Genotype and allele frequencies of MDR-1, TPMT, NAT2, GST and SULT1A1 in the Sudanese and Bahraini population

عند SULT1A1 و MDR1,TPMT,NAT2,GST التكرار الجيني والأليلي للجينات السودانين والبحرينين

A Thesis Submitted for Fulfillment Requirements for the degree of Doctor of Philosophy (PhD) in Molecular Biology

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DEDICATION


وَٱخۡفِضۡ لَهُمَا جَنَاحَ ٱلذُّلِّ مِنَ ٱلرَّحۡمَةِ
وَقُل رَّبِّ ٱرۡحَمۡهُمَا
كَمَا رَبَّيَانِي صَغِيرًا

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ABSTRACT

The goal of this study was to determine the frequencies of important allelic variants in the TPMT, NAT2, GST, SULT1A1and MDR-1 genes in the Sudanese and Bahraini population and compare them with the frequencies in other ethnic populations. Genotyping was carried out in a total of 300 unrelated Bahraini and 142 unrelated Sudanese subjects.TPMT*2 was detected using an allele-specific polymerase chain reaction (PCR) assay.TPMT*3C and NAT2 variants (*5, *6 and *7) were detected using an allele specific real-time PCR assay. Detection of GSTM1and GSTT1 null alleles was performed simultaneously using a multiplex PCR assay. Finally, a PCR-restriction fragment length polymorphism assay was applied for the determination of TPMT*3A(*3B), SULT1A1*2 and MDR-1(3435T) variants. Genotyping of TPMT revealed frequencies of (0.0) and (0.0) for TPMT*3A and TPMT*3C for Sudanese, and frequencies of (0.0) and 0.036 for Bahraini, respectively. No TPMT*2 or *3B was detected in the analyzed samples. The frequencies of specific NAT2 alleles were 0.1, 0.569, 0.288 and 0.044 for *4 (wild-type), *5 (341C), *6 (590A) and *7 (857A), for Sudanese , and 0.15 ,0.405 ,0.397 ,0.047 for Bahraini respectively. GSTM1 and GSTT1null alleles were detected in 54.4% and 42.0% for Sudanese, 49.7% and 28.7% for Bahraini, respectively.SULT1A1*2were detected at a frequency of 0.26 for Sudanese and 0.42 for Bahraini. Finally, the frequencies of the wild-type allele

(3435C) and the (3435T) variant in the MDR-1gene were found to be 0.74 and 0.24 for Sudanese 0.58 and 0.42 for Bahraini, respectively. We found that Bahraini population more closely resemble Caucasians and Sudanese population more closely resemble African. The purpose of our future studies is to investigate for new polymorphisms, which could be relatively unique to the Bahraini and Sudanese population.

الخلاصة

الهدف من هذة الدراسة هو دراسة التكرار الجيني والأليلي للجينات عند السودانيين والبحرينين *SULT1A1* و *MDR1,TPMT,NAT2,GST* ومقارنتها مع شعوب العالم. تم أخذ 300 عينة من البحرينين و 142 عينة من السودانيين ليس لديهم أي اعراض مرضية. وباستخدام جهاز تفاعل وباستخدام جهاز الوقت *TPMT*2* البلمرة المتسلسل لتحديد التكرار الاليلي ل و,*TPMT*C3* الحقيقي لتفاعل البلمرة المتسلسل لتحديد التكرار الاليلي ل وباستخدام تقنية متعدد. *NAT2*7* و *NAT2*6* و *NAT2** و *NAT2*4* *5 و GSTT1 تفاعل البلمرة المتسلسل لتحديد عدم وجود التكرار الاليلي لكل من واخيرا باستخدام الانزيمات لقطع الناتج من تفاعل جهاز البلمرة.GSTM1 و *TPMT*A3* و *MDR1* المتسلسل لتحديد التكرار الاليلي لكل من وكانت نتايج التكرار الجيني الاليلي ل.*ASULT1* و *TPMT*B3* *1*2* هي صفر,صفر للسودانيين وصفر ,0.036 *TPMT*3C* و *TPMT*A3* و *NAT2** للبحرنيين علي التوالي .أما بالنسبة للتكرار الاليلي ل *4* هي 0.1 و 0.569 و 0.288 و 0.044 *NAT2*7* و *NAT2*6* و *NAT2** *5 للسودانيين و 0.15 0.405 و 0.397 و 0.044 للبحرنيين علي التوالي كانت 54.4% , *GSTT1* و *GSTM1* .وكانت نتايج عدم وجود الاليل 42.0% للسودانيين و 49.7% ,28.7% للبحرينيين علي التوالي.واما كانت 0.26 للسودانيين و 0.42 للبحرينيين . *SULT1A1*2* بالنسبة للاليل هي Tوللاليل المريض 3435 3435 C واخيرا كانت نتايج الاليل الطبيعي 0.24, 0.74 للسودانيين و 0.42, 0.58 للبحرينيين. وخلصنا الي ان سكان البحرين اكثر شبها بالقوقازيين وسكان السودان اكثر شبها بسكان أفريقيا. والهدف من هذه الدراسة في المستقبل ايجاد طفرة جينية يمكن ان تكون فريدة من نوعها نسبيا خاصة بسكان البحرين والسودان.

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ABBREVIATIONS

A	Adanine
ABCB1	ATP binding cassette subfamily b member 1
ABI	Applied biosystem
ASN	Asargine
ASP	Asapartate
APOE	Apolipoprotein e
C	Cytocine
CI	Confiedence interval
CNS	Central nervous system
CYPs	Cytochromes P450
DNA	Deoxyribonucleic acid
DHFR	Dihydrofolate reductase
DRD2	Dopamine receptor d2
EGFR	Epidermal growh factor receptor
FDA	Food and drug administration
G	Guanine
GSTM1	Glutathione s-transferase M1
GSTT1	Glutathione s-transferase T1
GWA	Genome wide association
HMG-COA	Hydroxy-3methyl glutaryl-coenzyme A
INDEL	Insertion / deletion
INR	International normalized ratio
KB	Kilo base
KRAS	Kirsten rat sarcoma
MDR1	Multi drug resistance
MiRNA	microRNA

MRNA	Messengrg RNA
MTHFR	Methylene tetrahydrofolate reductase
NADPH	Nicotinamide adenine dinucleotide phosphate
NAT2	N-acetyltransferase 2
OATP1B1	Organic anion-transporting polypeptide 1B1
OPRMI	Opioid receptor mu 1
PHARM-GKB	Pharmcogenetic database
RCF	Relative centrifugal force
RFLP	Restriction fragment length polymorphism
RPM	Round per minute
RS	Reference sequence
T	Thamine
TPMT	Thiopurine methyltransferase
SNP	Single nucleotide polymorphism
SULT1A1	Sulfotransferase family 1A member 1
VKORC1	Vitamine k-epoxide reductase complex

CHAPTER ONE

Pharmacogenomics is an important tool in personalized medicine. There is wide variability in the response of individuals to standard doses of drug therapy which can lead to therapeutic failures or adverse drug reactions. Ethnic differences in response to drugs have been related to polymorphisms of key enzymes and proteins that affect the safety and efficacy of a drug in the individual patient. Genes provide our bodies with instructions for making enzymes, which help break down drugs in our systems, allowing our bodies to benefit from the medicine. Differences in our enzymes can affect how our bod metabolizes a drug and how long the drug stays in our bodies and thus, how well drugs may work in an individual .

For thiopurine S-methyltransferase (TPMT), four alleles have been associated with low or absent enzyme activity. These alleles are: *TPMT*2* (G238C), *TPMT*3A* (G460A and A719G), *TPMT*3B* (G460A) and *TPMT*3C* (A719G). TPMT-deficient patients showed severe haematopoietic toxicity when they are treated with standard doses of thiopurine drugs . TPMT-deficient patients with acute lymphoblastic leukaemia could be successfully treated with 5–15% of the conventional dose of mercaptopurine . McLeod et al., 1999 found that about 0.33% of the population is TPMT deficient and approximately 10% have intermediate activity of TPMT enzyme due to heterozygosity at the *TPMT* locus .

N-acetyltransferase 2 (NAT2) activity is controlled by genetic polymorphisms, and the individual phenotypes can be classified as rapid, intermediate, or slow acetylators according to their acetylation activity . NAT2 metabolizes a range of drugs including isoniazid, procainamide, and some polycyclic amines such as sulphonamides and hydralazines . Polymorphisms at positions 341, 580, and 857 of the *NAT2* gene have been observed in several human populations and characterize the major *NAT2* defective alleles (*NAT2*5*, *NAT2*6 and NAT2*7*, respectively). The impact of ethnicity is well known, with more than 90% being slow acetylators in some Mediterranean populations .

The glutathione S-transferases (GSTs) are a superfamily of enzymes that catalyse the conjugation of xenobiotics and endogenous substrates with glutathione. They play an important role in the inactivation and occasionally the activation of many drugs and xenobiotics . Large differences between populations are also seen for the GSTs. One member of the mu class gene family (*GSTM1*) has been shown to be polymorphic and is absent in 35–60% of individuals. Similarly, *GSTT1*, a member of the theta class gene family, is also polymorphic and absent in 10–65% of human populations .

Human sulfotransferases (SULTs) are a superfamily of multifunctional enzymes. Six cytosolic SULTs are involved in the metabolism of many drugs, xenobiotics, neurotransmitters and hormones . Previous biochemical

studies have demonstrated a large individual variation in the activity of platelet SULTs in humans . A large portion of this variability could be explained by a newly identified common polymorphism (a G to A transition) in the coding region of the *SULT1A1* gene (*SULT1A1*2*). This base change results in an arginine to histidine substitution at codon 213, and individuals homozygous for the *His* allele had only about 15% of the SULT activity in platelets compared with those with other genotypes .

P-glycoprotein (PGP), the product of the multidrug resistance (MDR-1) gene also plays an important role in the bioavailability of a wide variety of drugs, including the cardiac glycoside digoxin and anthracycline antibiotics antibiotics, vinblastine, daunomycin, and cyclosporine A . Recently, a C to T transition (C3435T) has been described in exon 26 of the *MDR-1* gene. The intestinal P-gp expression is much higher in individuals with the 3435CC genotype than those with the 3435TT genotype . The frequency of the homozygous CC genotype was highest among Africans and lowest in the South-west Asians .Heterozygous individuals displayed an intermediate phenotype .

The frequencies of the important allelic variants in the *TPMT*, *NAT2*, *GST*, *MDR1 and SULT1A1* genes have been extensively studied in many populations. The data showed ethnic variations in the distribution of these variants. However, no studies on these allelic variants were done regarding the Arabic populations, except for Egyptians . Therefore, we will

investigate the frequencies of the *TPMT*, *NAT2*, *GST*, *MDR1 and SULT1A1* polymorphisms in Sudanese and Bahraini individuals, providing a basis for future clinical studies concerning variability in the response and/or toxicity to drugs known to be substrates for these genes. The results may provide a framework for more rational use of drugs that are substrates for these genes. The data obtained may be useful in clinical pharmacogenetic investigations and epidemiological studies of these gene variations.

The present study aims to determine the frequencies of important allelic variants in the *TPMT*, *NAT2, GST, MDR1 and SULT1A1* genes in the Sudanese and Bahraini population. Also to compare these allelic requencies .with the frequencies in other ethnic populations

CHAPTER TWO

2.0 Litrure review

In a large patient population, a medication that is proven efficacious in many patients often fails to work in some other patients. Furthermore, when it does work, it may cause serious side effects, even death, in a small number of patients. Although large individual variability in drug efficacy and safety has been known to exist since the beginning of human medicine, understanding the origin of individual variation in drug response has proven difficult. On the other hand, the demand to overcome such variation has received more attention now than ever before. It is well documented that large variability of drug efficacy and adverse drug reactions in patients

is a major determinant of the clinical use, regulation, and withdrawal from market of clinical drugs and a bottleneck in the development of new therapeutic agents. Factors that cause variations in drug response are multifold and complex, some of which involve fundamental aspects of human biology, because a drug response directly affects well-being and survival. Genetic variation in humans was recognized as an important determinant of individual variability of drug response from clinical observations in late 1950s. In these cases, patients with very high or low plasma or urinary drug concentrations that correspond to a specific phenotype of a drug response were identified, and the biochemical traits leading to the variation of drug concentrations were found to be inherited. The observation that individual variation of a drug response is often larger among members in a population (population variability) than within the same person at different times (intrapatient variability) further supports inheritance as a major determinant of drug response . These clinical and population-based findings fostered the formation of pharmacogenetics to specifically address genetic contribution to individual variability in drug therapy. The human genome sequence provides a special record of human evolution that varies among populations and individuals. Sequence variations in drug target proteins, drug-metabolizing enzymes, and drug transporters can alter drug efficacy, drug side effects, or both to cause variable drug responses in individual patients . From This prospect, the

availability of the complete human genome sequence has made it possible to analyze the impact of variations of the human genome sequence on the pathogenesis of important diseases and the response to drug therapy at an accelerating rate in recent years. The rapid accumulation of knowledge on genome-disease and genome-drug interactions has also impelled the transformation of pharmacogenetics into a new entity of human genetics pharmacogenomics and, at the same time, provided a rationale for the hope that individualized medicine can be achieved in the near future. It is evident that both pharmacogenomics and individualized drug therapy are increasingly influencing medicine and biomedical research in many areas, including clinical medicine, drug development, drug regulation, pharmacology, and toxicology, a thematic reflection of the post genomic era of today's medicine .

2.1 Variation in Drug Responses

Genetic factors may account for between 20 to 95% of the observed variation in drug response between individuals. Drugs undergo five stages following administration:

1. Absorption into the body e.g. from the gut.
2. Distribution to their site of action in the body.
3. Target interaction e.g. binding to cellular receptors or ion channels.
4. Metabolic processing.

5. Excretion from the body.

Theoretically, genes that influence any stage of this pathway could affect the overall drug response. There are two main groups of genes that are important when studying variation in drug responses. The first are those influencing the pharmacokinetic properties of drugs, such as drug metabolizing enzymes and drug transporters, which affect how the drug is handled by the body. The second is those influencing pharmacodynamic properties of drugs, including drug targets such as enzymes, receptors and ion channels, and their associated pathways, which determine the drug's effect on the body. One example of a gene that affects pharmacokinetic drug properties is the ABCB1 gene, which encodes the MDR1 drug transporter; variants of ABCB1 are associated with resistance to the effects of drugs such as the anti-epileptic agent phenytoin. One example of a gene that affects pharmacodynamic drug properties is the CYP2C19 gene, which encodes the metabolic enzyme cytochrome p450. Variants of CYP2C19 are associated with a decreased level of responsiveness to omeprazole, used to treat peptic ulcers and other gastric complaints .

2.3 Pharmacokinetics and Pharmacodynamics

Interference with the therapeutic effects of a medicine can occur at one or more steps in the pathway for the drug. The terms pharmacokinetic and pharmacodynamics have been adopted to describe effects on the drug's

metabolism and on the drug target, respectively. Pharmacokinetic can include genetic influences on drug absorption, transport, and metabolism. Pharmacodynamics refers primarily to genetic variations in the drug target or surrounding pathways that influence the effects of the drug.

2.4 Pharmacokinetics

2.4.1 Drug transport

The active transport of a drug across biologic membranes involves a variety of transporters. Variation in a drug transporter producing a clinical impact is less common than is observed for other pharmacokinetic mechanisms. However, there are examples of variation in drug transport that can be attributed to adverse drug effects. One such example is the SLCO1B1 gene that encodes a polypeptide, OATP1B1, which mediates hepatic uptake of anionic drugs, including most HMG-CoA (3-hydroxy-3-methylglutaryl-coenzyme A) inhibitors . Statins are among the safest drugs; however, a relatively small percentage of treated individuals develop myopathy or, more rarely, rhabdomyolysis. Employing a genome-wide association study (GWAS), a variant in the SLCO1B1 gene (rs4363657) was found to be associated with statin-induced myopathy (60% of myopathy cases possessed the variant) . In addition to adverse effects, the dose of a drug

can also be affected by variation in a drug transporter. The P-glycoprotein, the product of the ABCB1 gene, is involved in the transport of a variety of drugs including digoxin, cyclosporine, and others. The bioavailability of digoxin can range from 67% to 87% depending on the genotype of the ABCB1 gene . Likewise, an ABCB1 haplotype comprised of five variants is associated with an almost two-fold difference in the effective dose of methadone in opioid-dependent subjects . Variability in drug transporters also contributes to resistance to a variety of medicines, most commonly observed in the treatment of a variety of cancers. Thus, although not commonly encountered, genetic alterations in genes for transport molecules are a significant contributor to treatment outcomes.

2.5 Drug metabolism

More commonly encountered sources of variability in drug disposition are genetic variations that affect enzymes involved in the activation or catabolism and removal of a drug (pharmacokinetics). Presently, the pharmacogenetic database, PharmGKB (http://www.pharmgkb.org) lists 301 genes implicated in the metabolic disposition of drugs. On the basis of the nature of the chemical modification, drug metabolizing enzymes can be categorized into oxidative enzymes (phase I) and conjugative enzymes (phase II). Most common among phase I reactions are the cytochrome P-450 (CYP) enzymes. These are monooxygenases of which more than 30 isoforms have been described; they have been estimated to metabolize over

80% of pharmaceuticals, either in the elimination of the active form of the drug or in activation of a prodrug. A prodrug is a pharmacologically inactive compound that requires chemical modification into its active form. Prodrugs can be subclassified by the site of bioactivation, intracellular or extracellular, such as in digestive fluids or in peripheral circulation. These reactions are typically catalyzed by phase I enzymes; variation in the genes for these enzymes can result in sub-optimal prodrug activation. An example is the antiplatelet drug clopidogrel, which requires the P-450 enzyme CYP2C19 for activation. The gene encoding the CYP2C19 enzyme is polymorphic, leading to reduced or increased function, depending on the respective genotype. Two reduced function alleles CYP2C19*2 and CYP2C19*3 produce enzymes with reduced capacity for prodrug activation and have been reported to be associated with an increase in cardiovascular events. Several investigations have estimated an increased risk ratio for cardiovascular events from 1.53 to 3.69 for carriers of loss-of-function alleles compared with individuals with two copies of the wild-type allele (CYP2C19*1/CYP2C19*1) . On the basis of the purported increased risk for ischemic events for carriers of the loss-of-function alleles, the FDA issued a black box warning about the reduced effectiveness of clopidogrel for individuals with those genotypes. The FDA warning included the recommendation for alternative anti-platelet therapy or increased clopidogrel dosing for those individuals. However, following the issuance

of the black box warning, conflicting findings regarding the effects of the loss-of-function genotypes on clinical events for patients enrolled in the cure and active A trials were reported . In addition to the activation of prodrugs, the cytochrome P-450 enzymes are frequently active in the inactivation and elimination of therapeutics as well. For example, warfarin is the currently most studied cardiovascular drug for pharmacogenetic effects. *S*-warfarin, the pharmacologically active isomer, is metabolized mainly to its inactive forms, 7-hydroxywarfarin and 6-hydroxywarfarin by CYP2C9. Thus, the elimination kinetics of warfarin is largely dependent on the availability and activity of the CYP2C9 microsomal enzyme. Pharmacogenetic studies have shown that the CYP2C9*2 variant (Cys144/Ile359) and the *3 variant (Arg144/Leu359) are associated with significantly reduced stable warfarin dose . Functional studies revealed that gene expression of the *2 and *3 variants was similar to that of wild-type, but the kinetics of substrate oxidation were slower . Thus, the reduced oxidative capacity of the CYP2C9 variants underlies impaired warfarin elimination ultimately resulting in a lower dose to achieve therapeutic anticoagulation . Genetic variability in the drug targets, receptors, or molecules of signal transduction can affect the dose, effectiveness, and tolerability of drugs. Numerous observations have related genetic variation in a drug target to clinical response or adverse events. Commonly recognized examples include the mu opioid receptor (OPRM1) and the

vitamin K epoxide reductase complex 1 genes (VKORC1) genes. A polymorphism in OPMR1 causes the substitution of aspartate (Asp) for asparagine (Asn) at position 40 in the amino acid sequence of the receptor, eliminating an N-glycosylation site. This modification confers greater affinity for the endogenous opioid β-endorphin leading to activation of the G-protein-activated inwardly rectifying potassium channel with altered response to opioid treatment. Commonly studied, the vitamin K epoxide reductase complex 1 gene (VKORC1) encodes the enzyme that is responsible for reducing vitamin K 2, 3-epoxide to the active form and is involved in the gamma-carboxylation required for activity of several coagulation factors. The enzyme is the molecular target for warfarin. Early studies identified 10 non-coding single nucleotide polymorphisms (SNPs) within the gene. Linkage studies combined the 10 SNPs into haplotypes, one associated with a low warfarin dose and another, a high dose with 2-3-fold dose differences between the two . A recent compilation of multiple sources including databases and literature citations lists 703 genes potentially susceptible to pharmacodynamics effects . Indeed, PharmGKB lists 837 genes with potential pharmacodynamics effects. This clearly illustrates the profound effect that variation in drug target can impart, and it provides potential targets for future drug development .

