-	-	-	-	-	?	?	-	-					8.4
5	-	-	-	-	-	x	x	x	x					3.4
6	-	-	-	x	x	x	x	x	-					7.7
7	-	-	-	-	-	x	x	-	-					7.9
8	-	-	-	-	-	x	x	-	x					9.3
9	-	-	x	-	-	x	x	-	x					30.5
10	-	-	-	-	-	x	x	-	x		x		x	8.8
11	-	-	-	-	-	x	x	-	-	x		x		5.1
12	-	-	-	-	-	x	x	-	-					23.6
13	-	-	-	-	-	x	x	x	x		x			5.1
14	-	x	-	-	-	x	x	-	-					13.3
15	x	x	-	-	-	x	x	-	-		x			7.1
16	-	-	-	-	-	x	x	-	-	-	x			4.2
17	x	x	-	-	-	x	x	-	-			?	x	6.8
18	x	x	-	-	-	x	x	-	-	x	x			10.6
19	-	-	-	-	-	x	x	-	-		x			10.1

* These SNPs are localized in introns. [a] Data from Hombhanje *et al.*, 2005. The sequencing was made without knowledge of the pharmacokinetic data. Only after the analysis was finished the match was made with the information provided by Prof. Akira Kaneko (Karolinska Institutet).

Study VIII - The role of GSTM1 and T1 in uncomplicated malaria

The antioxidant system, which includes gluthatione S-transferases, plays an important role in malaria. The deletion of two of these enzymes, GSTM1 and GSTT1 was analysed in the context of uncomplicated *P.falciparum* malaria. We found an extremely significant higher frequency of *GSTT1* null genotype in the control patients when compared with malaria patients (45.4% vs 21.5%) (Table 11). No difference was observed in the case of *GSTM1*, as can be seen by the *odds ratio* observed for each case (*GSTM1* - 0.978 (0.773–1.24) and *GSTT1* - 0.328 (0.259–0.417)).

These results suggest that GSTs may have a role in malaria susceptibility, especially GSTT1 since the lack of this gene seems to be a protective factor in uncomplicated malaria. Accordingly, some data show that drugs that decrease the oxidative stress can increase the parasitemia in malaria patients (Brandts *et al.*, 1997).

The hypothesis that GSTT1 deletion may be a protective factor in uncomplicated malaria is supported by the fact that GSTT1 is highly expressed in erythrocytes (Schroder *et al.*, 1996), while GSTM1 is not present (having serve in this exploration as a putative negative control). The expression of GSTT1 enzyme in the erythrocytes helps to decrease the levels of oxidative stress, probably by the conversion of peroxides that can damage the cell membrane. If the gene is lacking, by consequence the enzyme is not expressed and is not possible to conjugate the GSH with ROS, leading to higher levels of oxidative stress. This can further lead to non viability of the erythrocyte, and/or might contribute to the elimination of parasited RBCs through the oxidative stress associated to the phagocytosis processes. In fact, and possible due to its haemoglobin based metabolism, parasite is very sensitive to ROS during its intra-erythrocyte cycle, a

characteristic which is thought to contribute to the efficacy of antimalarial drugs, such as CQ or AQ.

The lacking of an association between GSTM1 genotype and uncomplicated malaria served as a control of the results obtained, since GSTM1 is expressed in lymphocytes but not in the erythrocytes (Wang *et al.*, 2000). An interesting fact is the description of GSTM1 expression in brain (Takahashi *et al.*, 1993), which can be related to the results observed in the case of severe malaria (Kavishe *et al.*, 2006).

Table 11 – The distribution of GST genotypes and *odds ratio*

Genotype*	Control Patients (n=370)		Malaria Patients (n= 1515)		OR (95% CI)	*p-value*
	n	Freq	n	Freq		
GSTM1						
Positive	233	0.630	962	0.635		
Null	137	0.370	553	0.365	0.978 (0.773–1.24)	0.8982
GSTT1						
Positive	202	0.546	1190	0.785		
Null	168	0.454	325	0.215	0.328(0.259–0.417)	<0.0001
GSTM1/GSTT1						
Positive	305	0.824	1370	0.904		
Both Null	65	0.176	145	0.096	0.497(0.361–0.682)	0.0002

*The subjects were classified as either positive - when at least one copy of the gene was present - or null genotypes.

