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THE IMPACT OF GENETICS, ENVIRONMENTAL, AND GEOGRAPHICAL FACTORS ON INTER-INDIVIDUAL AND INTER-ETHNIC DIFFERENCES IN CYP2C9-CATALYSED DRUG METABOLISM

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THE IMPACT OF GENETICS, ENVIRONMENTAL, AND GEOGRAPHICAL FACTORS ON INTER-INDIVIDUAL AND INTER-ETHNIC DIFFERENCES IN CYP2C9-CATALYSED METABOLISM
THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

Inter-individual and inter-ethnic variations in drug metabolism are creating an obstacle in providing efficient and effective drug treatments especially for drugs with narrow therapeutic windows. This thesis is contributes to the understanding of the molecular mechanism of these variations.

Paper I is an introduction to the inter-individual and inter-ethnic differences in CYP2C9 catalysed drug metabolism. Wide inter-individual differences were observed in the metabolic ratio of losartan to E-3174 metabolite even among individuals who were not carrying defective alleles *CYP2C9*2* and *CYP2C9*3*. Inter-ethnic variation was observed in the metabolism of losartan between CYP2C9 wild type Koreans and Swedes. An allelic variation in the intronic region between exon 8 and 9 of *CYP2C9*, *IVS8-109A>T* was observed to cause a lower *CYP2C9* activity in Swedes but not Koreans.

Paper II studied the combination of age, *CYP2C9* genotype, ethnicity, smoking habit, weight and sex as a predictor of CYP2C9 metabolic ratio variability. Ethnicity was the main significant factor influencing between subject-variability in CYP2C9 enzyme activity. Additionally, *CYP2C9* genotype and smoking were significant contributors to the variation. Grouping the subjects based on their ethnicity, we found that CYP2C9 genotype is a major predictor for both Koreans and Swedes (27% and 40% of the variability respectively). The smoking effect was non-significant in the Swedes but remains as a factor in the Koreans. The reason behind the smoking effect in Koreans is unidentified.

Paper III investigated the possibility of Behcet's disease as a *CYP2C9* inducer. A Swedish ultra-rapid CYP2C9 metaboliser was diagnosed with this disease and this study tested the possibility in Turkish healthy subjects to have a lower CYP2C9 activity than Behcet's disease patients. Interestingly, the Behcet's disease patients were shown to have a significantly low CYP2C9 metabolic activity. The factors of genetics, medication and inflammation-related biomolecules are suspected to have caused this down-regulation. We did not find evidence of *CYP2C9* genotype and typical Behcet's disease medication, colchicine having any influence on the observed low CYP2C9 metabolic activity. It is very possible that inflammation response agent caused this inhibitory effect on CYP2C9 activity.

Paper IV investigated the effect of the P450 oxidoreductase *(POR)*28* variant on the metabolic activity of CYP2C9. We screened all Swedish and Koreans *CYP2C9*1/*1* subjects for *POR* 5, *13* and **28*. No subject was found to carry **5* or **13*. Interestingly, Swedish individuals who carry *POR*28* allele were observed to display a 1.40 fold increase in CYP2C9 enzyme activity compared to none-*POR*28* carriers. We screened the ultra-rapid metaboliser for this variant and she was also a carrier of this variant. More studies should be done to investigate the effect of other SNPs in POR gene to the metabolic activity of drug metabolising enzymes.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following publications:

I. **Hatta FH**, Teh LK, Helldén A, Hellgren KE, Roh HK, Salleh MZ, Aklillu E, Bertilsson L (2012) Search for the molecular basis of ultra-rapid CYP2C9-catalysed metabolism: relationship between SNP IVS8-109A>T and the losartan metabolism phenotype in Swedes Eur J Clin Pharmacol 68:1033-1042

II. **Hatta FH**, Lundblad M, Ramsjö M, Kang JH, Roh HK, Bertilsson L, Eliasson E, Aklillu E (2015) Differences in CYP2C9 genotype and enzyme activity between Swedes and Koreans of relevance for personalized medicine: role of ethnicity, genotype, smoking, age, and sex. OMICS 6: 346-353

III. Goktaş MT, **Hatta F**, Karaca O, Kalkisim S, Kilic L, Akdogan A, Babaoglu MO, Bozkurt A, Helldén A, Bertilsson L, Yasar U (2015) Lower CYP2C9 activity in Turkish patients with Behçet's disease compared to healthy subjects: a down-regulation due to inflammation? Eur J Clin Pharmacol 71:1223-1228.

IV. **Hatta FH** and Aklillu E. The effect of P450 oxidoreductase (POR)*28 variant allele on CYP2C9 metabolic activity in Swedish and Korean healthy subjects. *New varsion of manucsript accepted by OMICS 28th October 2015*. OMI-2015-0159.R1