2.6 Individual variability in drug therapy

2.6.1 Pharmacokinetic variability

The term ‘pharmacokinetic variability’ refers to variability in the delivery of a drug or metabolite(s) to target molecules, and is traditionally subdivided into the processes of absorption, distribution, metabolism and elimination, or, collectively, drug disposition (Figure 1). Since Garrod’s original postulate, and the description of a familial component in N-acetylation, DNA variants have been described that contribute to variability in specific pathways of drug disposition, with important clinical consequences. Examples include: N-acetylation, drug oxidation by CYP2D6, CYP2C9 or CYP2C19, conjugation by thiopurine methyltransferase, glucuronosyltransferases or sulphotransferases and cleavage by pseudocholinesterase. Although familial aggregation of unusual responses to drugs has often been the first hint of the existence of clinically important variants in drug-metabolizing enzymes, modern genetic approaches have found several variants in single genes. For example, over 70 variants in the CYP2D6 gene have been described, some of which lead to loss of function. Homozygotes, which comprise 7% of Caucasian and African-American populations, are rendered so-called ‘poor metabolizers’ on this basis. Such loss-of-function alleles are very uncommon among Asian populations, in which, however, alleles causing reduction of function have been described. At the other end of the catalytic spectrum are individuals with multiple functional copies of the gene,

known as ‘hyper-extensive metabolizers’, who constitute up to 20% of some African populations. Variability in the frequency and, indeed, the types of allelic variant among ethnic populations is a common theme in contemporary genetics that could well underlie ethnic-specific beneficial and adverse drug responses (Figure 2). As a general principle, the problem of DNA variants contributing to aberrant drug metabolism becomes most evident for drugs that have only a narrow margin between the dosages that are required for efficacy, and those that are associated with serious toxicity (such as is the case with cardiovascular or oncology drugs), as well as drugs that have only a single main pathway for elimination. Drugs whose biotransformation to inactive metabolites is CYP2D6 dependent (for example, some tricyclic antidepressants or β-adrenergic blockers) cause side effects more often among poor metabolizers, and lack of efficacy among hyper extensive metabolizers. Conversely, drugs such as codeine, which undergoes CYP2D6-dependent biotransformation to form its more

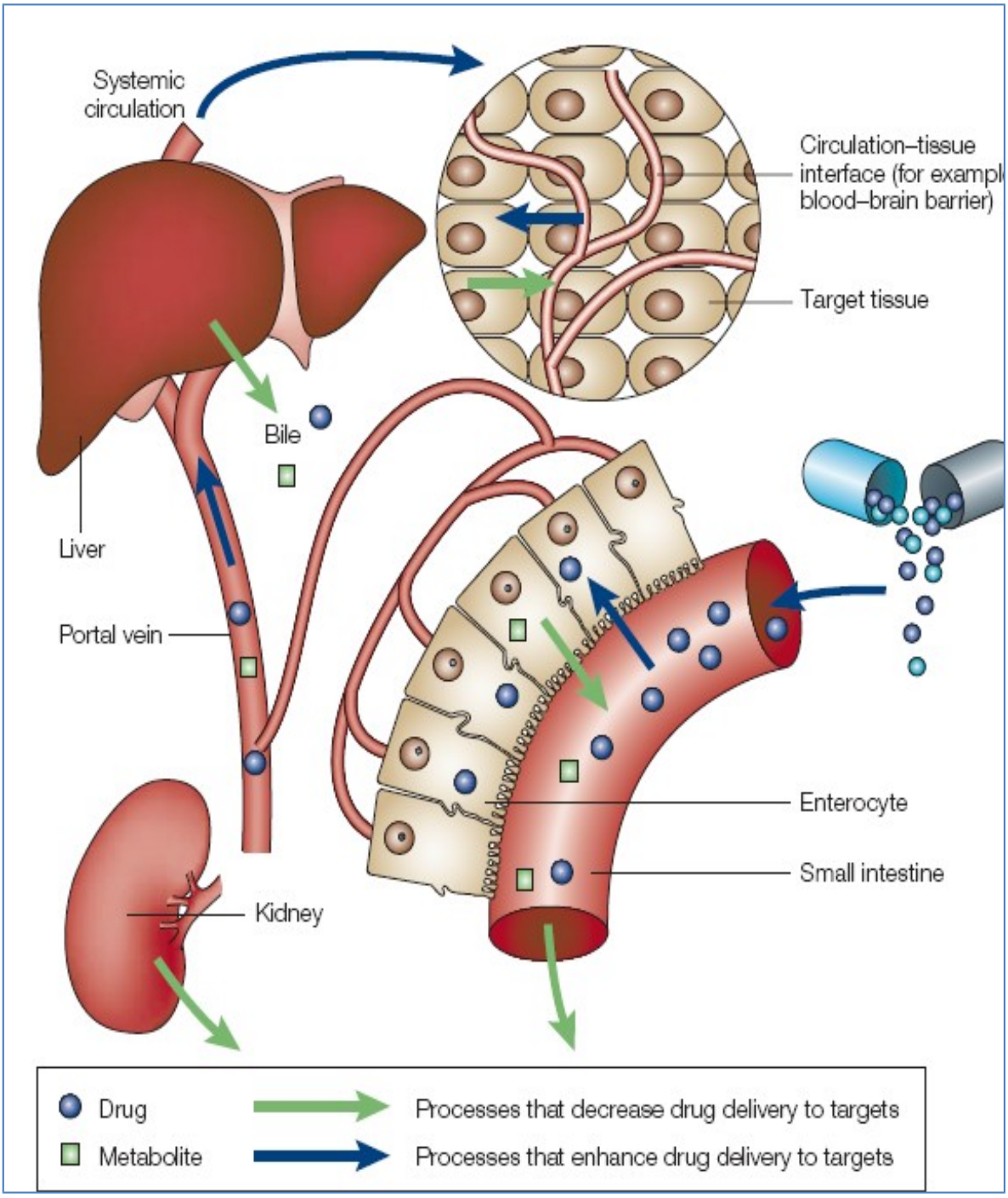

Figure 1: Determinants of drug delivery to target sites

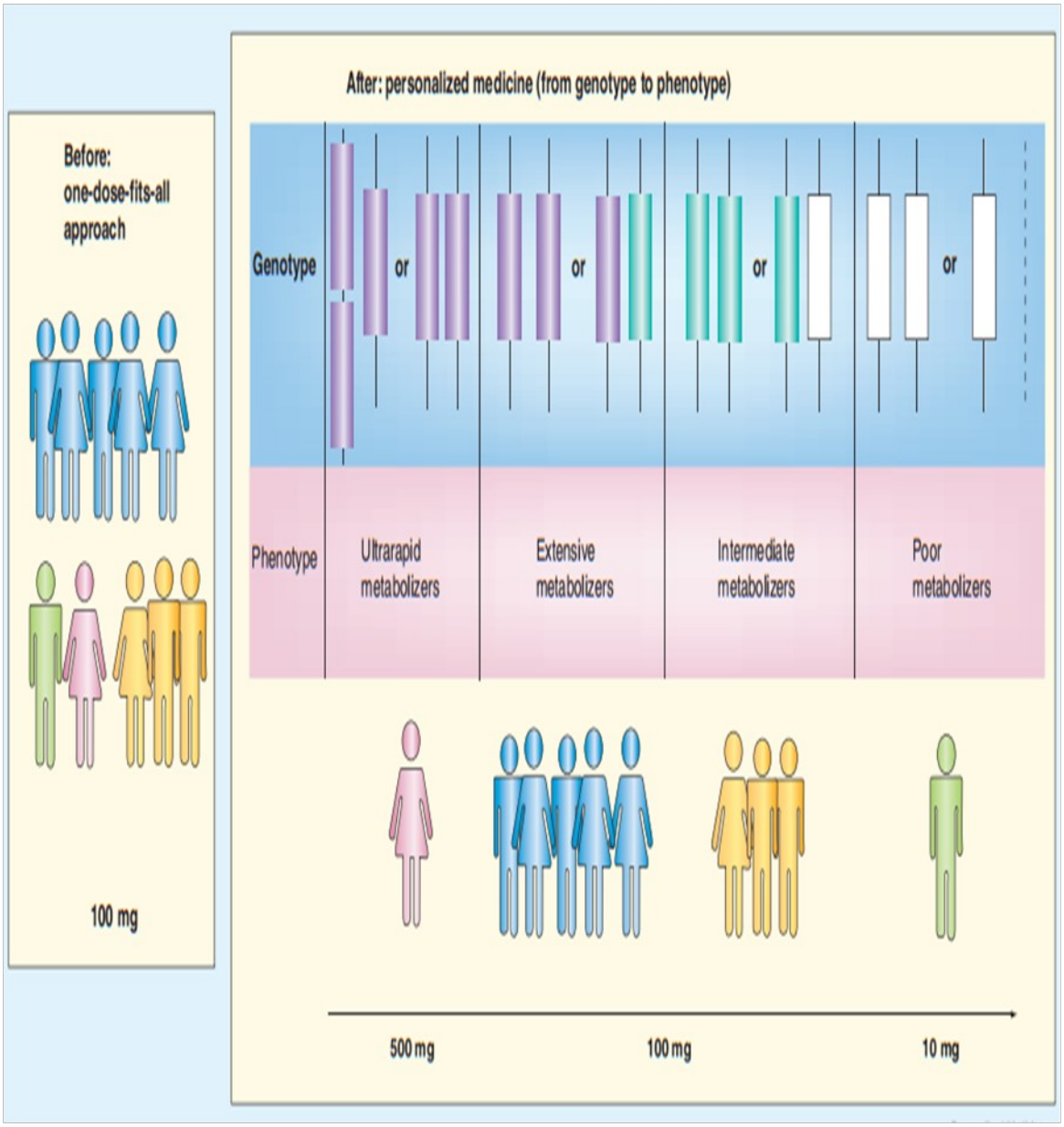

Figure 2: Personalized medicine **(WWW.FUTUREMEDICINE)**

Active metabolite (morphine), can have a lack of efficacy in poor metabolizers, and exaggerated effects among hyper extensive metabolizers.

A minority of individuals with ‘aberrant ‘metabolism makes up the subset that is generally identified in clinical investigation, and it is in this group that aberrant drug responses are most commonly seen early during drug therapy. A second, increasingly recognized, problem in the disposition of a drug that uses a single main pathway is the potential for drug–drug interactions. So, inhibition of CYP2D6 by co-administration of serotonin re-uptake inhibitors or tricyclic anti-depressant drugs29, or inhibition of CYP3A4 by co-administration of erythromycin or ketoconazole, can cause adverse drug effects, which occur during chronic drug therapy and are therefore a risk in most subjects that have ‘normal’ metabolism. Although the concept of genetic variants in the proteins that accomplish drug metabolism is a relatively mature one, several new areas in drug disposition are emerging. One is the increasing recognition that drug uptake into and efflux from, intracellular sites are accomplished by specific drug transport molecules, and that these also exhibit pharmacologically important allelic variability. For example, the integrity of the blood–brain barrier is now known to arise, not just from tight junctions in the capillary endothelium in this region, but also from expression of the drug-efflux transporter P-glycoprotein on the luminal surface of these cells, which thereby limits the access of drugs to the brain. Another area of active enquiry is the transcriptional regulation of normal proteins, which can be highly variable because of allelic variants in regions of DNA that regulate expression.

Variations in the function or expression of genes encoding factors, such as Nuclear Orphan Receptors, that control the transcription of the genes encoding drug-metabolizing enzymes and transporters, could also contribute to variable drug actions .

2.7 Pharmacodynamic variability

Whereas the term pharmacokinetics describes the relationship between the drug dose and the resulting plasma and tissue drug concentrations, ‘pharmacodynamics’ refers to the relationship between the drug concentration and its effect. Individuals with identical plasma and tissue drug concentrations vary in their responses, indicating that pharmacodynamic mechanisms could contribute a second important component to variable drug actions (Figure 3). Methods to evaluate such variability in response are less well standardized than those used to study drug disposition. In general, pharmacodynamic variability can arise from two distinct mechanisms. In the first mechanism, a drug exerts a variable effect because the specific molecular target on which it acts has some (often genetically determined) variability. For instance, the APOE genotype determines the extent of choline acetyltransferase expression, and has been linked to the response to therapy with tacrine, a choline acetyltransferase inhibitor that is used in the treatment of mild to moderate Alzheimer’s disease. The second, more generic, form of pharmacodynamic variability is the variability of the broader biological context a function of

the expression of tens or hundreds of genes in which the interaction between a drug and its target molecules takes place. As an example, β-blockers have been shown to be especially beneficial in a group of patients at high risk of heart failure who are homozygous for an intronic deletion in the angiotensin-converting enzyme (ACE) gene (the DD genotype), which encodes a key enzyme in the renin–angiotensin system, even though β-blockers do not act directly on the ACE gene itself . More generally, each polymorphism that mediates the development or severity of a human disease can be viewed as a candidate for modulating the responses of drugs that are used to treat that disease .

2.7.1 Factors affecting individual drug response

Genetic and non-genetic factors affect individual variability of a drug response by modulating the dose-response curves of drug efficacy and drug toxicity of patients. Clinical outcome is altered if drug dose is not adjusted accordingly (Figure 4). Genetic factors generally cause permanent changes in protein functions, whereas environmental and physiological factors and their impact on drug response are transient in most cases. Drug target

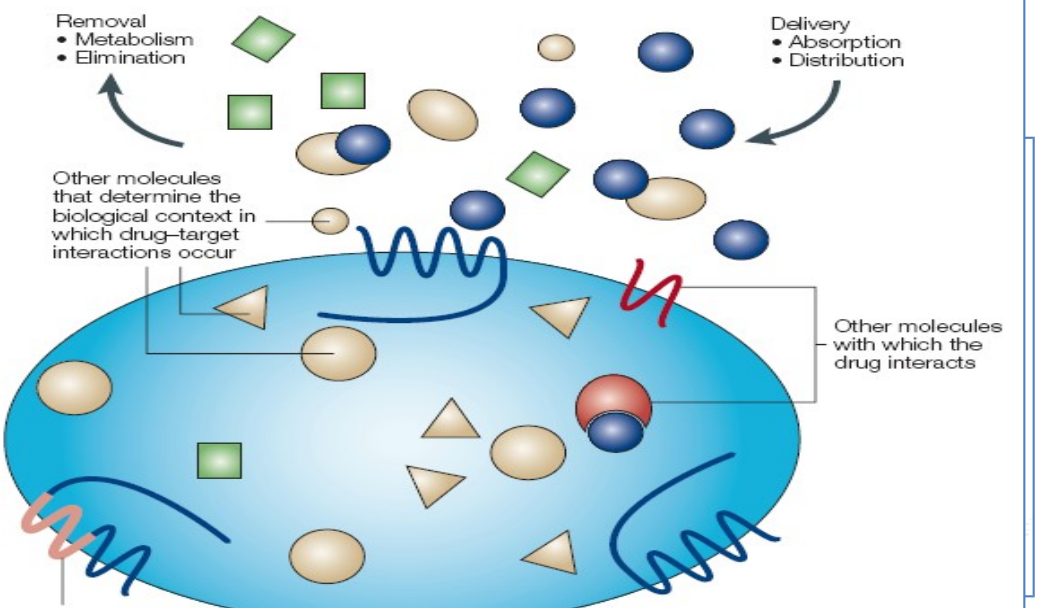

Figure 4: Therapeutic-index

Figure 3: Determinants of drug action at the target site

http://www.medpedia.com/

responsible for an adverse drug reaction can be the same as, or different from, the therapeutic target of the drug, resulting in on-target or off target side effects. Variations in drug pharmacokinetics alter the concentration of a toxic drug or metabolite in the target tissue to cause variable toxicity.

Some genetic variations affect drug efficacy and drug safety indirectly by modulating the biological context in which a drug reaction occurs. At the molecular level, genetic variations can change the structure of a target protein via mutations in the coding region of the gene or the amount of the protein expressed by modulating gene regulation, both of which ultimately

alter the function of the protein or the rate and kinetic constants in the case of an enzyme. Mutations can also modulate gene expression by way of epigenetic regulation. Structural changes of receptors or enzymes may affect drug-receptor or drug-enzyme interaction and, consequently, drug response. Genetic polymorphisms of drug-metabolizing enzymes and transporters can affect the absorption, distribution, metabolism, and elimination of drugs and thereby modulate their plasma and target tissue concentrations. Defective DNA repair enzymes reduce the ability of cells to repair mutations induced by alkylating chemotherapeutic agents. Mutations that alter the structure or reduce the amount of the enzymes involved in the biosynthesis of glutathione are likely to reduce the intracellular content of glutathione, which is critical in protecting cells from oxidative stress and reactive intermediates commonly encountered in adverse drug reactions. Environmental chemicals, coadministered drugs, dietary constituents, tobacco smoking, and alcohol use are all known to induce or inhibit P450s, other drug-metabolizing enzymes, and drug transporters; to alter drug efficacy; and to induce drug-drug and drug-chemical interactions and drug side effects. Large individual variability in the induction and inhibition of human P450 enzymes has been well documented . Environmental factors may also interact with drug targets to produce antagonism or synergy with drugs to alter drug therapeutic effects or toxicity Physiological factors, including age, sex, disease states, pregnancy, exercise, starvation, and

circadian rhythm, can also contribute significantly to individual variations of the pharmacokinetic and pharmacodynamic properties of administered drugs. Some physiological traits are also genetic generally polygenic in nature, such as sex, body weight, and susceptibility to chronic diseases , (Figure 5).