Further research is ongoing or planned (Malaria Research Unit, Karolinska Institutet) in order to obtain a more complete picture of the influence of GSTT1 in malaria pathology.

SUMMARY AND CONCLUSIONS

- *CYP3A4* and *MDR1* SNPs were analysed in the Portuguese population for the first time in an extensive and coordinated way. The degree of linkage between 3435 C>T and 2677 G>T presents significant differences when compared with previous reports.

- The analysis of *CYP3A4*1B* and NAT2 191 G>A in Guinea-Bissau revealed high allelic frequencies (72.0% and 19.2%, respectively). This numbers follow the trend of a high prevalence of both variants in populations of African origin.

- The study of the genetic variability in *CYP2C8* in populations of different ethnic origins showed significant differences in the prevalence of the studied alleles. *CYP2C8*2* revealed to be significantly more frequent in the African populations (Guinea-Bissau – 26.6%, Sao Tome and Principe – 9.0% and Zanzibar – 13.9%) when compared with Colombia (3.8%) and Portugal (1.2%). *CYP2C8*3* showed the inverse trend with the Portuguese presenting the highest frequency (19.8%), followed by the Colombians (7.1%) and after the African populations (Sao Tome and Principe – 4.0%, Zanzibar – 2.1%). *CYP2C8*4* is the less frequent allele with a frequency of 6.4% in the Portugal, 1.6% in Colombia, 1% in Sao Tome and Principe and 0.6% in Zanzibar. In Thailand none of the studied alleles were present and in Guinea-Bissau *CYP2C8*3* and *CYP2C8*4* are absent.

- *CYP1A1* 2455 A>G was shown to be present in 2.4% of the studied population, in the line of previous reports that points to its low prevalence in the African populations. Concerning the analysis of *CYP1B1* polymorphism a high variability was observed in terms of SNP, genotype and haplotype.

- *CYP3A5* 6986 A>G reveals a high prevalence in the Caucasian Portuguese population (93%) in a clear contrast with the numbers observed in Africa (Guinea-Bissau – 22% and Sao Tome and Principe – 42%). Thailand showed an intermediate frequency of 57%. The role of CYP3A5 in the metabolism of antimalarials (also in anti-HIV/AIDS and anti-TB drugs) is being clarified, an important step as in Africa the predicted high frequency of expressors of this gene may may lead to low therapeutic efficacy of its drug substrates.

- The PXR SNPs analysed are present in high frequencies, particularly in African populations. Different degrees of linkage were observed between the SNPs analysed in the different populations characterized. The studied SNPs are reported to be linked with alterations in transcription rates and protein levels, so the role of the alterations in the metabolism of the different antimalarials should be clarified.

- CYP1A1 was preliminary identified as the enzyme responsible for the *in vitro* metabolism of DEAQ. The analysis of *CYP1A1* genetic variation contribution to the pharmacokinetic variability did not reveal a precise gene alteration/PK parameters association; although the available data suggests that marked interindividual variation of the later is associated with higher genetic diversity. This point for the possible presence of pivotal unknown genetic associations not unveils by our approach in this work.

- The GSTT1 null genotype is suggested to play a protective role in malaria susceptibility, with a significantly high frequency in the control group (45.4% *vs.* 21.5%, *OR* = 0.328 (0.259–0.417)).

Taking into account the variability shown for the different countries, in particular in Africa, the role of the human host in malaria therapeutics should be more considered. In the African countries clinical trials should include not only the genotyping of *P. falciparum* in order to assess the level of resistance, but also of the human host. These studies could be useful in the evaluation of the role of genetic polymorphism in the therapeutic outcome, not only in terms of efficacy of the antimalarial treatment but also in the identification of secondary effects associated with toxicity and/or drug-drug interactions. This last issue is also of great importance in the African scenario where normally malaria, TB and HIV/AIDS are concomitant diseases.

The pharmacogenetic of a population can potentially influence the pharmacokinetics parameters of a drug. The alteration of PK parameters can led to the existence of subtherapeutic concentrations of drugs and this can modudulate the development of resistance. A better knowlgement of the pharmacogenetic of the human population can give a help in the long fight against one of the most important parasitic diseases – MALARIA.

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