CONTENTS

LIST OF ABBREVIATIONS

AhR	Aryl hydrocarbon receptor
BD	Behçet's disease
CAR	Constitutive active/androstane receptor
C/EBPb	CCAAT-enhancer-binding proteins beta
CYP2C8	Cytochrome P450, Family 2, Subfamily C, Polypeptide 8
CYP2C9	Cytochrome P450, Family 2, Subfamily C, Polypeptide 9
CYP2C19	Cytochrome P450, Family 2, Subfamily C, Polypeptide 19
CYP2D6	Cytochrome P450, Family 2, Subfamily D, Polypeptide 6
CYP3A4	Cytochrome P450, Family 3, Subfamily A, Polypeptide 4
DNA	Deoxyribonucleic acid
LD	Linkage equilibrium
MR	Metabolic ratio
NSAIDs	Non steroidal anti-inflammatory drugs
OC	Oral contraceptive
PAH	Polycyclic aromatic hydrocarbons
PCR	Polymerase chain reaction
POR	P450 (cytochrome) oxidoreductase
PXR	Pregnane X receptor
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
SNP	Single nucleotide polymorphism
URM	Ultra-rapid metaboliser
UTR	Untranslated region
VKOR/C1	Vitamin K Epoxide Reductase/Complex, Subunit 1

1 INTRODUCTION

1.1 A SHORT HISTORY OF PHARMACOGENETICS

The discovery of the cytochrome P450 was first reported in 1955 [1, 2]. The enzyme was described to be able to oxidise xenobiotic compounds and is abundant in the endoplasmic reticulum of the liver cells [2]. It was named cytochrome P450 because it had a maximum absorption at 450 nm in pig and rat microsomes [3, 4]. Parallel to the discovery of the cytochrome P450, advances in pharmacology studies witnessed Kalow and Staron characterise serum-cholinesterase deficiency in a subject with succinylcholine apnoea in 1957 [5] followed by the conceptualisation that inheritance explains many individual differences in the efficacy and toxicity of drugs [6]. In his paper, he found that hereditary gene-controlled enzymatic factors determine why certain individuals become 'sick', whereas others are not affected by identical drug exposure. This paper may be one of the first evidence -based reports on genetic-based inter-individual variability in drug response.

Friedrich Vogel came up with the word 'pharmacogenetics' in 1959 [7]. Working on a book at the same time, Werner Kalow published a historically important systematic account of pharmacogenetics in 1962. The monograph entitled *Pharmacogenetics — Heredity and the Response to Drugs* recorded genetic variations of significance from numerable hosts, insects, bacteria and also humans. These genetic variations were recorded in relations to their response to environmental chemicals [8]. Since then, the advances in pharmacogenetics studies have been moving forward in positive light. The ultimate creation that revolutionised the world of genetic studies, the polymerase chain reaction (PCR) technology came up in a very timely manner. Two years after the announcement of the novel method of amplifying DNA in 1985, the first nomenclature for the P450 supergene family was announced. From then on, studies on the important superfamily of drug metabolising enzymes began to speed up. Restriction fragment length polymorphism (RFLP) enables the *CYP2D6*3* and **4* to be discovered amongst poor debrisoquine metabolisers. The important finding of copy number variation in the gene was reported by two groups working together lead on a patient who had an extremely high oxidation capacity of antidepressants [9-11]. To date, *CYP2D6* is still one of the most studied CYP450 enzymes.

1.2 THE CASE OF A CYP2C9 ULTRA-RAPID METABOLISER

Fast forward to recent times, a group in Karolinska Institutet [12] reported an interesting case of a woman who was an ultra-rapid metaboliser of phenytoin. The patient was a 59 years old Caucasian who weighted 50kg. She was previously diagnosed with Behçet's disease due to progressive systemic inflammations among others and was on multiple immunomodulation therapies. She later on developed recurrent epilepsy and was prescribed phenytoin. Her compliance was questioned as her plasma phenytoin level was barely detected. Given two to three times higher dosage (600-700mg daily) than recommended (4-5mg/Kg), this patient still did not reach the desirable plasma therapeutic level of between 40-80μmol/L. On two separate events, this patient was hospitalised due to CNS toxicity symptoms after being

prescribed fluconazole. Both events were similar in that she developed severe adverse reaction to phenytoin soon after receiving the CYP2C9 inhibitor. Knowing that CYP2C9 and CYP2C19 metabolise phenytoin, the patient was genotyped for defective alleles for both genes. She was a non-carrier to any of the known defective alleles. The research group tested her with CYP2C9 phenotyping probe drug losartan. She was found to have a very high CYP2C9 activity by having the losartan/E-3174 metabolic ratio (MR) of less than 0.13. Comparing this patient to the general Swedish healthy volunteers phenotyped for losartan/E-3174 ratio, she is an extremely fast metabolic outlier (Figure 1).

This curious case of this patient inspired the beginning of this thesis. CYP2C9 is also a well-studied metabolic enzyme that is responsible of metabolising up to 13% of clinically important drugs [13]. However, all the known defective alleles found in the gene cause a lower metabolic activity. It is important to find the molecular reason behind the ultra-rapid CYP2C9 variant, if there is any. This case is also the example of an inter-individual variation in CYP2C9 catalysed pharmacokinetics. Unlike CYP2D6, there is no established cut-off MR between the poor metaboliser (PM), extensive metaboliser (EM) and the ultra-rapid metaboliser (UM) in CYP2C9. An inter-ethnic variation in CYP2C9 losartan metabolism was also observed.