2.8 Types of Genetic Variants

A polymorphism is a variation in the DNA sequence that is present at an allele frequency of 1% or greater in a population. Two major types of sequence variation have been associated with variation in human phenotype: single nucleotide polymorphisms (SNPs) and insertions/deletions (indels). In comparison to base pair substitutions, indels are much less frequent in the genome and are of particularly low frequency in coding regions of genes. Single base pair substitutions that are present at frequencies of 1% or greater in a population are termed single nucleotide polymorphisms (SNPs) and are present in the human genome at approximately 1 SNP every few hundred to a thousand base pairs, depending on the gene region. SNPs in the coding region are termed cSNPs. cSNPs are further classified as non-synonymous (or missense) if the base pair change results in an amino acid substitution, or synonymous (or sense) if the base pair substitution within a codon does not alter the encoded amino acid. Typically, substitutions of the third base pair, termed the wobble position, in a three base pair codon, such as the G to A

substitution in proline, do not alter the encoded amino acid (Figure 5). Base pair substitutions that lead to a stop codon are termed Nonsense mutations. In addition, about 10% of SNPs can have more than two possible alleles (e.g., a C can be replaced by either an A or G), so that the same polymorphic site can be associated with amino acid substitutions in some alleles but not others. Polymorphisms in noncoding regions of genes may occur in the 3' and 5' untranslated regions, in promoter or enhancer regions, in intronic regions, or in large regions between genes, intergenic regions. Polymorphisms in introns found near exon-intron boundaries are often treated as a separate category from other intronic polymorphisms since these may affect splicing, and thereby affect function. Noncoding SNPs in promoters or enhancers may alter cis or transacting elements that regulate gene transcription or transcript stability.

Noncoding SNPs in introns or exons may create alternative exon splicing

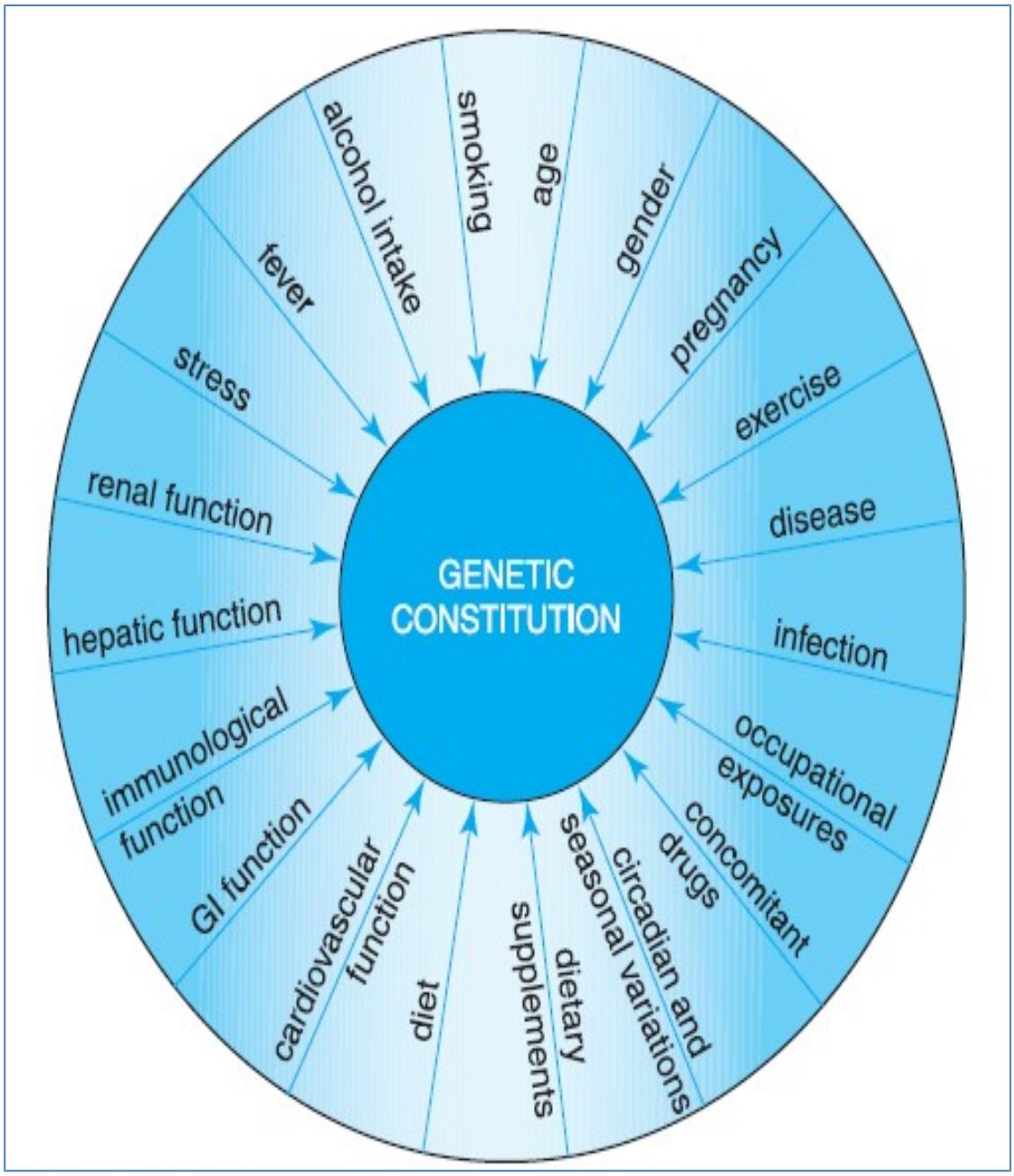

Figure 5: Exogenous and endogenous factors contribute to variation in drug response

sites, and the altered transcript may have fewer or more exons, or shorter or larger exons, than the wild-type transcript. Introduction or deletion of exonic sequence can cause a frame shift in the translated protein and thereby change protein structure or function, or result in an early stop codon, which makes an unstable or nonfunctional protein. Because 95% of the genome is intergenic, most polymorphisms are unlikely to directly affect the encoded transcript or protein. However, intergenic polymorphisms may have biological consequences by affecting DNA tertiary structure, interaction with chromatin and topoisomerases, or DNA replication. Thus, intergenic polymorphisms cannot be assumed to be without pharmacogenetic importance. A remarkable degree of diversity in the types of insertions/deletions that are tolerated as germline polymorphisms is evident. A common glutathione-S-transferaseM1 (GSTM1) polymorphism is caused by a 50-kilobase (kb) germline deletion, and the null allele has a population frequency of 0.3 to 0.5, depending on race/ethnicity. Biochemical studies indicate that livers from homozygous null individuals have only ~50% of the glutathione conjugating capacity of those with at least one copy of the GSTM1 gene .

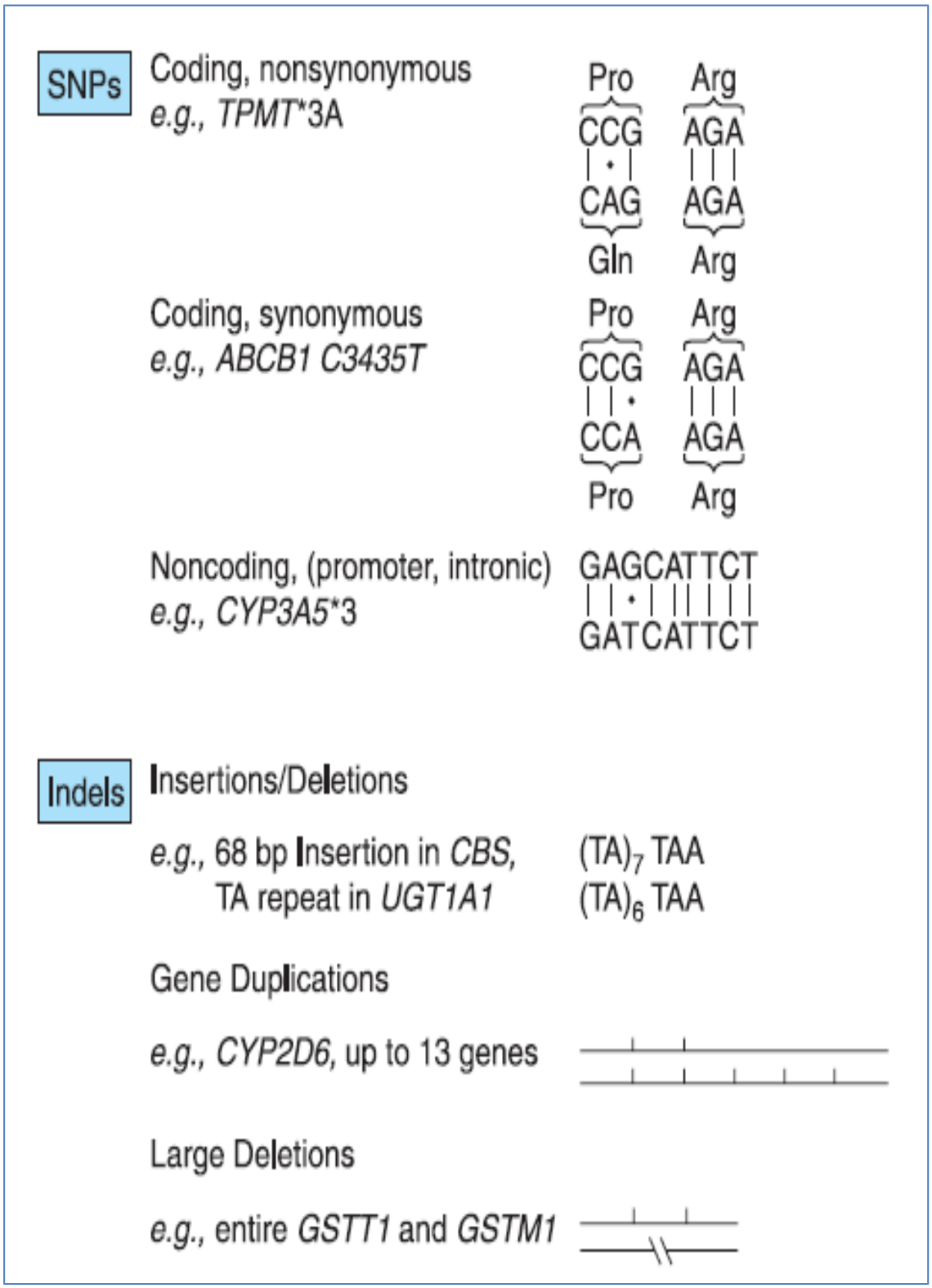

Figure 6: Molecular mechanisms of genetic polymorphism
(Mary V. Relling and Kathleen M. Giacomini-Pharmacogenetics/Chapter4)

The number of TA repeats in the UGT1A1 promoter affects the quantitative expression of this crucial glucuronosyl transferase in liver; although 4 to 9 TA repeats exist in germline inherited alleles, 6 or 7 repeats constitute the most common alleles. Cystathionine β-synthase has a common 68 base pair insertion/deletion polymorphism that has been linked to folate levels. Although in many of these cases the local sequence context of these insertions/deletions strongly suggests mechanisms underlying the genomic alterations (*e.g.,* homologous recombination sites bracket the GSTM1 deletion), high allele frequencies are maintained due to Mendelian inheritance. A haplotype, which is defined as a series of alleles found at a linked locus on a chromosome, specifies the DNA sequence variation in a gene or a gene region on one chromosome. For example, consider two SNPs in ABCB1, which encodes for the multidrug resistance protein, P-glycoprotein. One SNP is a T to A base pair substitution at position 3421 and the other is a C to T change at position 3435. Possible haplotypes would be T_{3421} C_{3435}, T_{3421} T_{3435}, A_{3421} C_{3435}, and A_{3421} T_{3435}. For any gene, individuals will have two haplotypes, one maternal and one paternal in origin, which may or may not be identical. Haplotypes are important because they are the functional unit of the gene. That is, a haplotype represents the constellation of variants that occur together for the gene on each chromosome. In some cases, this constellation of variants, rather than the individual variant or allele may be functionally important. In others,

however, a single mutation may be functionally important regardless of other linked variants within the haplotype(s). (From PHARMACOGENETICS-Chapter 4).

2.9 Human genetics in drug response

Genetic variation is likely to contribute substantially to the variation in drug response observed across human populations. The field of pharmacogenomics, which seeks to relate genetic variability to variability in human drug response, has evolved considerably from candidate gene studies to studies of variation across whole genomes of human populations containing individuals who exhibit a range of responses to different drugs. The initial successes in the field were often the identification of genetic variants within drug metabolizing genes that had large effects on sensitivity to a given drug. The field has since broadened in scope to encompass regulatory mutations, and refined techniques have enabled the identification of mutations with smaller effect sizes. Whereas early pharmacogenomics studies sought primarily to identify associations between common genetic variation and drug response, more recent approaches have begun to identify mRNAs, miRNAs, and other downstream events that are influenced by genetic variation and may underlie variation in pharmacologic responses. A primary aim of pharmacogenomics has been to uncover novel human genetic variants that affect therapeutic response phenotypes and to identify the genes responsible for those phenotypic differences. The ultimate goal of the field

has been to use an understanding of these relations to devise novel personalized pharmacological treatment strategies that maximize the potential for therapeutic benefit and minimize the risk of adverse effects for any given medication. The potential cost savings (via increased drug efficacy) and decreased morbidity and mortality (via increased drug safety) is immense. Advances in DNA sequencing and polymorphism characterization technologies have enabled the field to evolve from the sole reliance on hypothesis driven approaches to the use of discovery oriented, genome-wide approach that requires fewer a priori assumptions regarding genetic variants. Candidate gene approaches resulted primarily in the identification of genetic variants in drug-metabolizing genes with large effects on toxicity or response; however, many genome wide association studies (GWAS) have identified novel associations between drug response and genetic variants with unknown functional relevance and often with relatively small effect sizes (Figure 7), .

2.9.1 Genomic variation

The genetic mechanisms that give rise to pharmacogenetic effects recapitulate all forms of genomic variation. The 1000 Genomes Project is an international collaboration that has the goal of creating a map of human genetic variation. A recent data release from the project included 15 million SNPs, 1 million insertion/deletion variants, and 20,000 structural variations 1000 Genomes Project (Consortium et al., 2010). As one might expect,

genomic changes resulting in modified drug effects have been found to include all of these more common types of genetic variation. In addition, other mechanisms contribute to the regulation of gene expression and translation. These include epigenetic modifications, miRNA interference, and copy number variation.

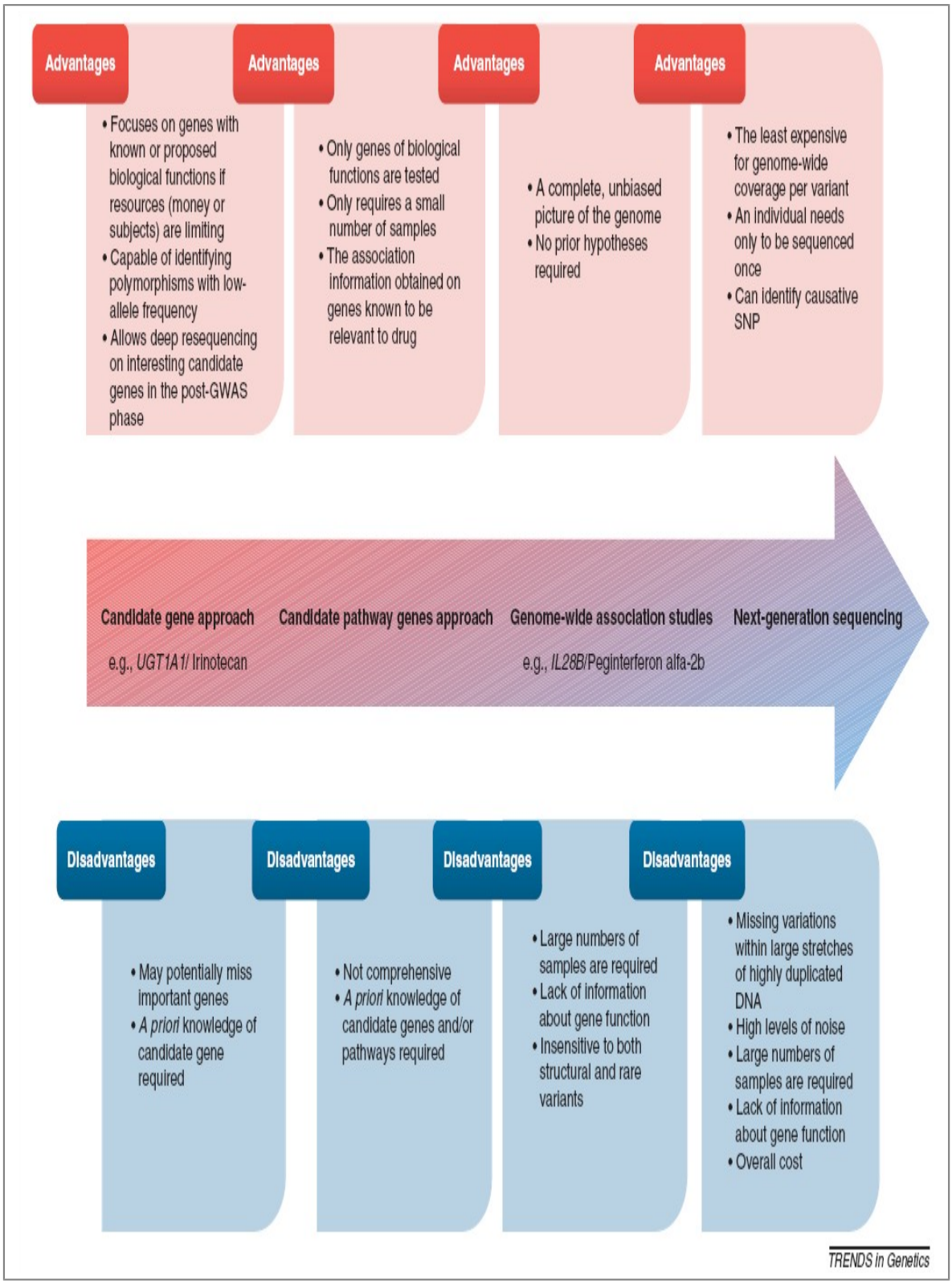

Figure 7: The evolution of pharmacogenomics

2.9.2 Sequence variation

The most frequent form of genomic variation leading to modified drug response is sequence variation, particularly polymorphism at the level of the single nucleotide (SNP). The frequency of SNP-associated pharmacologic effects parallels the overall frequency of single nucleotide variation. SNPs can occur in coding or non-coding regions; in the former case, they can be synonymous producing no change in the coded amino acid, or non-synonymous causing an amino acid substitution .

2.9.3 Non-synonymous sequence alterations

It is expected that a change in the amino acid sequence of a protein has the potential to alter the function of the protein. However, experimental demonstration of altered protein function can be laborious and is not available for the majority of pharmacogenetically active SNPs believed to alter the primary protein sequence. The well-studied model of warfarin metabolism affords an excellent model for altered protein function as a result of an alteration in coding sequence. CYP2C9 metabolizes warfarin as well as other compounds through an eight-step cycle that involves interaction of CYP2C9 with the substrate and with the NADPH-cytochrome P-450 reductase. Crespi and Miller (1997) found that the *CYP2C9*2* (R144C) variant has altered binding properties to the P-450 reductase; downstream metabolic steps are largely unaffected, attributing the slowed rate of warfarin metabolism to the altered P-450:P-450-

reductase binding. Unfortunately, the physiologic effects resulting from gene variants leading to amino acid substitutions have not been examined at this level. As a result, the effects of non-synonymous variants in many genes are unknown or simply inferred.

Synonymous and non-coding sequence alterations

By comparison to non-synonymous variations, the physiologic effects of sequence variations that do not predictably alter the primary sequence of a protein are less easily understood. The simplest explanation for a synonymous coding variation associated with a physiologic effect is that the synonymous variant is in linkage disequilibrium with a functional variant. The ABCB1 3435C>T polymorphism, for example, is an exonic synonymous variant that has been associated with the level of expression of the ABCB1 gene and translated protein. Interestingly, that variant has been associated with the transport of several drugs, despite maintaining the primary amino acid sequence. Association studies have produced equivocal results as to the functional capacity of the *ABCB1* 3435C>T polymorphism. Using allelic coupling (phase) analysis and a tagging SNP approach, one group of investigators were able to identify two *cis*-acting promoter variants that, in probability, underlie the observations associated with 3435C>T. Two mechanisms have been identified through which synonymous changes directly impact translation. These are: (1) codon bias with reduced translation efficiency, and (2) mRNA destabilization. One

must recall that the genetic code is degenerate; that is, for each amino acid, there are up to six codons. The six possible codons are not used randomly; rather, there is actually biased use of codons within a gene. The use of one preferred codon for a particular amino acid leads to the predominance of the corresponding single species of tRNA expressing the anti-codon. Mutation of the favored codon to a different, albeit synonymous codon, selects for a different tRNA that may have reduced availability or be unexpressed due to lack of use. This situation leads to inefficient translation and the reduced availability of newly synthesized protein . The second documented mechanism through which a synonymous change can mediate an effect on translation is through destabilization of mRNA and alteration of mRNA folding. An example of this is the synonymous variation (957C>T) in the human dopamine receptor D2 gene (*DRD2*), which does not alter the protein coding or regulatory regions, but cells with that variant produce reduced protein levels compared to translation of the wild-type gene .

Investigation of the effects of the variation on the tertiary structure of the mRNA revealed the synonymous variation did affect RNA folding and stability; these changes resulted in reduced availability of the receptor mRNA and translated protein . These documented mechanisms codon bias and mRNA destabilization dispel some earlier assumptions of the insignificance of synonymous variation in gene function. Moreover, these

findings emphasize the complexity of genetic sequence variation and their corresponding physiologic effects. Undoubtedly additional physiologic consequences of “silent mutations” will be discovered.

2.9.4 Variations affecting mRNA splicing

Variations in splice sequences of pre-mRNA have also been described that have impact on drug therapy. Probably the best studied, and one of the first pharmacogenetic observations, is the thiopurine methyltransferase gene (TMPT). Thiopurine drugs are commonly used to treat lymphoblastic leukemia as well as autoimmune disease. These agents are metabolized by *S*-methylation of the active intermediate, a reaction catalyzed by thiopurine methyltransferase (TPMT). It has been known for decades that patients who inherited very low levels of TPMT activity are at greatly increased risk for thiopurine-induced toxicity (myelosuppression) when treated with that class of drugs. One genetic anomaly that leads to risk for myelosuppression is the TMPT*4 variant that alters the splice site of intron 9 of the gene. The G to A transition creates two aberrant mRNA transcripts, one with a frameshift in exon 10, and the other with inclusion of an intronic segment with a premature stop codon. Both non-functional transcripts lead to markedly reduced enzyme and risk for thiopurine-mediated toxicity . In this example, a non-coding genetic variation disrupts the fidelity of gene transcription thereby preventing the normal elimination of a drug. However, other regulatory mechanisms that reduce the amount of gene

transcript or gene product can have the same effect. It is of note that TMPT genotyping prior to treatment is recommended.

2.9.5 Variations affecting Signaling pathways

Genetic variation in downstream pathways from the drug target can also have effects on drug efficacy. A well-studied model for mutation in a signaling pathway is the epidermal growth factor receptor (EGFR)-KRAS signaling pathway. EGFR is activated by binding of a specific ligand to the receptor's extracellular domain causing phosphorylation of the receptor's cytoplasmic tyrosine kinase domain and activation of intracellular effectors such as the G protein, KRAS . Many cancers are associated with the activation of growth factors specific for EGFR, and between 30% to 85% of human colon tumors have genetic/chromosomal aberrations associated with EGFR expression . Cetuximab is a recombinant monoclonal antibody directed against the extracellular EGFR domain, thereby preventing the activation of the receptor and downstream signaling pathways. Following its initial approval, treatment failures were found to be common. It was hypothesized that activating mutations in KRAS downstream of EGFR signal initiation would make the pathway insensitive to EGFR antagonists. In 2006, a study of 30 patients with metastatic colorectal cancer treated with cetuximab found that mutations in the KRAS gene were present in 13 of 19 non-responders and 0 of 11 drug responders . Numerous subsequent studies replicated these early findings, and it is now accepted that blocking

the EGFR receptor with cetuximab is ineffective for patients with downstream *KRAS*-activating mutations. The FDA, in July 2009 approved changes to the drug label to include information about treatment of patients with KRAS mutations.

2.9.6 Variations affecting miRNA

Any process that produces a change in the coding, expression, or translation of a drug-related gene can have a potential pharmacogenetic effect. One example of a newly recognized mechanism impacting drug response is miRNA regulation of translation. miRNAs are short non-coding RNA sequences (approximately 22 nucleotides long) that bind to complementary sequences in the 3′ ends of gene-expressed transcripts and mark them for degradation or translation inhibition . The number of identified human miRNAs continues to grow; the human genome may encode as many as 1,000 miRNAs with the ability to control the transcription of 50-60% of known and predicted human genes . The study of miRNA is a somewhat new discipline, but the importance of these small molecules in the development of the organism and in its health and disease is becoming appreciated. From a human health perspective, the role of miRNAs in cancer has been the most studied. For example, one early investigation measured the activity of over 200 miRNA genes and correlated the resultant biosignature to the pathologic classification of chronic lymphocytic leukemia . The potential for utilizing this approach to

determine the best therapy begins to emerge. Inquiry into applications of miRNA to pharmacogenetics seems to be a natural direction for investigations although completed studies are sparse. A few examples of miRNA control of drug targets or drug metabolizing intermediates have recently appeared. A SNP identified many years ago in the 3′ untranslated region of the dihydrofolate reductase gene (DHFR) was initially shown be associated with the level of expression of the gene. Recently, it was found that the naturally occurring SNP is located near the microRNA-24 (miR-24) binding site in the 3′ regulatory region of the gene. The binding of miR-24 to the regulatory sequence did not occur in the presence of the polymorphism, and the subsequent overexpression of the enzyme in the absence of miR-24 regulation was determined to be the mechanistic cause of methotrexate (a dihydrofolate reductase inhibitor) resistance . A second example involves the first description of a cytochrome P-450 enzyme under the regulatory control of a miRNA. The P-450 enzyme, CYP1B1, is involved in an NADPH-dependent electron transport pathway in which it oxidizes a variety of structurally unrelated compounds, including steroids, fatty acids, and xenobiotics. A study by , identified a complementary sequence for miR-27b in the 3′ untranslated region of the CYP1B1 gene. Using a reporter assay construct of the regulatory sequence and a luciferase reporter assay, they were able to demonstrate an inverse association between the levels of the CYP1B1 protein and the level of the inhibitory

miR-27b. The authors speculated that the loss of miR-24 may be the cause of the dysregulated CYP1B1 in some tumors. A novel approach to identifying potential miRNA targets is to use informatics tools to identify possible miRNA binding sites in known genes . Using this approach, a team of investigators found highly conserved extended binding sites for miR-133 and miR-137 in the VKORC1 gene. This prediction has significant impact given the influence VKORC1 has on warfarin metabolism and is worth further exploration. Another bioinformatics model identified a binding site for miR-22 in the 3′ UTR of the MTHFR gene, which is a key enzyme in folate homeostasis and metabolism . The use of the bioinformatics approach holds much promise in combining the rapidly expanding realms of pharmacogenetics and microRNA biology.

2.10 Pharmacoepigenetics

Epigenetic modification refers to processes that modify DNA or chromatin structure in a manner that alters the level of expression of genes but not the DNA sequence itself. Chemical processes that fall into the realm of epigenetics include DNA methylation and post-translational modifications of histones such as the addition of methyl, phosphate, and acetyl groups. These modifications influence the overall chromatin structure and the availability of gene regulatory regions to transcription machinery. The combination of hypomethylated DNA and acetylated histones are characteristic of a transcriptionally active gene. The converse of this

configuration describes a gene that is transcriptionally silent . In some instances, there is a pharmacoepigenetic basis for the development of drug resistance in cancer cells. An interesting illustration was provided by in vitro experiments involving the selection of drug resistant clones by exposing breast cancer cells to physiologic concentrations of doxorubicin. Surviving clones were found to over-express mRNA and protein for the ATP-dependent transporter ABCG2 suggesting that the over-expressed transporter was linked to drug resistance. The investigators used chromatin immunoprecipitation to demonstrate hyper-acetylation of the histone 3 (H3) in close proximity to the promoter of the ABCG2 gene. Hyperacetylation was accompanied by reduced histone deacetylase binding in that region and stronger attachment of the RNA polymerase. Whereas deacetylation of histone proteins functions to silence gene transcription, hyperacetylation enhances RNA polymerase binding and is consistent with the observed dysregulated transcription/translation of that gene . In a clinical study of drug resistance, aberrant methylation patterns were observed for 9 of 14 genes examined in 75 drug resistant breast tumors. Moreover, absent methylation of the ABCB1 promoter correlated with disease progression during doxorubicin treatment . Presently, the examples of pharmacoepigenetics are few but convincing. It is anticipated that with further understanding of non-sequence based modification of the genome,

additional examples of chromatin modifications with therapeutic significance will emerge.

2.11 Copy number variation

DNA copy number variation (CNV) has been described for over twenty years, but the surprising prevalence of CNV in the human genome has been only recently recognized . CNVs defined as duplications or deletions of genomic segments of 1 kb or larger may account for as much as 12% inter-individual genomic variability . Although duplication or deletion of a gene has the potential for significant physiologic impact, the recognized pharmacogenetic effects of large chromosomal changes have been relatively few in number. Perhaps the most noteworthy example is the cytochrome P-450 enzyme, CYP2D6, the first observed example of genetic polymorphism among the P-450 superfamily of enzymes . CYP2D6 is a gene encoding for an important drug-metabolizing enzyme, one thought to be essential for the biotransformation of up to 25% of currently approved medications including β-blockers, antiarrhythmics, opioids, and a number of antidepressant and antipsychotic agents. The gene is highly polymorphic, and the number of duplications ranges from 0-12. Driven by copy numbers of the *CYP2D6* gene, individuals can be categorized into

poor metabolizers, extensive metabolizers, and ultra-rapid metabolizers. Poor metabolizers lack enzyme function as the result of a null allele (CYP2D6*5 variant), a frameshift mutation (CYP2D6**3* and CYP2D6**6*), or a splicing defect (CYP2D6**4*). Approximately 5-14% of Caucasians, 0-5% Africans, and 0-1% of Asians lack CYP2D6 activity . Rapid metabolizers carry multiple copies of the gene (CYP2D6**2XN*). The increased enzymatic activity in individuals carrying one or more duplications enhances the degradation of drugs, which may cause subtherapeutic drug concentrations at typical dosages. Many antidepressants, for example, are metabolized by CYP2D6, and there is a distinct relationship between the number of CYP2D6 genes and the rate of metabolism of antidepressants .

2.12 Ethnic Diversity

Polymorphisms differ in their frequencies within human. Among coding region SNPs, synonymous SNPs are present, on average, at higher frequencies than nonsynonymous SNPs. Thus, for most genes, the nucleotide diversity, which reflects the number of SNPs and the frequency of the SNPs, is greater for synonymous than for nonsynonymous SNPs. This fact reflects selective pressures (termed negative or purifying selection), which act to preserve the functional activity of proteins, and therefore the amino acid sequence. Frequencies of polymorphisms in ethnically or racially diverse human populations have been examined in

whole genome scanning studies . In these studies, polymorphisms have been classified as either cosmopolitan or population (or race and ethnic) specific. Cosmopolitan polymorphisms are those polymorphisms present in all ethnic groups, although frequencies may differ among ethnic groups. Cosmopolitan polymorphisms are usually found at higher allele frequencies in comparison to population-specific polymorphisms. Likely to have arisen before migrations of humans from Africa, cosmopolitan polymorphisms are generally older than population-specific polymorphisms. The presence of ethnic and race-specific polymorphisms is consistent with geographical isolation of human populations. These polymorphisms probably arose in isolated populations and then reached a certain frequency because they are advantageous (positive selection) or more likely, neutral, conferring no advantage or disadvantage to a population. Large-scale sequence studies in ethnically diverse populations in the United States demonstrate that African Americans have the highest number of population-specific polymorphisms in comparison to European Americans, Mexican Americans, and Asian Americans. Africans are believed to be the oldest population and therefore have both recently derived, population-specific polymorphisms, and older polymorphisms that occurred before migrations out of Africa. Consider the coding region variants of two membrane transporters identified in 247 ethnically diverse DNA samples (Figure 7). The figure shows nonsynonymous and synonymous SNPs; population-specific

nonsynonymous cSNPs are also indicated in the figure. The multidrug resistance associated protein, MRP2, has a large number of nonsynonymous cSNPs. There are fewer synonymous variants than nonsynonymous variants, but the allele frequencies of the synonymous variants are greater than those of the nonsynonymous variants. By comparison, DAT, the dopamine transporter, has a number of synonymous variants but no nonsynonymous variants, suggesting that selective pressures have acted against substitutions that led to changes in amino acids. In a survey of coding region haplotypes in 313 different genes in 80 ethnically diverse DNA samples, most genes were found to have between 2 and 53 haplotypes, with the average number of haplotypes in a gene being 14. Like SNPs, haplotypes may be cosmopolitan or population specific and about 20% of the over 4000 identified haplotypes were cosmopolitan . Considering the frequencies of the haplotypes, cosmopolitan haplotypes actually accounted for over 80% of all haplotypes, whereas population-specific haplotypes accounted for only 8% .

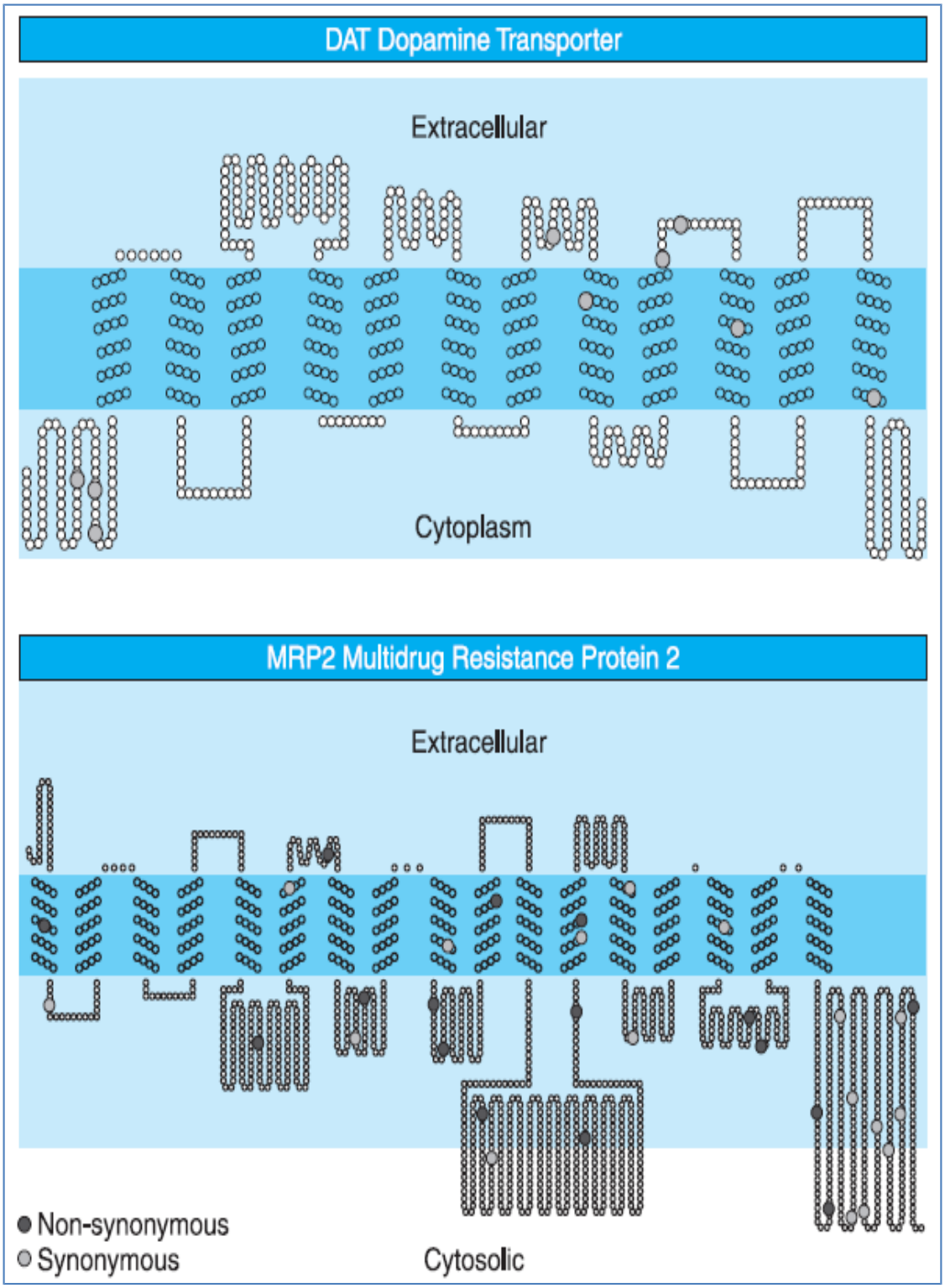

Figure 8: Coding region polymorphisms in two membrane transporter
Source: Shu *et al.*, 2003

Race has been reported to contribute to variability in dosing requirements for warfarin in anticoagulation, with African Americans requiring higher doses and Asians requiring lower doses than whites. CYP2C9 is the enzyme primarily involved in warfarin PK. Variant CYP2C9 genotypes are associated with an increased risk of major hemorrhage, and the frequency of variant genotypes is significantly higher in European Americans than in African Americans . Variations in the VKORC1 gene that encodes for the pharmacologic target of warfarin, vitamin K epoxide reductase, contribute to differences in sensitivity to warfarin. One of the variants in VKORC1 associated with lower dose requirements is 1639 G>A and the AA genotype is found in warfarin-sensitive patients. The frequency of the warfarin-sensitive AA genotype is higher in Chinese than in whites, with frequencies of 82% in Chinese and 14% in whites . A genomic basis found in recent mechanistic studies supports the previously observed ethnic differences in warfarin dose requirements. This serves as an example of the need to understand potential reasons for pharmacokinetic or pharmacodynamic variability in clinical pharmacology or phase II studies so that dosing can be adjusted for relevant populations in later clinical studies.