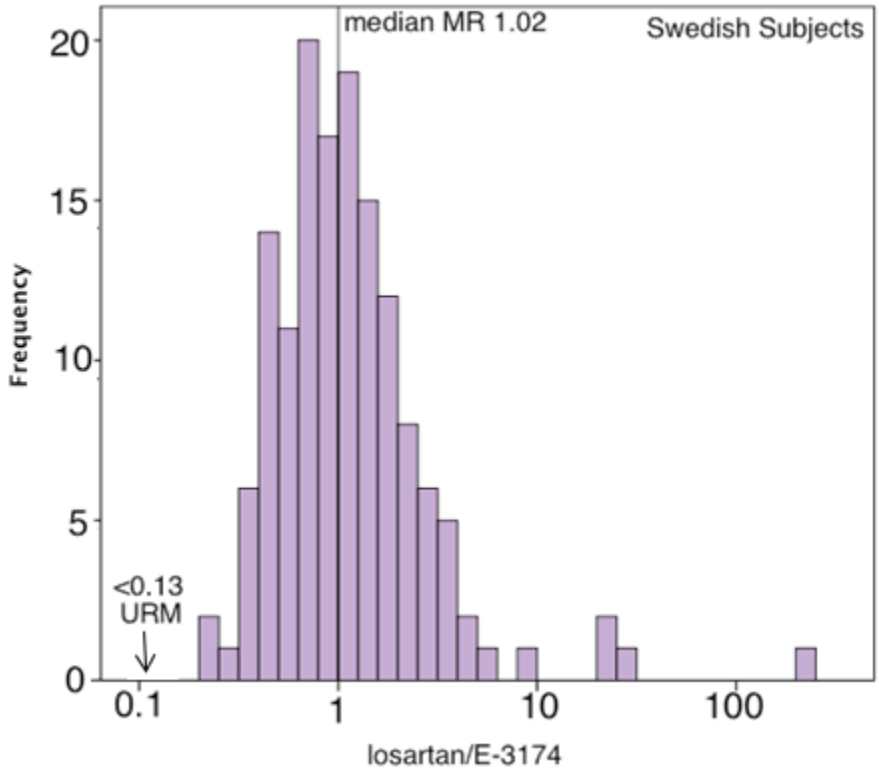

Figure 1. *Histogram showing metabolic ratio (MR) of losartan/E-3174 in 146 Swedish healthy subjects and an ultra-rapid metaboliser*. Subjects are not on oral contraceptives. The MRs are log transformed, 'URM' indicates the ultra-rapid metaboliser. MR=1 is indicated as an arbitral vertical line.

1.3 THE PHARMACOGENETICS OF *CYP2C9*

The cytochrome P450 isoenzyme CYP2C9 plays an important role in the metabolism of many therapeutically important drugs such as phenytoin [14], S-warfarin [15], tolbutamide [16], losartan [17], and non-steroidal anti-inflammatory agents (NSAIDs) [13] *in vitro* and/or *in vivo*. Inter-individual differences in CYP2C9 metabolic activity have been linked to dosing difficulty that might lead to ineffective treatments or worse, toxicity [18]. Factors such as genetics, age, stimulatory and inhibitory interactions are the usual cause for the variability of drug elimination.

1.4 DETERMINANTS OF CYP2C9 METABOLIC ACTIVITY

The need to move from 'one size fits all' dosage regiments to a more personalised, tailor-made type of therapy has long been a dream in the clinical pharmacogenetics world. The inter-individual variation in drug response is typically monogenic and inherited traits [19]. However, some cases of drug metabolism variability can be a result of a more complex factor. The genetics influence on the pharmacokinetics and pharmacologic effects of medications are determined by how important the impact of the variant allele is to the mechanism of action [19]. Although the most of the clinically important genotypes have been known, the CYP2C9 still display a very wide inter-individual differences in activity, even within individuals who do not carry any of the defective allele. Inter-ethnic differences in *CYP2C9* allele frequencies among most of the world's major populations have been well described [20-22].

1.4.1 *CYP2C9* genotypes

The first *CYP2C9* single nucleotide polymorphisms (SNPs) identified ware *CYP2C9*2* (*Arg*144*Cys*) and *CYP2C9**3 (Ile359Leu). These alleles are most commonly present in Caucasians and Africans but *CYP2C9**2 appear to be absent in Asians [23]. These allelic variants encodes for an enzyme with decreased activity *in vitro* and *in vivo* as compared with the 'wild type' allele *CYP2C9*1* (*Arg*144 *Ile*359) [24-26]. Due to the inter-ethnic variability in enzyme activity and the differences in distribution of the two alleles, a number of population-based genotyping studies have been conducted to identify other polymorphisms in different ethnic groups. These studies gave rise to the findings of among others *CYP2C9*4* (*Ile*359*Thr*) that seemed to occur exclusively in Japanese individuals [27], but was absent in the Koreans. Also *CYP2C9*5* and *CYP2C9*6* were found in a small group of African-Americans [28] and black Africans. To this date, almost 60 different *CYP2C9* SNPs have been reported (http://www.cypalleles.ki.se/cyp2c9.htm). Not all of these SNPs have a clinically significant effect. But those that do were found to cause a decrease in CYP2C9 activity. So far, there is no SNP in the *CYP2C9* gene that is found to increase its activity *in vivo*.