2.12.1 Warfarin Pharmacogenomics

Many drugs have proven efficacy in the treatment and prevention of cardiovascular disease. Not uncommonly, these drugs have narrow therapeutic indexes that are influenced by genetic variation — a hallmark of drugs for which pharmacogenomic approaches are likely to provide substantial clinical benefit. The anticoagulant agents warfarin and clopidogrel are high on the list of widely prescribed cardiovascular drugs with narrow therapeutic indexes. The pharmacogenomic features of these drugs illustrate the rapid evolution of our understanding of the role of inheritance in the variation in drug efficacy and the risk of adverse drug reactions. In the case of both agents, the application of classic candidate-gene pharmacogenetics has identified important genomic markers of variation in efficacy and adverse reactions, observations that were subsequently confirmed in genomewide association studies. The FDA acted quickly on these data by relabeling warfarin and adding a warning box on the labeling for clopidogrel. Data supporting the clinical utility of routine use of pharmacogenetic testing for both these drugs are evolving. Warfarin is the most widely prescribed oral anticoagulant in North America and much of Europe. Despite the availability of the international normalized ratio (INR), a laboratory test that is universally used to measure the anticoagulant effect of warfarin, serious adverse responses, including hemorrhage and undesired coagulation, continue to complicate therapy,

making warfarin one of the drugs most often responsible for emergency room visits. Chemically, warfarin is a racemic mixture (i.e., one that is composed of two enantiomorphic isomers). S-warfarin is three to five times as potent as R-warfarin as an anticoagulant, has a shorter half-life, and is metabolized predominantly by a cytochrome P-450 enzyme, CYP2C9. Two common CYP2C9 allozymes have only a fraction of the level of enzyme activity of the wild-type allozyme CYP2C9*1: 12% for CYP2C9*2 and 5% for CYP2C9*3. More than a decade ago, it was reported that patients who required a low final dose of warfarin on the basis of INR values often carried one or two of these two common CYP2C9 variant alleles and were at increased risk for hemorrhage during warfarin therapy, presumably because they metabolized the drug more slowly. Those observations were confirmed, but it quickly became clear that the presence of CYP2C9 polymorphisms did not explain most of the variation in the final warfarin dose. Pharmacogenetic studies of warfarin changed dramatically in 2004 when the target for warfarin based anticoagulants, vitamin K epoxide reductase complex subunit 1 (VKORC1), was identified, and single-nucleotide polymorphisms (SNPs) in VKORC1 were shown to be associated with the dose of warfarin required to achieve a target INR value. In 2009, a genome wide association study looked for associations between several hundred thousand SNPs and warfarin dose in about 1000 Swedish patients who were taking warfarin. The results showed two major signals in

and around CYP2C9 and VKORC1 (Figure 8. 1A). When the authors removed the effects of those signals through multiple regression adjustment, they observed an additional signal, implicating another cytochrome P450 gene (CYP4F2) (Figure 8. 1B). CYP4F2 was subsequently shown to catalyze vitamin K oxidation . The variant CYP4F2 allozyme shows decreased ability to catalyze the reaction, and as a result persons who carry the relevant genetic variant in CYP4F2 might require an increase in the warfarin dose (Figure 8. 1C). CYP2C9, VKORC1, and CYP4F2 have also been implicated in a genome wide association study of the administration of acenocoumarol, an anticoagulant related to warfarin. Taken together, CYP2C9 and VKORC1 genotypes explain about 30 to 40% of the total variation in the final warfarin dose. These observations raise the possibility that testing patients for variations in CYP2C9 and VKORC1 might provide information that could enhance clinical algorithms currently used to guide the administration of warfarin. To examine the potential clinical utility of testing for CYP2C9 and VKORC1 genotypes, in addition to INR monitoring and routine use of clinical algorithms, the International Warfarin Pharmacogenetics Consortium recently investigated the anticoagulant response to warfarin, as well as CYP2C9 and VKORC1 genotype data, for about 4000 persons of various ancestral origins .

2.12.2 Pharmacogenetic Measures

What are pharmacogenetic traits and how are they measured? A pharmacogenetic trait is any measurable or discernible trait associated with a drug. Thus, enzyme activity, drug or metabolite levels in plasma or urine, blood pressure or lipid lowering produced by a drug and drug-induced gene expression patterns are examples of pharmacogenetic traits. Directly measuring a trait (e.g., enzyme activity) has the advantage that the net effect of the contributions of all genes that influence the trait is reflected in the phenotypic measure. However, it has the disadvantage that it is also reflective of nongenetic influences (*e.g.*, diet, drug interactions, diurnal or hormonal fluctuation) and thus, may be "unstable." For CYP2D6, if a patient is given an oral dose of dextromethorphan, and the urinary ratio of parent drug to metabolite is assessed, the phenotype is reflective of the genotype for CYP2D6.

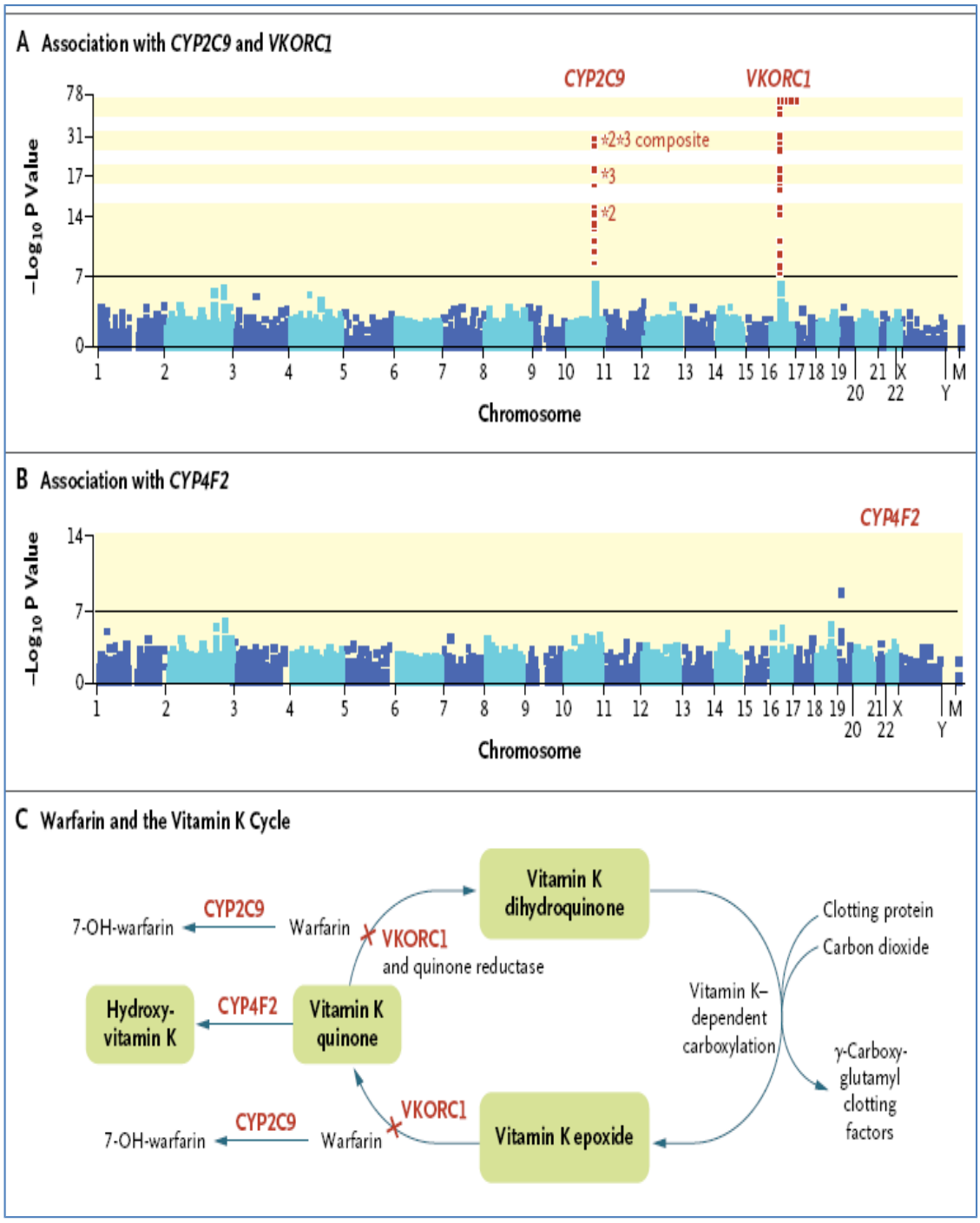

Figure 9: Warfarin pharmacogenomics

Source: Liewei et al., 2011

However, if dextromethorphan is given with *quinidine*, a potent inhibitor of CYP2D6, the phenotype may be consistent with a poor metabolizer

genotype, even though the subject carries wild-type CYP2D6 alleles. In this case, quinidine administration results in a drug-induced haplo-insufficiency, and the assignment of a CYP2D6 poor metabolizer phenotype would not be accurate for that subject in the absence of quinidine. If a phenotypic measure, such as the erythromycin breath test (for CYP3A), is not stable within a subject, this is an indication that the phenotype is highly influenced by nongenetic factors, and may indicate a multigenic or weakly penetrant effect of a monogenic trait. Because most pharmacogenetic traits are multigenic rather than monogenic (Figure 9), considerable effort is being made to identify the important genes and their polymorphisms that influence variability in drug response. Most genotyping methods use germline DNA, that is, DNA extracted from any somatic, diploid cells, usually white blood cells or buccal cells (due to their ready accessibility). DNA is extremely stable if appropriately extracted and stored, and unlike many laboratory tests, genotyping need be performed only once, because DNA sequence is generally invariant throughout an individual's lifetime.

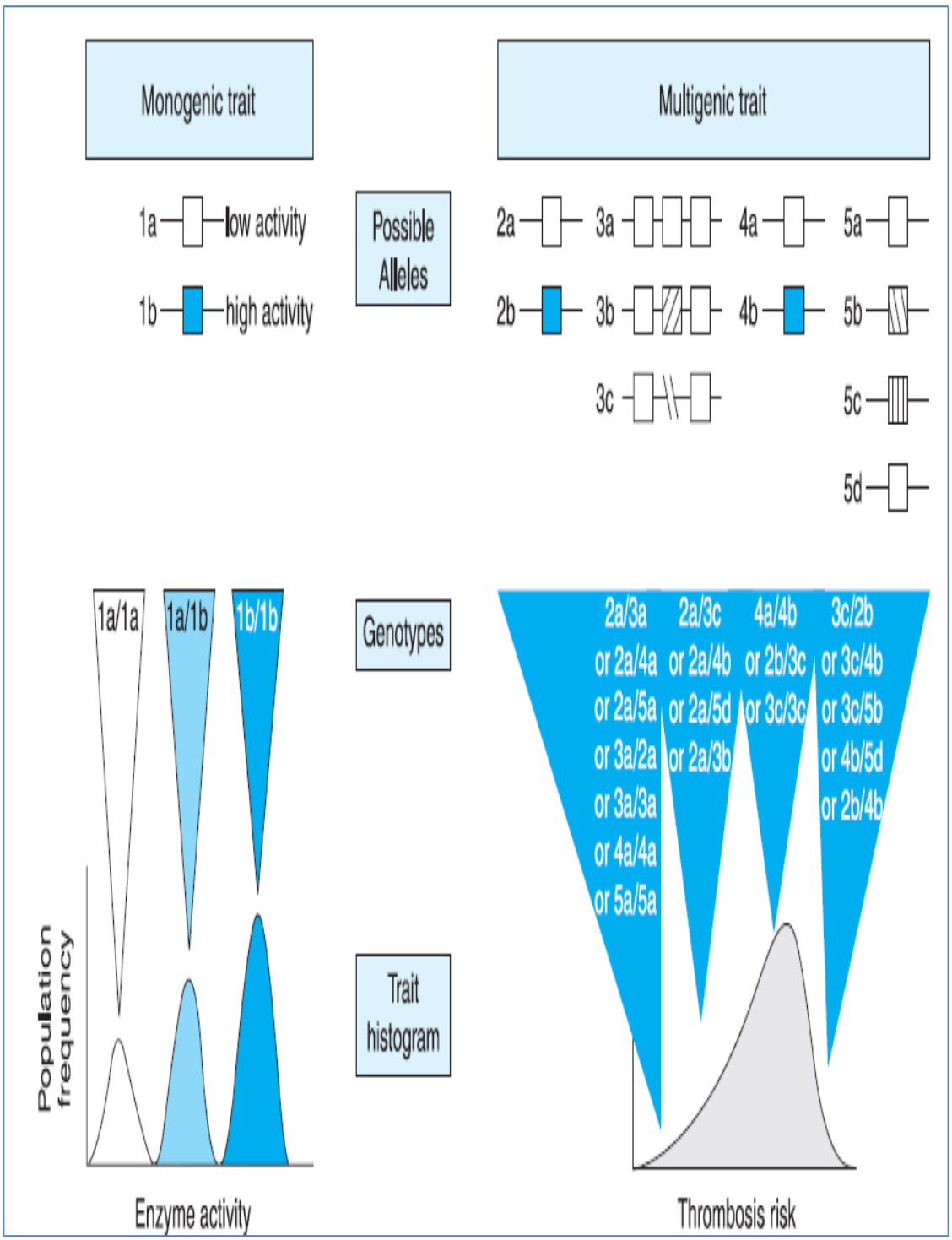

Figure 10: Monogenic and multigenic pharmacogenetics traits

Source: Shu et al.,2003

Genotyping tests are directed at each specific known polymorphic site using a variety of strategies that generally depend at some level on the specific and avid annealing of at least one oligonucleotide to a region of DNA flanking or overlapping the polymorphic site. Because genomic variability is so common (with polymorphic sites every few hundred nucleotides), “cryptic” or unrecognized polymorphisms may interfere with oligonucleotide annealing, thereby resulting in false positive or false negative genotype assignments. Full integration of genotyping into therapeutics will require high standards for genotyping technology, perhaps with more than one method required for each polymorphic site. One method to assess the reliability of genotype determinations in a group of individuals is to assess whether the relative number of homozygotes to heterozygotes is consistent with the overall allele frequency at each polymorphic site. *Hardy-Weinberg equilibrium* is maintained when mating within a population is random and there is no natural selection effect on the variant. Such assumptions are described mathematically when the proportions of the population that are observed to be homozygous for the variant genotype (q2), homozygous for the wild-type genotype (p2), and heterozygous (2*p*q) are not significantly different from that predicted from the overall allele frequencies (p = frequency of wild-type allele; q = frequency of variant allele) in the population. If proportions of the observed three genotypes, which must add up to one, differ significantly from those

predicted, it may indicate that a genotyping error may be present. Because polymorphisms are so common, haplotype (the allelic structure that indicates whether polymorphisms within a gene are on the same or different alleles) may also be important. Thus, experimental methods to unambiguously confirm whether polymorphisms are allelic have proved to be feasible but technically challenging. Most investigators use statistical probability to assign putative or inferred haplotypes; *e.g.*, because the two most common SNPs in TPMT (at 460 and 719) often are allelic, a genotyping result showing heterozygosity at both SNPs will have a >95% chance of reflecting one allele wild-type and one allele variant at both SNP positions (resulting in a "heterozygote" genotype for TPMT). However, the remote prospect that each of the two alleles carries a single SNP variant, thereby conferring a homozygous variant/deficient phenotype, is a theoretical possibility. (From PHARMACOGENETICS-Chapter 4)

2.12.3 Candidate Gene versus Genome-Wide Approaches

Because pathways involved in drug response are often known or at least partially known, pharmacogenetic studies are highly amenable to candidate gene association studies. After genes in drug response pathways are identified, the next step in the design of a candidate gene association pharmacogenetic study is to identify the genetic polymorphisms that are likely to contribute to the therapeutic and/or adverse responses to the drug. There are several databases that contain information on polymorphisms and

mutations in human genes, which allow the investigator to search by gene for polymorphisms that have been reported. Some of the databases, such as the Pharmacogenetics and Pharmacogenomics Knowledge Base (PharmGKB), include phenotypic as well as genotypic data. Because it is currently not practical to analyze all polymorphisms in a candidate gene association study, it is important to select polymorphisms that are likely to be associated with the drug-response phenotype. For this purpose, there are two categories of polymorphisms. The first are polymorphisms that do not, in and of themselves, cause altered function of the expressed protein (*e.g.*, an enzyme that metabolizes the drug or the drug receptor). Rather, these polymorphisms are linked to the variant allele that produces the altered function. These polymorphisms serve as biomarkers for drug-response phenotype. However, their major shortcoming is that unless they are in 100% linkage with the causative polymorphism, they are not the best markers for the drug response phenotype. The second type of polymorphism is the causative polymorphism, which directly precipitates the pheno- type. For example, a causative SNP may change an amino acid residue at a site that is highly conserved throughout evolution. This substitution may result in a protein that is nonfunctional or has reduced function. Whenever possible, it is desirable to select polymorphisms for pharmacogenetic studies that are likely to be causative. If biological information indicates that a particular polymorphism alters function, for

example, in cellular assays of nonsynonymous variants, this polymorphism is an excellent candidate to use in an association study. A potential drawback of the candidate gene approach is that the wrong genes may be studied. Genome-wide approaches, using gene expression arrays, genome-wide scans, or proteomics, can complement the candidate gene approach by providing a relatively unbiased survey of the genome to identify previously unrecognized candidate genes. For example, RNA, DNA, or protein from patients who have unacceptable toxicity from a drug can be compared with identical material from identically treated patients who did not have such toxicity. Patterns of gene expression, clusters of polymorphisms or heterozygosity, or relative amounts of proteins can be ascertained using computational tools, to identify genes, genomic regions, or proteins that can be further assessed for germline polymorphisms differentiating the phenotype. Gene expression and proteomic approaches have the advantage that the abundance of signal may itself directly reflect some of the relevant genetic variation; however, both types of expression are highly influenced by choice of tissue type, which may not be available from the relevant tissue; for example, it may not be feasible to obtain biopsies of brain tissue for studies on CNS toxicity. DNA has the advantage that it is readily available and independent of tissue type, but the vast majority of genomic variation is not in genes, and the large number of SNPs raises the danger of type I error (finding differences that are false-positives). Nonetheless,

technology is rapidly evolving for genome wide surveys of RNA, DNA, and protein, and such approaches hold promise for future pharmacogenomic discoveries .

2.13 Pharmacogenetics in Clinical Practice

Despite considerable research activity, pharmacogenetics is rarely utilized in clinical practice. There are three major types of evidence that should accumulate in order to implicate a polymorphism in clinical care (Figure 10): screens of tissues from multiple humans linking the polymorphism to a trait; complementary preclinical functional studies indicating that the polymorphism is plausibly linked with the phenotype; and multiple supportive clinical phenotype/genotype studies. Because of the high probability of type I error in genotype/phenotype association studies, replication of clinical findings will generally be necessary. Although the impact of the polymorphism in TPMT on mercaptopurine dosing in childhood leukemia is a good example of a polymorphism for which all three types of evidence are available, proactive individualized dosing of thiopurines based on genotype has not been widely incorporated into clinical practice. Most drug dosing takes place using a population “average” dose of drug. The clinical outcome of such adjustments has not been studied .

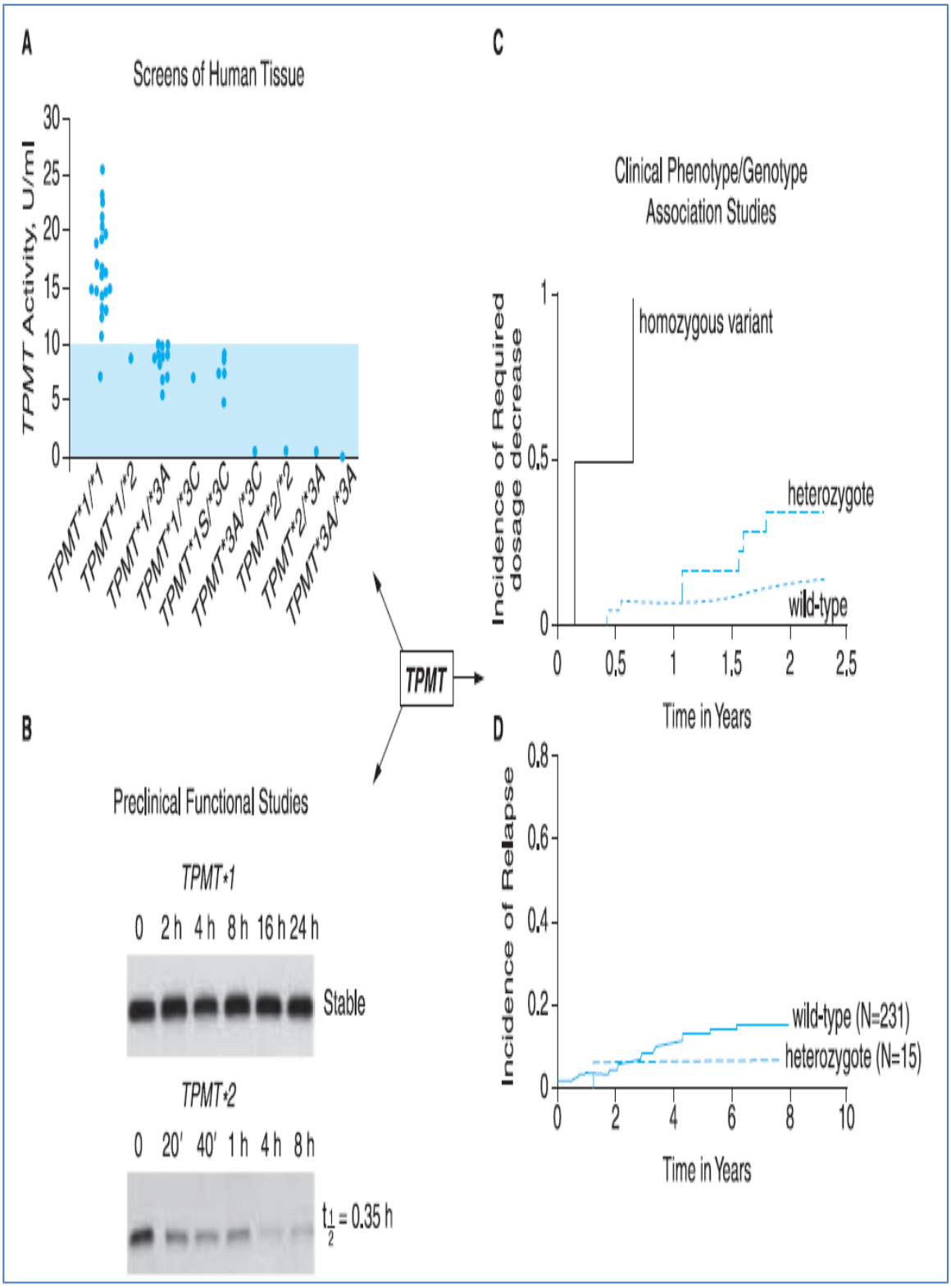

Figure 11: The primary types of evidence in pharmacogenetics

Source: Relling et al.,1999

2.14 Drug Toxicity

The medicine will move from being largely reactive to being predictive, personalized, preventive and participatory. Personalized medicine will focus on the integrated diagnosis, treatment and prevention of disease in individual patients. This will tailor drug therapy at a dosage that is most appropriate for an individual patient, with the potential benefits of increasing the efficacy and safety of medications . P-gp, encoded by MDR1gene, is an ATP-dependent efflux pump which can drive intracellular toxins and drug metabolites of the cell. Single-nucleotide polymorphisms in MDR1 gene are associated with variation in the P-gp expression. Lower expression of P-gp would reduce the protection for cells and may contribute to disease susceptibility . Although P-gp expression is highly variable between individuals, the molecular basis for this variation was not clear until recently, with the description of functional (C3435T) SNP in exon 26 of the MDR-1 gene . The silent mutation C3435T is the most commonly reported polymorphism linked to different responses of patients to various MDR1 substrates. The frequency of this mutation is not uniformly distributed among diverse population, but ethnic and geographical differences were observed . There was a significant relationship of the silent C3435T SNP with intestinal P-gp expression levels. Individuals who were homozygous for the 3435T variant had

significantly decreased intestinal P-gp expression . As P-gp has an important role in the bioavailability of a wide variety of drugs, including anticancer, antihypertensive, antiarrythmics, antiviral, glucocorticoids, antibiotics, immunosuppressants, antidepressants, neuroleptics, opioids and many others . So, these drugs must be used cautiously in those subjects.MDR1 gene encodes P-gp which functions as a physiologic intestinal barrier against drug absorption including clopidogrel and digoxin. Clopidogrel, an antiplatelet drug, reduces the cardiovascular complications in patients with coronary artery disease. The response to clopidogrel varies greatly among patients. Simon . reported that acute myocardial infarction patients on clopidogrel carrying 3435T variant of MDR1 presented more than five times the rate of adverse events of patients with wild-type genotype. Also observed that the prevalence of 3435T variant of MDR1 was associated with decrease clopidogrel response in patients with acute .myocardial infarction

CHAPTER THREE

3.0 METHODS

Genotyping was carried out in a total of 300 Bahraini subjects from blood donors, central laboratory at Salmaniya Medical Center. The samples will be collected from unrelated individuals under institutionally approved

internal review board protocols with informed consent and114 unrelated healthy Sudanese from Khartoum, the capital of Sudan. TPMT*2 was detected using an allele-specific polymerase chain reaction (PCR) assay. TPMT *3C and NAT2 variants (*5, *6 and *7) were detected using an allele-specific real-time PCR assay . Detection of GSTM1and GSTT1null alleles was performed simultaneously using a multiplex PCR assay. Finally, a PCR-restriction fragment length polymorphism assay was applied for the determination of TPMT *3A(*3B) , SULT1A1*2 and MDR-1 (3435T) variants .

3.1 **DNA Extraction**

Total genomic DNA was extracted from leukocyte-rich buffy coat layer, using Qiagen mini-spin column technique, according to manufacturer's specifications (Qiagen, Hilden, Germany). This kit isolates total genomic DNA from variety of biological samples, and relies on cell lysis using strong chaotropic agent (guanidium thiocyanate), followed by DNA binding to a QIAamp silica-gel membrane (contained in a microspin column), and passage of non-DNA contaminants through the membrane. Purified DNA is subsequently eluted with nuclease-free water, and stored at 4°C.

3.1.1 Extraction

Proteinase K (20 µl of 1 mg/ml solution) was added to 200 µl of buffy coat cells in 1.5 ml microcentrifuge tube, followed by addition 200 µl of

guanidine Thiocyanate-containing lysis solution. The mixture was mixed by pipetting several times using a wide-bore 1000 µl pipet tip, followed immediately by pulse-vortexing for 15 sec (to collect droplets at the micotube walls), and incubation for 10 min at 56°C in dry bath. During incubation, one mini-spin column was placed in a 2-ml labeled collection tube. Absolute ethanol (200 µl of 96-100%) was then added to the lysates, which were vortexed for 15 sec.

3.1.2 DNA Binding

The lysates were transferred to the micro-spin column, and centrifuged for one min at 6,000 g (8,000 rpm) in an Eppendorf 5415D microcentrifuges. Relative centrifugal speed/force (RCF) was calculated as per: $RCF = 1.12 \times r \times (rpm/1000)^2$; where r = radius of rotor bucket (in mm), and rpm = revolution per minute. The flow-through contents were then discarded by emptying the collection tube, the column was then reintroduced to a new back collection tube.

3.1.3 Washing

Wash solution (500 µl) was added to the mini-spin column, which was re-centrifuged for 3 min at 13,000 g at room temperature. The flow-through contents were discarded, and the column was reintroduced back to the collection tube, and washed with 70% ethanol (500 µl) by centrifugation at 13,000 g for 30 sec at room temperature. The collection tube was then

discarded, and the mini-spin column was then transferred into a clean sterile RNase/DNase-free 1.5 ml microcentrifuge tube for elution of DNA.

3.1.4 Elution

The elution buffer (TE buffer or nuclease-free water, 250 µl) were applied directly to the mini-spin column, incubated for one min at room temperature, and centrifuged for 1 min at 6,000 g to recover purified DNA. DNA was then stored indefinitely at 4°C pending analysis.

3.2 Genotyping by Real Time PCR Analysis

TPMT*3C (A719G), NAT2 variants (*5 (T341C), NAT2*6 (G590A) and NAT2*7 (G857A) were detected using an allele genotyping were done by real-time polymerase chain reaction (PCR), using the Applied Biosystem (ABI) StepOne PCR System (Table 2). This was based on the detection of fluorescent signals produced proportionally during each PCR cycle. The main advantage of SNP detection by real-time PCR (as opposed to PCR-RFLP analysis) was the relatively short turn-around time for data acquisition and analysis (less than two hours for standard assays, and only 40 min for quick runs (fast chemistries), and ease of genotype discrimination (genotype clusters) (Fig 12). The genotyping required two steps: cycling (PCR amplification) followed by endpoint detection of fluorescent signals.

3.2.1 Methodology

Specific fluorescent-dye-labeled probes were obtained for each allele, which contain different fluorescent reporter dyes for allelic discrimination (Fig. 13). TaqMan® minor groove binder (MGB) probes were used for the StepOne systems, each containing: A reporter dye at the 5′ end of each probe (VIC® linked to the 5′ end of Allele 1, and FAM™ linked to the 5′ end of the Allele 2).

- A nonfluorescent quencher (NFQ) at the 3′ end of the probe (Fig. 13).

For every run, negative and positive controls were included. The former included water (or elution buffer) instead of the DNA template, and should yield no amplification. Optionally, positive controls were included in the initial runs. These consisted of samples with known genotypes (allele 1 homozygotes, allele 2 homozygotes, and heterozygotes).

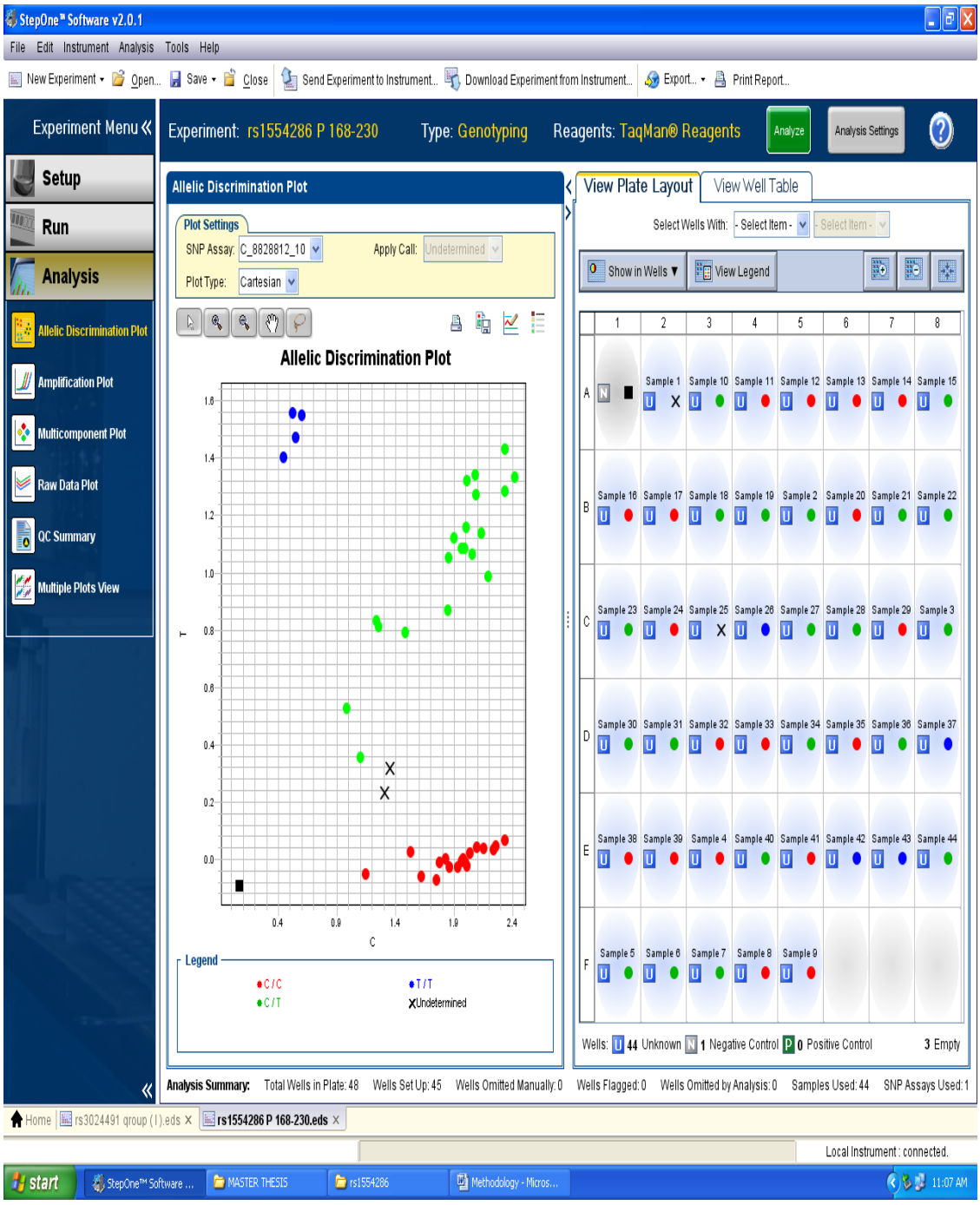

Figure 12: Genotype discrimination (genotype clusters by real time PCR)

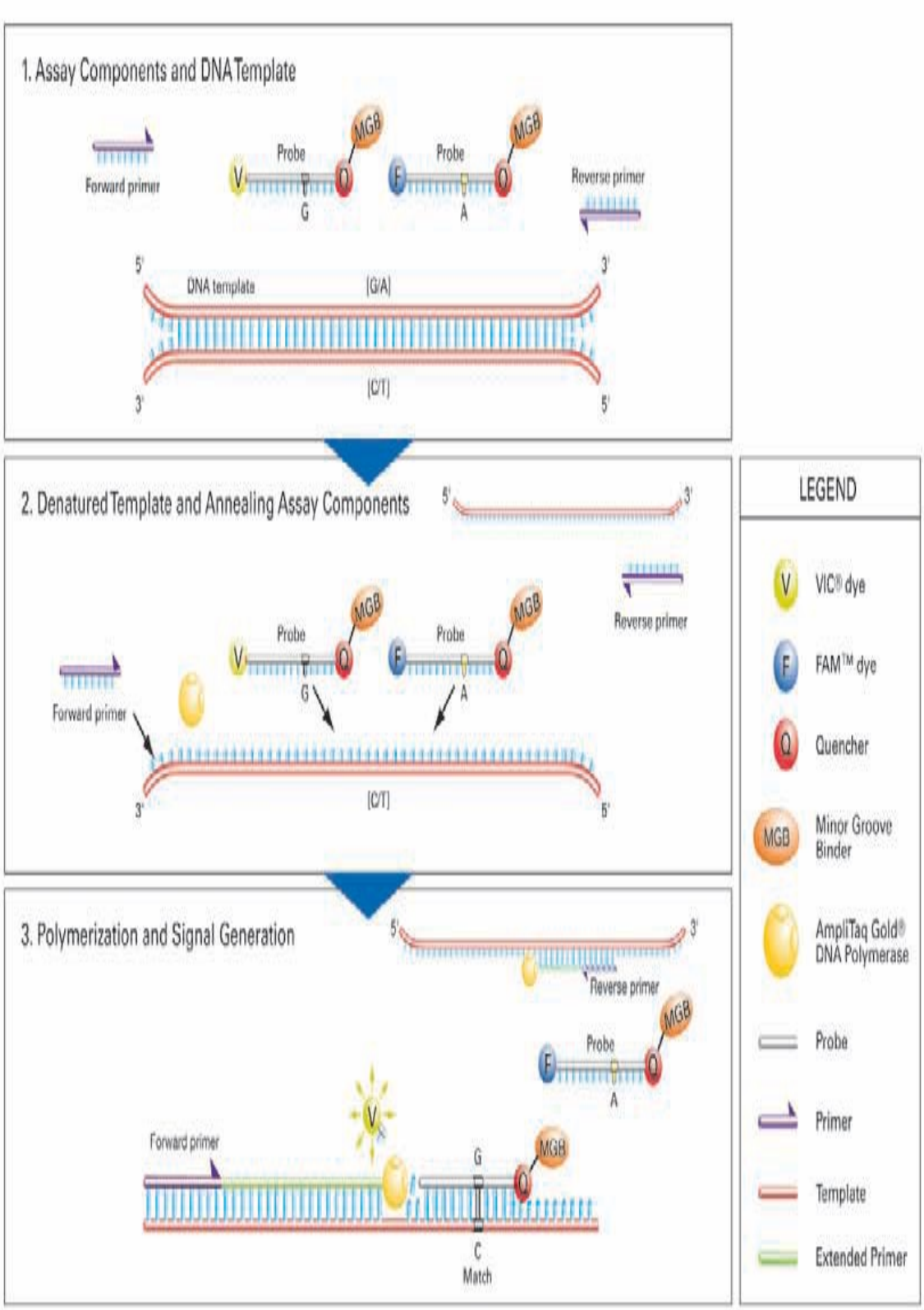

Figure 13: Specific Fluorescent-Dye-Labeled Probes

3.2.2 Allelic Discrimination

Allelic discrimination was achieved by the selective annealing of TaqMan® MGB probes. During PCR, each probe anneals specifically to its complementary sequence between the forward and reverse primer sites. AmpliTaq Gold® DNA polymerase (5' nuclease) cleaves only probes that hybridize to the matching allele sequence. In turn, cleavage separates the reporter dye from the quencher, thereby increasing fluorescence of the reporter. Generation of fluorescence signals during PCR indicates positive allele, and mismatches between probe and allele reduce the efficiency of probe hybridization (Fig. 14), and AmpliTaq Gold DNA polymerase displaces mismatched probe rather than cleaves it (to release reporter). Results are interpreted as per:

- Homozygotes (samples having only allele 1 or allele 2).
- Heterozygotes (samples having both allele 1 and allele 2).