1.4.2 Gene-gene interaction

A well-studied example of gene-gene interaction involving CYP2C9 is on warfarin metabolism. Warfarin is catalysed by CYP2C9 [29]. Ironically, individuals of Asian origin require a lower warfarin dose despite the low frequency of defective CYP2C9 alleles in Asians compared to Caucasians [30]. Warfarin is an anti-coagulant that inhibits vitamin K epoxide reductase (VKOR) [31]. *VKORC1*, a subunit of *VKORC* gene is the target of warfarin [32]. Some genetic variations in the *VKORC1* gene were found in warfarin-resistant patients [33]. Multiple studies have been done on the dynamics of *CYP2C9* and *VKORC1* genotypes in determining warfarin dosage. A study demonstrated that the combination of both *CYP2C9* and *VKORC1* genotype is determinant of warfarin dosage requirements. It was shown that although Asians carry less of the defective *CYP2C9* gene, they are mostly (80%) carriers of *VKORC1 -1639 A* allele. On the other hand, almost 95% of Caucasians carry the *-1639 G* allele. The *-1639 G* allele was seen to increase the activity of VKORC1 by 44% [34]. The difference in frequencies of CYP2C9 defective alleles and the *VKORC1 -1639 A>G* allele was also concluded in a Malaysian study [35]. This group has developed an algorithm to account for *CYP2C9* and *VKORC1* genotypes in relation to warfarin dosage. They also calculated that the correlation of age and the genotypes contributes up to 37% of warfarin dosage variability. A study on Caucasian subjects found that genotypes of *VKORC1* primarily and *CYP2C9* account for up to 40% of warfarin dose variability [36].

Another example of gene variation that directly affects the CYP2C9 enzyme activity is the P450 (cytochrome) oxidoreductase (*POR*). POR plays a role in drug hydroxylation by assisting electrons transfer from reduced nicotinamide adenine dinucleotide phosphate (NADPH) to the CYP P450 enzymes [37, 38]. An *in vitro* study was conducted by comparing wild type *CYP2C9/POR* expression to combinations of *CYP2C9*2* and *CYP2C9*3* with POR allelic variations *A503V, Q153R, A287P* and *R457H* [39]. Using known CYP2C9 probe namely flurbiprofen, diclofenac, and tolbutamide*, A503V (POR*28)* and *Q153R (POR*13)* resulted in an increase of CYP2C9 activity, and *A287P (POR*5)* and *R457H (POR*2)* showed a reduced activity compared to wild type *POR* [39]. However, the effect of the *POR* variant *A503V* varies from one CYP 450 enzyme to another. For example, de Jonge et al., [40] reported an increase of CYP3A5 activity in individuals with the *POR*28* allele, while Sandee et al. [41] showed that this allele caused a decreased CYP2D6 activity.

1.4.3 Induction or inhibition by xenobiotics

Co-administration of drugs can directly or indirectly influence the regulation of CYP2C9. Some drugs can act as enzyme inducers, whereas others as inhibitors. Competitive inhibition can occur between inhibitor and substrate targeting for the same binding site of an enzyme. Non-competitive binding can either cause inhibition or induce the production of the enzyme through interaction with the promoter or transcription factor-binding sites. For example, fluconazole is a well-known potent inhibitor of CYP2C9 [42] as well as many flavonoids [43]. We have observed that fluconazole inhibits phenytion metabolism by CYP2C9 in the ultra-rapid metaboliser mentioned earlier. Rifampin, on the other hand was observed to

induce the enzyme via activation of the *pregene X receptor (PXR)* [44]. CYP2C9 is mainly regulated by PXR and constitutive *androstane receptor (CAR)* binding sites. Drugs such as fluconazole and other azole antifungals have been shown to inhibit CYP2C9 activity *in vitro* and/or *in vivo* [45]. Anti-gout drug colchicine was also shown in vitro to cause a lower CYP2C9 activity [46].

1.4.4 Disease

Disease is also a determinant of CYP2C9 activity. In most in vitro studies, disease-related biomolecules caused a disruption in the regulation of CYP2C9 or any other CYP450 enzymes in general. Recently, a study has found *CYP2C9* is the direct binding target of inflammation-associated micro RNA-130 in the gene's 3'-UTR [47]. The group reported a lower CYP2C9 activity as an effect of the binding *in vitro*. Behçet's disease is a systemic immune disease; therefore, it would be very interesting to see the effect of such diseases *in vivo*. A report on mRNA expression of *CYP2C9* and *CYP2C19* in human hepatocytes showed cytokines IL-6 and TGF to cause a significant decrease of 30% to 50% in their expression [48]. Besides that, several other reports have shown that immune responsive agents are involved in the down regulation of CYP2C9.

1.4.5 Other factors

Intrinsic-physiologic factors such as age, weight and sex were seen to be weak predictors of warfarin dosage determination [49]. Some reports have found that age might have a higher priority in the determination of warfarin dosage [50]. Weight is not seen as a significant determinant of warfarin metabolism in a few studies [35, 51]. So far, reports on the effect of sex on the metabolism of CYP2C9 have been drug-oriented. For example, S-ibuprofen metabolism by CYP2C9 was seen to have a sex-related effect [52]. Other reports denied the sex-based effect of CYP2C9 [53]. The effects of extrinsic factors such as smoking and diet on CYP2C9 activity were also extensively studied but to date, they studies have been very broad in spectrum and often inconclusive [54-56].

2 AIMS

The general aim of this study was to further understand the pharmacokinetic variability of CYP2C9 catalysed drug metabolism.

The specific aims of the included studies were:

Study I:

To find out whether if there is any genetic polymorphism in *CYP2C9* that might cause ultra-rapid metabolism.