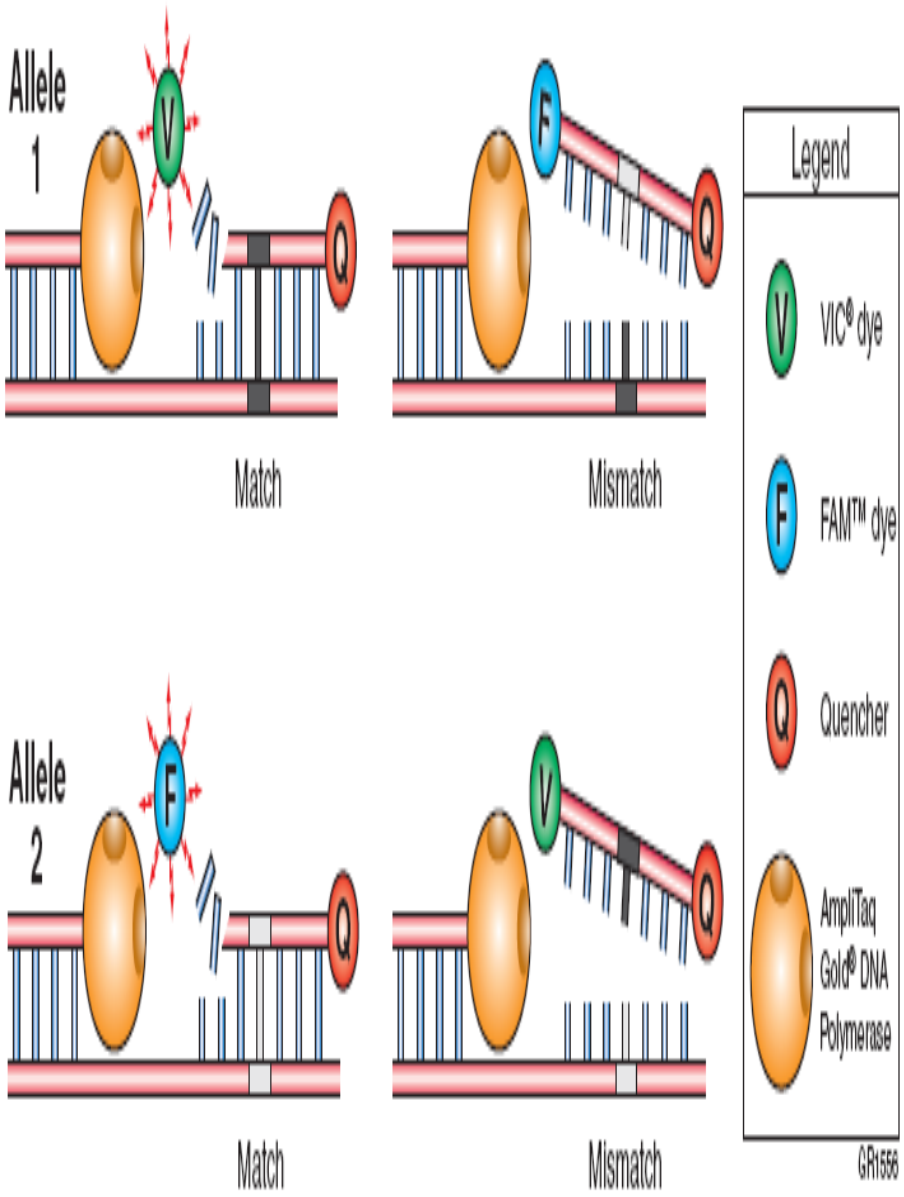

Figure 14: Real Time PCR Allelic Discrimination (Applied Biosystem)

3.2.3 Genotyping

The primers and the probes for (TPMT*3C (A719G), NAT2*5 (T341C), NAT2*6 (G590A)and NAT2*7 (G857A))are given in (Table 2).

3.2.4 Real-Time PCR Setup:

The assays were carried out in a total volume of 6 µl, which contained 1.5 µl DNA template, and 4.5 µl TaqMan genotype master mix (2.5 µl TaqMan 2× mix, 1.875 µl nuclease free water, and 0.125 µl 40× SNP primer mix) (Applied Biosystem, Foster City, California, USA).Cycling conditions comprised 2 steps: pre- and post-PCR steps. Pre-PCR (hold step) stage was performed at 60 °C for 30 sec and 95°C for 10 min (to activate the polymerase). Cycling conditions consisted of 40 cycles of denaturation (92°C for 15 sec), and combined annealing/extension (60 °C for 1.0 min). This was followed by a holding stage at 60°C for 30 sec (Post-PCR read) (Fig. 15).

TPMT*2 (G238C) The genotyping of each individual for the G238C mutation was performed using an allele specific polymerase chain reaction (PCR) assay as previously described by Yates et al. . TPMT*3A (*3B) (G460A) genotyping of each individual for the G460A mutation was performed using a PCR assay using primers (Table 1).

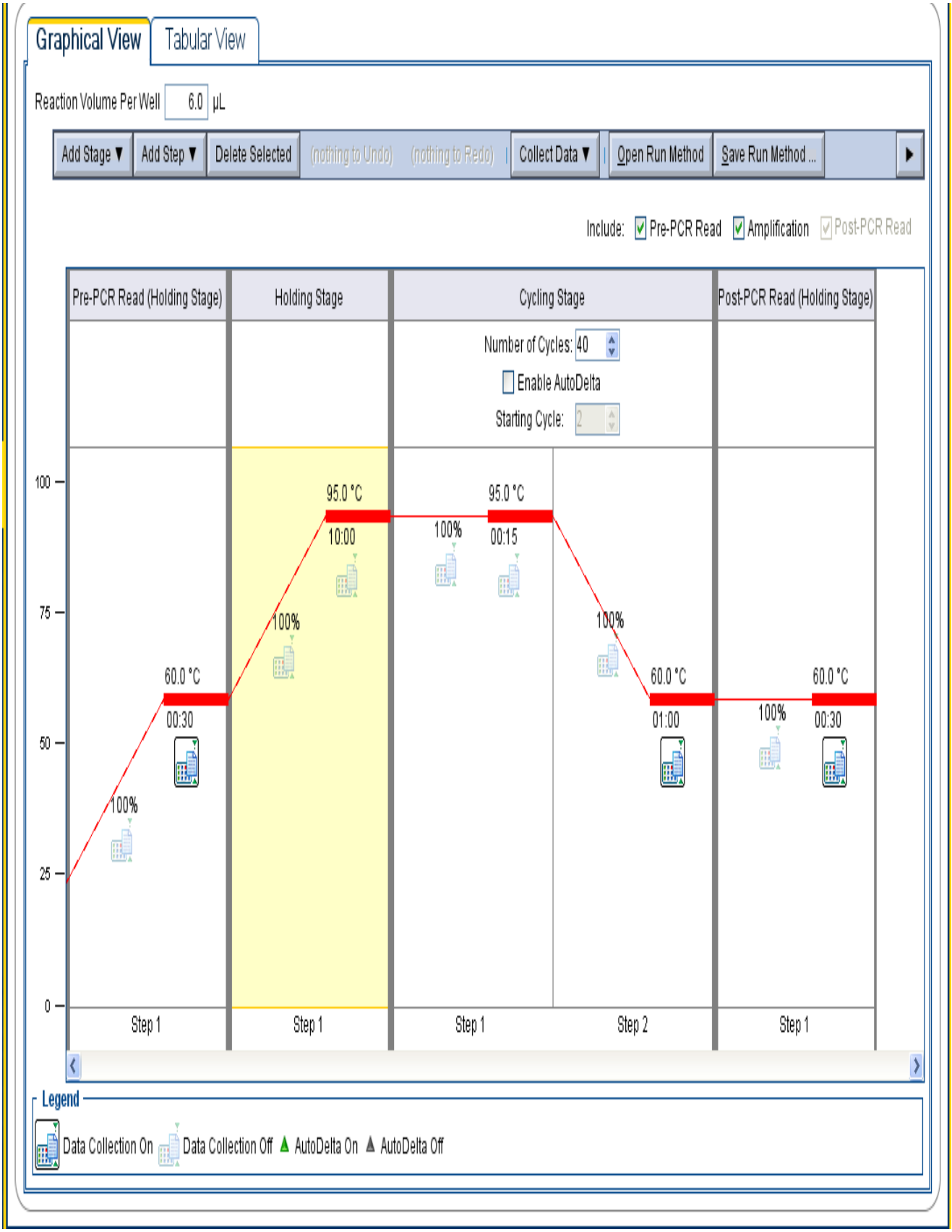

Figure 15: Real-Time PCR conditions

However, 250 ng of patient DNA was used as a template, and buffer J (Invitrogen) (which contained Tris hydrochloride (pH 9.5), 60 mmol/L; ammonium sulfate, 15 mmol/L; and magnesium chloride, 2.0 mmol/L) was used. The PCR product was desalted by filtration through a Centricon 30 filter (Amicon, Inc., Beverly, Massachusetts) and then digested with *Mwo* I (New England Biolabs, Beverly, Massachusetts) for 1 hour at 60 °C. Digested products were analyzed by gel electrophoresis. Mwo I digestion of wild-type DNA yields fragments of 267 and 98 base pairs, whereas DNA containing the G460A mutation is not digested and yields an uncleaved fragment of 365 base previously described PCR-restriction fragment length polymorphism assay (RFLP) . In brief, a 694-bp fragment containing nucleotide 460 was amplified and digested with the restriction enzyme MwoI. The wildtype allele, but not the mutant allele, contains a MwoI restriction site allowing RFLP analysis of the digested products after their separation on a 3% agarose gel. Digestion of the 694-bp fragment at .the MwoI site yielded fragments of 443 and 251 bp

3.3 Multiplex PCR

The genetic polymorphism analysis for the GSTM 1 and the GSTTI genes was determined simultaneously in a single assay using a multiplex PCR approach, with CYP1A1 gene as internal control to document successful PCR amplification. GST (M1 and T1 null alleles) The determination of GSTM1, GSTT1 null alleles was done simultaneously in a single assay using a multiplex PCR approach as described by . The presence or absence of GSTM1 and GSTT1 genes was detected by the presence or absence of a band at 215 bp (corresponding to GSTM1) and a band at 480 bp (corresponding to GSTT1). A band at 312 bp (corresponding to CYP1A1gene) was always present and was used as an internal control to document successful PCR amplification (figure 18). SULT1A1 (G638A) The SULT1A1 gene is one of three very closely related sulfotransferase genes located on chromosome 16; the open reading frames derived from SULT1A1 and its neighbours SULT1A2 and SULT1A3 share more than 94% identity. Thus the primer pair was chosen specifically to amplify the appropriate region from SULT1A1 using intron sequences flanking exon VII where sequence identity was lower, as described in the method of . After specific amplification of a 281-bp fragment of DNA including exon VII of the SULT1A1 gene, PCR products were digested with HaeII. Fragments were then resolved on 3% agarose gel. Digestion of the amplified sequence resulted in the formation of two fragments with lengths of 104 and 177bp. Complete and partial digestion indicated the genotypes

SULT1A1*1/*1 and SULT1A1*1/*2, respectively. Lack of digestion indicated the genotype SULT1A1*2/*2(figure 18).

MDR-1 (C3435T) In the present study, we have designed a PCR-RFLP assay and successfully applied it for the analysis of the C3435T mutation of the MDR-1 gene(Table 1).A 231-bp fragment containing nucleotide 3435 was amplified with 0.67 Um of MDR-1F MDR-1F . PCR amplification consisted of an initial denaturation step at 94 ∞C for 5 min followed by 33 cycles of denaturation at 94 ∞C for 30 s, annealing at 56 ∞C for 20 s, and extension at 72 ∞C for 30 s. The final extension step was performed at 72 ∞C for 5 min. All these steps were carried out using a Perkin-Elmer (Shelton, Connecticut, USA) thermocycler. The PCR products were digested with the restriction enzyme Sau3AI at 37C overnight. The digested products were then separated on a 3% agarose gel. The wild-type allele, but not the mutant allele contains a Sau3AI restriction site allowing RFLP analysis of the digested products. Digestion of the 231-bp fragment at the Sau3AI site yielded two fragments of 163 and 68bp. Throughout the genotyping assays, positive controls (samples with known genotypes) were not included. Instead, all the subjects were genotyped twice for all the tested mutations, which make the possibility of genotyping error less likely (figure 16).

Table 1: **Genotyping SNPs Primer**

TPMT*3A (G460A)
Forward primer (5'-ATAACAGAGTGGG-GAGGCTGC-3')
Reverse primer (5'-CTAGAACCCA-GAAAA AGTATAG-3')
TPMT*2A (G238C)
Forward primer 5′-GTA TGA TTT TAT GCA GGT TTG-3′ OR
Forward primer 5′-GTA TGA TTT TAT GCA GGT TTC-3′
Reverse primer 5′-TAA ATA GGA ACC ATC GGA CAC-3′
GSTM1
Forward primer 5′-GAACTCCCTGAAAAGCTAAAGC-3′
Reverse primer 5'-GTTGGGCTCAAATATACGGTGG-3′
GSTT1
Forward primer 5′-TTCCTTACTGGTCCTCACATCTC-3′
Reverse primer 5'-TCACCGGATCATGGCCAGCA-3′
CYP1A1
Forward primer 5′-GAACTGCCACTTCAGCTGTCT-3′
Reverse primer 5'-CAGCTGCATTTGGAAGTGCTG-3′
SULT1A1
Forward primer 5-′GTTGGCTCTGCAGGGTTTCTAGGA-3
Reverse primer 5'-CCCAAACCCCCTGCTGGCCAGCACCC-3
MDR-1
Forward primer 5'- ACTCTTGTTTTCAGCTGCTTG-3'
Reverse primer 5'-AGAGACTTACATTAGGCAGTGACTC-3'.

Table 2: Summary of primer pairs [wild-type (WT) or mutant (MT) and common (CM) primers] and TaqMan probe (TM)sequences (5’-3’)

TPMT*3C (A719G)
WT: TATGTCTCATTTACTTTTCTGTAAGTAGTT
MT: TATGTCTCATTTACTTTTCTGTAAGTAGTC
TM: AGACAGTCAATTCCCCAACTTTTATGTCGTTCTT
CM: TTAACATGTTACTCTTTCTTGTTTCA
NAT2*5 (T341C)
WT: CCTTCTCCTGCAGGTGACCTT
MT: CCTTCTCCTGCAGGTGACCTC
TM: TTACATTGTCGATGCTGGGTCTGGAAGCTC
CM: AGGCTGCCACATCTGGGAG
NAT2*6 (G590A)
WT: GTATTCATAGACTCAAAATCTTCAATTGTCC
MT: GTATTCATAGACTCAAAATCTTCAATTGTCT
TM: TTCTGTCAAGCAGAAAATGCAAGGCACCTG
CM: CCAGATGTGGCAGCCTCTAGAA
NAT2*7 (G857A)
WT: TTTTGTTCCTTATTCTAAATAGTAAGGGACC

MT: TTTTGTTCCTTATTCTAAATAGTAAGGGACT
TM: TCACCAGGTTTGGGCACGAGATTTCT

3.4 Statistical analysis

Data were compiled according to the genotype and allele frequencies estimated from the observed numbers of each specific allele. The frequency of each allele in our subjects is given together with the 95% confidence interval (CI).Differences in allele frequencies between Sudanese, Bahrani and other ethnic populations were measured using the c2 test and Fisher's exact test. A *P*-value <0.05 was considered to be statistically significant throughout the population comparisons. The Hardy-Weinberg equilibrium was assessed by an exact test provided by the Arlequin program.

CHAPTER FOUR

4.0 RESULTS

Table 3: Allele frequencies of TPMT in various ethnic groups

Population	Allele frequency				Reference
	N	TPMT*2	TPMT*3A	TPMT*3C	
Sudanese	80	0	0.006	0.008	**This study**
Bahraini	265	0.008	0.043	0.003	**This study**
Egyptians	200	0	0.003	0.013	
American	282	0.002	0.032	0.002	
British	199	0.005	0.045	0.003	
French	191	0.005	0.057	0.008	
Italian	206	0.005	0.039	0.01	
Saami-Norwegian	194	0	0	0.033	
Norwegian	66	0	0.034	0.003	
Japanese	192	0	0	0.015	
Chinese	192	0	0	0.023	
South west Asians	99	0	0.01	0	
Kenyan	101	0	0	0.054	
Ghanaian	217	0	0	0.076	
African-Americans	248	0.004	0.008	0.024	

N, Number of subjects. †South West Asians included people from India, Pakistan, Sri Lanka and Nepal.

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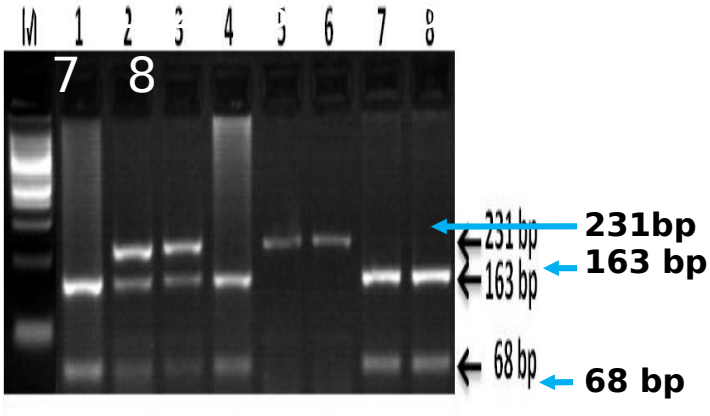


Figure 16: Electrophoresis patterns for MDR1 genotypes by PCR-RFLP based assay.

M is a 100 bp DNA marker. Samples 1, 4, 7 and 8 are homozygous CC genotype (Has Sau3AI restriction site). Samples 5 and 6 are homozygous TT genotype (Lack Sau3AI restriction site). Sample 2 and 3 are heterozygous CT genotype

Table 4: **Genotype & allele frequencies of MDR-1 (C3435T) polymorphism in various ethnic groups**

Population	n	Genotype %			Allele frequencies		Reference
		C/C	C/T	T/T	C	T	
Sudanese	131	52.7	42	5.3	0.74	0.26	
Bahraini	184	34.8	19.5	45.7	0.58	0.42	
Egyptian	200	34	51.5	14.5	0.6	0.4	
British	190	24	48	28	0.48	0.52	
Jordanian	116	24	60	32	0.46	0.54	
German	188	28	48	24	0.52	0.48	
Saudi	96	37	38	26	0.55	0.45	
Japanese	50	34	46	20	0.57	0.43	
Chinese	132	32	42	26	0.53	0.47	
Filipino	60	38	42	20	0.59	0.41	
South west Asian	89	15	38	47	0.34	0.66	
Kenyan	80	70	26	4	0.83	0.17	
Ghanaian	206	66	34	0	0.83	0.17	
African – Americans	88	68	31	1	0.84	0.16	

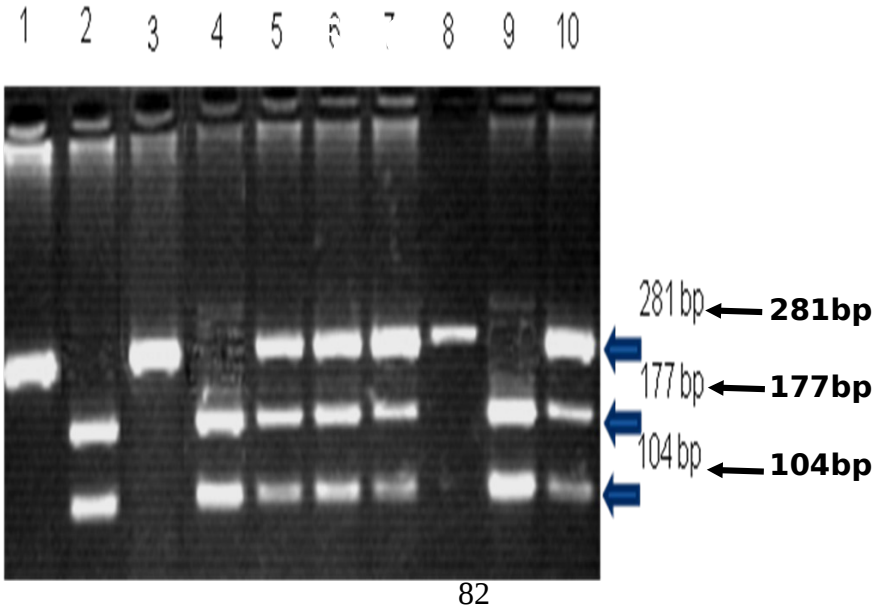
N, Number of subjects. Differences in allele frequencies were measured using Fisher's exact test. NS, There were no significant differences ($P > 0.05$).