To investigate the effect of four intronic SNPs on the metabolism of losartan by CYP2C9 in Korean and Swedish healthy subjects.

Study II:

To investigate the effect of age, *CYP2C9* genotypes, ethnicity, weight, smoking and sex on CYP2C9 metabolic activity of Koreans and Swedes.

Study III:

To investigate if Behçet's disease causes ultra-rapid metabolism comparing losartan MR in Turkish healthy subjects and Behçet's disease patients.

Study IV:

To observe the impact of the *POR*28* genotype on CYP2C9 metabolic activity in Korean and Swedish healthy subjects.

3 SUBJECTS AND METHODS

3.1 SUBJECTS

The study population consists of 148 healthy Swedish volunteers from Karolinska University Hospital, Huddinge, Sweden, 146 healthy Korean volunteers from Inha University Hospital, Incheon, Korea, 96 Turkish healthy volunteers from Hacettepe University Hospital, Ankara, Turkey. The 52 Behçet's disease (BD) patients and 9 other patients who were prescribed with colchicine were recruited from the Department of Rheumatology at the Hacettepe University Hospital, Ankara, Turkey. All subjects on oral contraceptives (OC) were excluded from the study.

Some of the healthy Turkish subjects (n=73) used in this study have been published in 2004 [57]. Together with the 52 BD patients, 23 new healthy subjects were recruited to show that there is no significant difference in the losartan metabolic ratios (MR) between the previously recruited group of healthy Turkish subjects and the recently recruited group. The two groups were than merged to form the 96 healthy Turkish subjects.

Studies I, II and IV were approved by the local ethics committee at Karolinska Institutet, Stockholm, Sweden and Inha University Hospital, Incheon, Korea while **Study III** was approved by the ethics committee at Hacettepe University Hospital, Ankara, Turkey and Karolinska Institutet, Stockholm, Sweden. All subjects were informed about the study and had given their consent before being recruited. These studies were performed in accordance with the Helsinki Declaration and The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) Guideline for Good Clinical Practice.

3.2 GENOMIC DNA

Genomic DNAs were stored in -20°C. The DNA samples were diluted to 50 ng/μL before they were analysed in the laboratory. Each of the samples was labeled numerically to hide the subjects' identity.

3.3 *CYP2C9* GENOTYPING

Genotyping for *CYP2C9*2* and *CYP2C9*3* was done using Taqman assays [26]. Genotyping for *CYP2C9*13* was done using PCR- Restriction Fragment Length Polymorphism (RFLP) PspGI [58]. The reference sequence of *CYP2C9 was* retrieved from The National Center for Biotechnology Information (NCBI) website (NCBI Reference Sequence: NG_008385.1). Due to a very high similarity of *CYP2C9* with other subfamilies of *CYP2C*, very specific polymerase chain reaction (PCR) primers for CYP2C9 are required. The primers were first designed based on the reference sequence and the sequence of the primers was then aligned back to the reference sequences of *CYP2C9, CYP2C19* and *CYP2C8* using an online software; Kalign (http://www.ebi.ac.uk/Tools/msa/kalign/) *CYP2C9*-specific primers sequences were determined. Nine sets of primers were designed

to amplify specifically exon 1 to exon 9 of *CYP2C9*, the intron-exons junctions, and ~2.5 Kbp upstream of the start codon (**Paper I**).

Four single nucleotide polymorphisms (SNPs) were screened through all the subjects. SNP 1 [*IVS1+83T>C* (rs9332104)] and SNP 4 [*IVS8-109A>T* (rs1934969)] were screened using PCR- RFLP while an allele-specific PCR was carried out to detect SNP 2 [*IVS2+73T>C* (rs9332120)] and SNP 3 [*IVS6+95A>G* (rs9332174)].

The exact positions of the target variants were identified and primers were designed based on the position of the SNP. First PCR was performed to amplify exons 1, 2, 6 and 8 of *CYP2C9* by using the sequencing primers (**Paper I**). Two sets of allele-specific primers were designed to specifically match SNP 2 and SNP 3. For SNP 1 and SNP 4, the PCR amplicon for exon 2 and 6 were digested with 10U of restriction enzymes *HpyCH4IV* (NEB) and *TfiI* (NEB) respectively. Initially, a gradient PCR was done to obtain the right condition to amplify all the nine *CYP2C9* regions. The conditions were described in **Paper I**.

After an overnight incubation at 37°C (SNP 1) or 4-h incubation at 65°C (SNP 4), the digested fragments were separated by 3% agarose gel electrophoresis. In this analysis, the completely digested fragments will be a positive variant, partly digested PCR products will be heterozygous and the wild-type samples will not be digested. SNPs 2 and 3 products were analysed using 2% agarose gel electrophoresis. The gels were pre-stained with ethidium bromide. A 100 bp DNA ladder was used as a reference for determination of the sizes of the amplicons.

3.4 *POR* GENOTYPING

Genotyping for *POR*5, *13,* and **28* alleles was carried out using TaqMan® drug metabolism genotyping assay and reagents for allelic discrimination (Applied Biosystems, USA). Genotyping was carried out using Quant Studio 12 K Flex Real-Time PCR system (Life Technologies Inc). The final volume for each reaction was 10 µl, consisting of TaqMan fast advanced master mix (Applied Biosystems, USA), TaqMan 20X drug metabolism genotyping assays mix (Applied Biosystems, USA), and genomic DNA. The PCR conditions are described in **Paper IV**.

3.5 CYP2C9 PHENOTYPING

CYP2C9 metabolic ratio (MR) analysis was carried out in an earlier study by giving subjects losartan 50 mg (Cozaar; Merck, Darmstadt Germany) as a single oral dose [17]. Losartan and CYP2C9-specific metabolite E-3174 were measured in urine collected for 8 hours following drug intake [17].

3.6 STATISTICAL ANALYSIS

Chi square test was used to compare the observed and expected allele frequencies according to the Hardy–Weinberg equilibrium. The MR was determined by dividing the molar concentration of losartan/metabolite E-3174. CYP2C9 MR was log-transformed before statistical analyses were performed. Data were expressed as median and geometric mean, 95% CI. Independent t-tests were done to compare log CYP2C9 MR between the two study populations. Non-parametric tests were performed to compare the median MR differences in **Paper I**.

Univariate linear regression analyses were used to identify the individual effect of covariates. Predictor variables that resulted in p-values < 0.2 in the univariate were entered into a stepwise multivariate regression analysis to identify significant predictors in the final model. Statistical analyses were performed using SPSS Statistics (IBM Corporation, USA) software, version 20.0. P-values of <0.05 were considered to be statistically significant.

4 RESULTS

4.1 SUBJECTS' DEMOGRAPHICS

Table 1 summarises the demographics of all subjects included in **Study I to IV.** A total of 146 Korean, 148 Swedish, 96 Turkish healthy subjects, and 52 Turkish Behçet's disease patients were participated. Swedish and Korean subjects were recruited for **Study I, II, and IV.** The healthy and Behçet's diseased Turkish subjects were recruited for **Study III**. It was observed that most of the BD patients, with 3 individuals in exception, were on anti-gout medication colchicine. Nine other patients with various inflammation-related illnesses (three with familial Mediterranean fever (FMF), two with BD, two with arthritis, one with osteoarthritis and one with oral aphthous lesions) were also recruited for **Study III** (demographics unavailable). Individuals who were on oral contraceptive (OC) were excluded from all of the studies due to its inhibitory effect on CYP2C9 enzyme activity [26].

Table 1. *Subjects' demographics according to ethnicity.* na; data unavailable

	Healthy subjects			**Turkish Behçet's Patients**
	Koreans	**Swedes**	**Turks**	**(Study III)**
	(Study I, II, IV)	**(Study I, II, IV)**	**(Study III)**	
Sex:				
Female	72	63	47	24
Male	74	85	49	28
Mean age in years (range)	24.9 (20-46)	30.5 (19-60)	32.7 (26-47)	41.5 (29-73)
Weight in kg (range)	62.1 (38-94)	71.8 (50-109)	72.2 (56-95)	72.6 (50-96)
Smoking	27	29	na	na

4.2 CYP2C9 GENOTYPES

Genotypes of the subjects in this study were at Hardy-Weinberg's equilibrium for all alleles. The *CYP2C9* gene of an ultra-rapid metaboliser (MR <0.13) [12] and three other control subjects who have normal CYP2C9 metabolism (MR=1) were sequenced. No SNPs were detected in the ultra-rapid metaboliser but the controls were identified with four SNPs; SNP 1*: IVS1+83T>C*, SNP 2: *IVS2+73T>C*, SNP 3*: IVS6+95A>G* and SNP 4: *IVS8-109A>T* (**Paper I**). All the study subjects were genotyped for *CYP2C9*2 (C430T), *3 (A1075C)* and four additional synonymous SNPs detected from the mentioned earlier controls. The Koreans

were screened for an additional genotype, *CYP2C9*13*. One Korean subject was found to carry *CYP2C9*13*, a *CYP2C9*-defective variant among Asian individuals (**Paper II**).

The CYP2C9 genotype frequencies of the Korean and Swedish subjects are shown in **Paper II**. It was observed that there are differences in the frequency of the allelic variations between Koreans and Swedes. Most of the Koreans were *CYP2C9*1/*1* (88%) whereas only 59% of Swedes carried the wild type allele (Fisher's test, P<0.0001). The *CYP2C9*2* allele was not detected in the Koreans but occurred at a frequency of 0.11 in the Swedish population. **Paper III** shows that the genotype frequencies for the Turkish healthy subjects and Behçet's disease patients are statistically similar (Fisher's test, P=0.14).

Paper I discusses the differences in CYP2C9 enzyme activity between Korean and Swedish healthy subjects who were non-carriers (denoted as *CYP2C9*1/*1*) for defective alleles *CYP2C9*2* and/or **3*. The effect of genetic polymorphisms on *CYP2C9* metabolic activity was tested by screening the four new SNPs against the *CYP2C9*1/*1* subjects. The result showed a significant difference in the frequency distributions of the polymorphic alleles between the Koreans and Swedes. Variants *IVS1+83T>C*, *IVS2+73T>C*, and *IVS6+95A>G* were significantly lower in Koreans (Fisher's test, P<0.0001) but not *IVS8-109A>T.* On the other hand, the genotype frequency of *CYP2C9*2*, **3*, and the four synonymous SNPs was not significantly different between the Swedes and Turks.