Table 5: **Allele frequencies of NAT2 in various ethnic groups**

Population	n	Allele frequency				Reference
		NAT2*4	NAT2*5	NAT2*6	NAT2*7	
Sudanese	114	0.1	0.569	0.288	0.044	**This study**
Bahraini	263	0.15	0.405	0.397	0.047	**This study**
Egyptians	199	0.215	0.497	0.26	0.028	
American	266	0.22	0.47	0.28	0.003	
German	844	0.244	0.465	0.27	0.013	
Portuguese	128	0.212	0.433	0.328	0.027	
Danish	242	0.254	0.473	0.25	0.023	
Scottish	96	0.203	0.49	0.271	0.036	
Swedish	70	0.194	0.507	0.278	0.021	
Japanese	79	0.641	0.019	0.23	0.11	
Chinese	70	0.473	0.057	0.31	0.16	
Korean	85	0.692	0.018	0.18	0.11	
Filipino	100	0.395	0.065	0.36	0.18	
Taiwanese	100	0.515	0.025	0.31	0.15	
Indians	61	0.257	0.33	0.38	0.033	
Polynesians	25	0.6	0.04	0.34	0.02	
Gabonese	52	0.356	0.404	0.221	0.019	
Hispanic –Americans	65	0.39	0.32	0.19	0.1	
African –Americans	214	0.43	0.295	0.23	0.045	

N, Number of subjects. †NAT2*4 represent the allele that did not contain any of the mutations analysed in our study, while NAT2*5, NAT2*6 and NAT2*7 represent the alleles that contained 341C, 590A and 857A mutations, respectively.

Figure 17: SULT1A1 PCR products after restriction digestion with HaeII on 3% agarose gel.



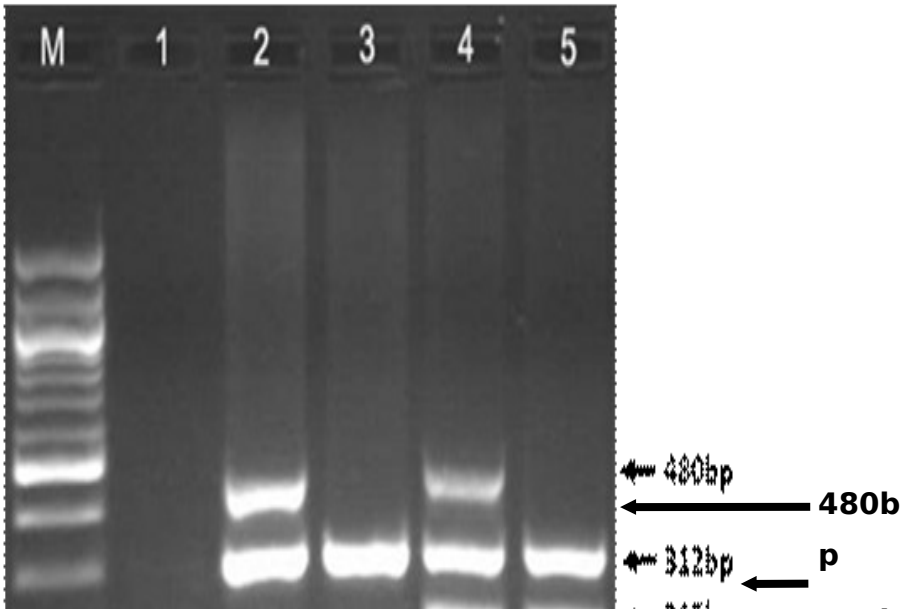
Lane 1, 3 & 8 = AA genotype; Lane 5, 6, 7 & 10 = GA genotype and Lane 2 & 4 = GG genotype

Table 6: **Allele frequencies of SULTIAI in various ethnic groups**

Population	Allele frequency			Reference
	N	***Arg**	***His**	
Sudanese	131	0.74	0.26	**This study**
Bahraini	184	0.58	0.42	**This study**
Egyptians	200	0.865	0.135	
American	150	0.69	0.31	
American	245	0.67	0.33	
German	300	0.63	0.37	
British	106	0.68	0.32	
Scottish	187	0.68	0.32	
Chinese	290	0.914	0.08	
Nigerian	52	0.73	0.27	
African-Americans	70	0.71	0.29	

n, Number of subjects. (Differences in allele frequencies were measured using Fisher's exact test.) †These studies were designed to detect only SULT1A1*2, so carriers of *3 or *4 alleles may be scored as carriers of SULT1A1*1 or *2. ‡These studies were designed to detect either *2 and *3 or *2**,** *3 **and** *4. For this table, the common allele SULT1A1*1 and the rare alleles *3 and *4 were combined with the allele SULT1A1*1 and assigned as *Arg.

Figure 18: PCR products analyzed on 1.5% agarose gel.



A band at 312 bp, corresponding to CYP1A1 gene, was used as an internal control. Lane 1 is a negative control. Lane 2 is an individual with GSTT1 present (480 bp) and GSTM1 null alleles. Lane 3 is individual with null alleles for both GSTT1 and GSTM1 genes showing only one band at 312 bp (internal control). Lane 4 is an individual with both GSTT1 and GSTM1 alleles present. Lane 5 is an individual with GSTT1 null and GSTM1 present (215 bp) alleles. M is a DNA molecular marker.

Table 7: **Frequency of GSTs null genotypes in various ethnic groups**

Population	Allele frequency				Reference
	n	%GSTM1 null	%GSTT1 null	%Double null	
Sudanese	114	54.4	42.	24.6	
Bahraini	167	49.7	28.7	14.4	
Lebanese	141	52.5	37.6	16.3	
Tunisians	186	63.4	37.1	21	
Egyptians	200	55.5	29.5	NA	
Saudi	513	54.6	25	17.2	
Turkish	133	51.9	17.3	NA	
Indians	198	20.7	18.2	NA	
Chinese	735	56.5	56.5	31.4	
Japanese	143	44.1	43.4	15.4	
Koreans	1051	53.8	54.3	29.1	
Cameroonians	126	27.8	46.8	NA	
Gambians	337	20.2	37.1	NA	
Ivory Cost	133	36.1	33.1	14.3	

* Significant when compared to Sudanese

CHAPTER FIVE

5.0 DISSUCION

5.0.1 MDR1

The present study investigated the frequency of the C3435T SNP in MDR1 gene among unrelated Bahraini and Sudanese subjects, and compared the results with data reported for other ethnic groups (Table 4). The results obtained in Bahrain showed genotype frequencies of 34.8%, 19.5%, and 45.7% for CC, CT and TT genotypes, respectively, while that obtained in Sudanese showed genotype frequencies of 52.7%, 42.0%, and 5.3% for CC, CT and TT genotypes, respectively. The frequencies of the wild C-allele (0.58) and the wild-genotype CC (34.8%) in Bahraini subjects were different significantly from that reported in Sudanese (0.74 and 52.7%). This could be due to higher frequency of the C-allele found in Sudanese population. Our results give the evidence that the frequencies of MDR1 alleles, and genotypes determined in Bahraini , are very similar to the respective frequencies observed in Jordanian and Saudi Arabians which may be attributed to their common Arab origin. Interestingly, the frequency of the wild C-allele in Bahraini subjects did not differ from that reported in Asian populations, apart from Japanese, including Iranian, Turkish,Indians, and Chinese and in European populations including British, Spanish, and French. However, the frequency of 3435T variant in Bahraini subjects, most often associated with a decreased P-gp activity, is significantly higher compared to populations of African origin (Kenyan, Ghanaian, Moroccan

and Egyptians). Significant differences in C3435T genotype distribution were seen between Bahraini and Ashkenazi Jews. As regards the Sudanese, the frequency of the wild CC genotype and C-allele were similar to that present in Africans (Moroccan, Kenyan) which may be attributed to their common origin. Interestingly, the frequency of the wild C-allele in Sudanese subjects did not differ from that reported Ashkenazi Jews. The Sudanese samples in our study were similar to a previous study done by . However, the frequencies of the wild C-allele (0.737) and the wild-genotype CC (52.7%) in Sudanese subjects were higher and significantly different from that reported in Asian populations (Bahraini, Saudi Arabians, Iranian, Turkish, Indians, Chinese and Japanese) and in European populations (British, Spanish, German and French).There was a significant relationship of the silent C3435T SNP with intestinal P-gp expression levels. Individuals who were homozygous for the 3435T variant had significantly decreased intestinal P-gp expression [13]. About 45.7% of Bahraini and 5.3% of Sudanese subjects were homozygous for the 3435T. Sudanese had at least one T allele, with lower P-gp level in small intestine. As P-gp has an important role in the bioavailability of a wide variety of drugs, including anticancer, antihypertensive, antiarrhythmic, antiviral, glucocorticoids, antibiotics, immunosuppressant's, antidepressants, neuroleptics, opioids and many others . So, these drugs must be used cautiously in those subjects.The substrate specificity of P-gp is altered by

synonymous and silent SNPs. It has been suggested that silent SNPs may affect protein translation rates hence influencing protein folding and activity. Therefore, silent SNPs that do not change the coding sequence of the protein may contribute to altered pharmacokinetics of substrate drugs and development of certain disease conditions .The Europeans and Asians had lower frequencies of the C-allele compared to that of Africans. The high frequency of the C-allele in Africans implies overexpression of P-gp. As overexpression of P-gp has been associated with altered drug absorption, therapy resistant, this SNP may provide a useful approach to individualize therapy and it may have important therapeutic and prognostic implications for use of P-gp dependent drugs in Sudanese.

5.0.2 GST

The frequencies of GSTM1 and GSTT1 null genotypes have been reported in diverse ethnic groups . Here we investigated the polymorphism at GST loci in Sudanese population. To our knowledge, this is the first study done on Sudanese population regarding the frequencies of GST deletion polymorphisms. The homozygosity for the GSTM1 deletion in Sudanese (54.4%) was generally comparable to those reported for Arabs from Bahrain (49.7%); Saudi Arabia (54.6%); Egypt (55.5%); Lebanon (52.5%) and Tunisia (63.4%) whereas no statistically significant differences were found (Table 7). An ethnic and geographic basis for variations in the distribution of GSTM1 null genotype among diverse population was

suggested [31,33]. The prevalence of individuals not expressing the GSTM1 enzyme due to a homozygous gene deletion is reportedly higher in Europeans and Asians, as compared to Africans . The frequencies of GSTM1 null genotypes vary from 38-67% in Europeans, 33-63% in Asians, and 16-36% in sub-Saharan Africans . The prevalence of GSTM1 homozygous deletion seen among Sudanese (54.4%) was comparable for Europeans from Great Britain, France and Germany. In addition, they were comparable to Asians, such as Turkish , Chinese , Koreans and Japanese (Table 7). Significant differences in GSTM1 null genotype distribution were seen with respect to Asians from India ($p < 0.001$) , and sub-Saharan Africans, including Gambians ($p < 0.0001$), Cameroonians ($p < 0.001$) , and from Ivory Coast ($p < 0.004$) [29] (Table 7). The frequency of GSTM1 null genotype among Sudanese was comparable to those reported for Arabs, European and Asian population (apart from Indians) but was significantly higher than those from sub-Saharan Africans.

The prevalence of GSTT1 null homozygotes in Sudanese (42.1%) was comparable to those reported for Arabs from Lebanon (37.6%) and Tunisia (37.1%) and was significantly higher than those reported for Bahrainis (28.7%, $p < 0.020$), Saudi Arabians (25%, $p < 0.001$) , and Egyptians (29.5%, $p < 0.023$) . The GSTT1 frequencies reported for Caucasians, Africans and Asians are 20%, 40% and 60%, respectively . The frequencies

of GSTT1 null genotype in Sudanese samples was similar to that of the sub-Saharan Africans as Gambians , Cameroonians , and from Ivory Coast (Table 7). The difference of frequency of GTSM1 and GSTT1 null alleles between Sudanese individuals and those of others is likely to be attributed to admixture of Sudanese population, and also to selection arising from varied exposures to toxic substances . The distribution of GSTM1 null deletion in Sudanese was higher, but that of the GSTT1 null genotype was comparable to that of sub-Saharan Africans. This can be explained by the ethnic mixture of the Sudanese, with influence from sub-Saharan Africans and Arabs. Our data showed that Sudanese are similar to Arabs, Europeans and Asians (apart from Indians) with regards to the frequency of GSTM1 null genotype, and to Asians (Chinese, Japanese and Koreans) and sub-Saharan Africans regarding the distribution of the GSTT1 null genotype .

5.0.3 TPMT

Population studies have demonstrated significant ethnic differences in the distribution of TPMT variant alleles among various ethnic groups (Table 3). TPMT*3A accounts for more than 80% of the variant alleles in Caucasians, but for only approximately 17% of the variant alleles in

African-Americans. However, TPMT*3C accounts for only 5% of the variant alleles in Caucasians,70% in African-Americans and 100% in the Japanese and Chinese. In the present study, we found that TPMT*3C ,TPMT*3A and TPMT*2 for the variant alleles detected in sudanese is (0.000)and for Bahraini (0.036) ,(0.000), (0.008)respectively. The frequency of TPMT*3C found in Bahraini (0.036) is significantly (P > 0.05) to the frequencies reported for sudanese(0.00) . In vivo studies indicate that homozygosity for TPMT*3C impairs metabolism of thiopurine drugs, possibly due to the intrinsic instability of the enzyme compared with that of TPMT*1 [29]. TPMT*3A, which causes the largest decrease in enzyme activity [29], was not detected in Sudanese and Bahrain .sudanese allele frequencies of TPMT*3A and TPMT*2 is similar Japanese, Chinese, Kenyan and Ghanaian (see table 3). Reported for South-west Asians (0.010) , and African-Americans (0.008) . However, *TPMT*3A* was found in significantly higher frequencies (P < 0.0001) in Caucasian populations, including Americans (0.032) , British (0.057) ,French (0.057) , Italians (0.039) and Norwegians(0.034) .In conclusion, the present study showed that the Sudanese have a relatively low frequency of TPMT mutant alleles. The low frequency of variant TPMT alleles in the Sudanese population infers that the Sudanese may have higher TPMT activity than other Caucasian or African-American populations.

5.0.4 SULT1A1

The frequency of the SULT1A1*2 allele the wild type(His) in sudanese is 0.74 in Bahrainis is 0.58 while the mutant allele (Arg) in Sudanese is 0.26 and in Bahraini is 0.42 . The frequency of the SULT1A1*2 allele did not differ significantly from Caucasians, Nigerians and African-Americans, but it is significantly higher than those of Chinese and Egyptians (table 6). The difference of frequency of SULT1A1 alleles between Sudanese and those of others is likely to be attributed to admixture of Sudanese population, and also to selection arising from varied exposures to toxic substances. Fifteen different alleles have been described for the SULT1A1 gene, including four that resulted in alterations in encoded amino acids (the wild-type allele (SULT1A1*1) and SULT1A1*2–**4* variants) . Comparison of the distribution of SULT1A1 alleles between various ethnic groups has been reported previously . The results of those studies showed striking ethnic differences in allele frequencies, with a SULT1A1*1 frequency that was highest in Chinese (0.914) and lowest in Bahraini subjects (0.58). Conversely, The frequency of the SULT1A1*2 Americans (0.294), but it was much lower in Chinese (0.080) than in the other two ethnic groups . The rare allele SULT1A1*4 was only detected in a sample of 150 Caucasian Americans with a frequency of 0.003 According to our results, the frequency of SULT1A1*2 was 0.26 in Sudanese and 0.42 which is significantly higher frequencies from Sudanese individuals. Our data

support the need for further investigation about this different pattern in the frequencies of the SULT1A1*2 variant.

5.0.5 NAT2

According to the rich literature regarding the ethnic variation in the distribution of defective NAT2 alleles all over the world [30–36], two major groups could clearly be distinguished according to the incidence of the NAT2*5 and NAT2*7 variants. Caucasians, Indians and Africans are very similar (> 0.290 and <0.050, respectively). However, the frequency of the NAT2*6 variant is only slightly different among various populations.In the present study, we found that NAT2 allele frequencies among the Sudanese and Bahraini were similar (Table 5). In Sudanese the majority of NAT2 alleles are composed of NAT2*4 (wild type), NAT2*5 and NAT2*6, which were found in our study in frequencies of 0.1, 0.569 and 0.288, respectively. We also found the NAT2*7 variant had a frequency of 0.044. In Bahraini the majority of NAT2 alleles are composed of NAT2*4 (wild type), NAT2*5 and NAT2*6, which were found in our study in frequencies of 0.15, 0.405 and 0.397, respectively. We also found the NAT2*7 variant had a frequency of 0.047 (Table 5).The predominance of the slow acetylator genotype in Sudanese and Bahraini populations (60.50%) conforms to findings amongst other populations: 52.60% in Caucasian-Americans, 60.00% in Germans [37], and 63.40% among Polish

children [38].In contrast, in the Japanese, Chinese, Koreans and Filipinos, the fast genotype is largely over-represented (up to 90.00%) .

5.1 CONCLOUSIN

This is the first report on the polymorphic distribution of the GSTM1, GSTT1, TPMT, MDR1 and SULT1A1 genotypes in Sudanese population. In this study , we provide a basis for future clinical studies concerning variability in the response and/or toxicity to drugs known to be substrates for GST, TPMT, NAT2, MDR1 and SULT1A1. Further detailed studies of GST, TPMT, NAT2, MDR1 and SULT1A1 variants could be helpful in understanding the roles of these variants as genetic susceptibility markers and its association drugs pharmacogenomics among Sudanese and Bahraini population. Sudan is located in northeastern part of Africa and Sudanese population show considerable ethnic and linguistic diversity. In this study, We found that Bahraini population more closely resemble Caucasians and Sudanese population more closely resemble African. The purpose of our future studies is to investigate for new polymorphisms, which could be relatively unique to the Bahraini and Sudanese population.

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