4.3 POR GENOTYPE

The distribution of the *POR*28* allele was in accordance to Hardy-Weinberg equilibrium in both Koreans and Swedes. Observation from **Study IV** showed that *POR*5* and **13* were absent in both study populations. There was a difference in the distribution of the *POR*28* allele between the Koreans (43%) and Swedes (30%) (Fisher's test, p=0.04). Table 2 shows the genotype and allelic frequency of *POR*28* in Swedes and Koreans. Overall, the frequency of subjects carrying the *POR*28* allele was 65% in Koreans and 49% in Swedes. The same trend was seen among the *CYP2C9*1/*1* subjects that were used for **Study IV**, where the *POR*28* allele frequency was higher in Koreans (43%) than the Swedes (29%).

Table 2. *CYP2C9 and POR genotypes for all subjects*. The Turkish subjects were not genotyped for *POR*5*, **13* and **28*. No *POR*5* or **13* variant was detected in any Korean or Swedish subjects. na; not available

Genotype	**Healthy subjects N (%)**			**Turkish Behçet's disease patients N (%)**
	Koreans	**Swedes**	**Turks**	
CYP2C9				
**1/*1*	128 (87.7)	87 (58.8)	51 (70.8)	31 (59.6)
**1/*2*	0	24 (16.2)	10 (13.8)	12 (23.1)
**1/*3*	17 (11.6)	29 (19.5)	9 (12.5)	8 (15.4)
**1/*13*	1 (0.7)	na	na	na
**2/*2*	0	2 (1.4)	1 (1.4)	0
**2/*3*	0	4 (2.7)	1 (1.4)	1 (1.9)
**3/*3*	0	2 (1.4)	1(1.4)	0
IVS1+83				
TT	142 (98.6)	82 (56.2)	53 (72.6)	43 (82.7)
TC	2 (1.4)	62 (42.5)	17 (23.3)	8 (15.4)
CC	0	2 (1.3)	3 (4.1)	1 (1.9)
IVS2+73				
TT	142 (98.6)	95 (65.1)	53 (72.6)	49 (94.2)
TC	2 (1.4)	44 (31.1)	16 (21.9)	3 (5.8)
CC	0	7 (4.8)	4 (5.5)	0
IVS6+95				
AA	142 (98.6)	88 (60.3)	53 (72.6)	42 (80.7)
AG	2 (1.4)	56 (38.4)	17 (23.3)	7 (13.5)
GG	0	2 (1.4)	3 (4.1)	3 (5.7)
IVS8-109				
AA	62 (43.1)	74 (50.7)	44 (60.3)	28 (53.8)
AT	62 (43.1)	54 (37.0)	22 (30.1)	20 (38.5)
TT	20 (13.8)	18 (12.3)	7 (9.6)	4 (7.7)
POR				
**28 CC*	45 (35.0)	45 (50.7)	na	na
**28 CT*	58 (43.6)	44 (43.1)	na	na
**28 TT*	30 (21.4)	6 (9.2)	na	na
Total	133 (100)	95 (100)		

4.4 GENETIC LINKAGE IN CYP2C9

Figure 2 shows the linkage association among the *CYP2C9* SNPs studied. There is a complete linkage predicted in the Koreans for *IVS1+83T, IVS2+73T,* and *IVS6+95A*. This result is almost predictable because the mutated alleles are very rare in this population. The Koreans also showed high D' value (0.64) between two wild type alleles in Block 2. There is a low degree of linkage observed between *IVS1+83T* with *IVS2+73T* (*D*'=0.37) and *IVS1+83T* with *IVS6+95A* (*D*'=0.54) in the Swedes indicating none of the other SNPs in Koreans or Swedes are in linkage to one another. The majority of Turkish healthy subjects were wild type (D'=0.73). The second most common haplotype for the Turks carried the mutated allele *IVS6+95G* (9%). The same observation was made in the Behçet's disease group.

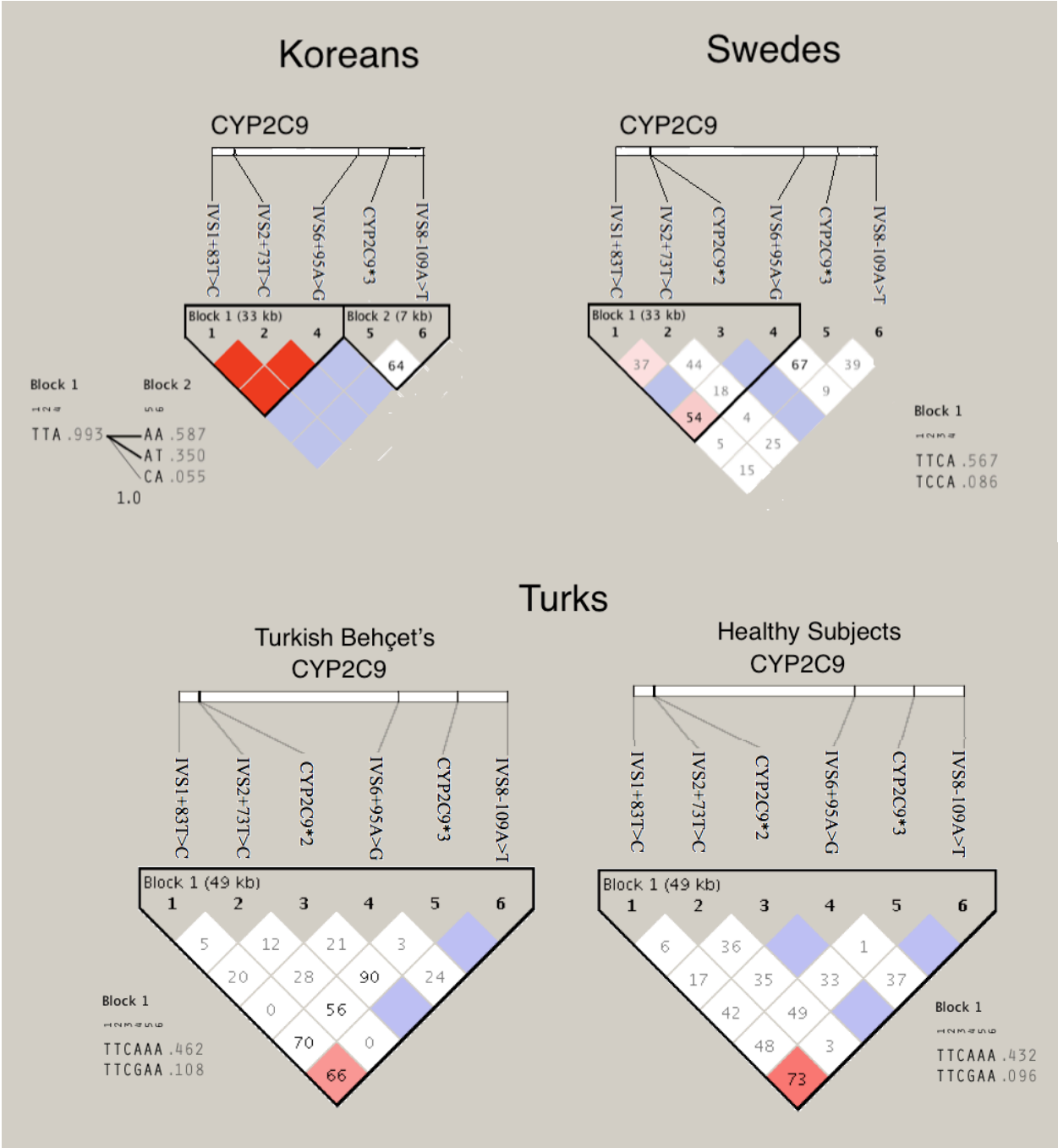

Figure 2. *The linkage disequilibrium (LD) among the studied CYP2C9 SNPs.* This figure predicts the linkage between the polymorphisms and the resulting LD blocks at the *CYP2C9* locus. LD block size is given as horizontal bars as calculated by Haploview [59].The LD blocks drawn below represent the LD blocks between the SNPs. Dark red boxes without a value denote a D'-value of 1.00; light blue boxes without a value denote a not determinable value. Numbers denote the 100-fold of the respective D'-values. The higher the value, the darker the fill colour of the boxes and the higher the LD.

4.5 THE EFFECT OF CYP2C9 GENOTYPES ON CYP2C9 PHENOTYPE

The metabolic ratio (MR) of losartan to its CYP2C9-specific metabolite E-3174 was measured in all subjects. There is a similarity in the MR of Swedes and Turks (Figure 2). However, Koreans display a significantly different MR profile, where they were observed to have a lower MR, indicating a more rapid metabolism of losartan to E-3174 by CYP2C9 compared to Swedes ($p<0.001$). After dividing the subjects into their genotype groups, significant differences were still observed between Koreans and Swedes in both the *CYP2C9*1/*1* ($P<0.001$) and *CYP2C9 *1/*3* groups ($P=0.001$) (**Paper II**, Fig 2).

There was also a significant difference in CYP2C9 enzyme activity observed between the Turkish healthy subjects and the BD patients ($P=0.002$). In contrast to the ultra-rapid metaboliser who also suffered from BD, the subjects who were in the BD patients groups showed a higher MR compared to the healthy subjects (**Paper III**).

4.6 THE EFFECT OF POR GENOTYPE ON CYP2C9 PHENOTYPE

Considering subjects with *CYP2C9*1/*1* genotypes only, the CYP2C9 MR was significantly lower in individuals who carried the *POR*28* allele among Swedes ($P=0.02$) but not Koreans ($P=0.68$). The geometric mean of MR between the subjects who carried the allele *POR*28* and subjects without the allele was significantly different in Swedes (0.64 vs 0.89, $P=0.002$). However no significant difference in CYP2C9 enzyme activity between the different POR*28 genotype groups was observed in the Korean population (0.56 vs 0.55, $P=0.68$) (**Paper IV**, Table 2).

The effect of *POR*28* genotype on CYP2C9 MR among Koreans was tested by excluding smoking individuals. No significant difference in CYP2C9 MR between *POR* wild-type and *POR*28* carrier was found among non-smokers (t-test, $p=0.97$). A similar observation was found in smokers (t-test, $p=0.38$). Result signified there is no effect of *POR*28* genotype on CYP2C9 enzyme activity in Korean subjects regardless of smoking status.

Multiple linear regression analysis showed that the effect of the *POR*28* genotype contributes up to 8% (step 1, ANOVA $p=0.02$) of variation in Swedes. Together with sex, the variables are up to 11% (step 2, ANOVA $p=0.03$) responsible for CYP2C9 activity variation in this group.

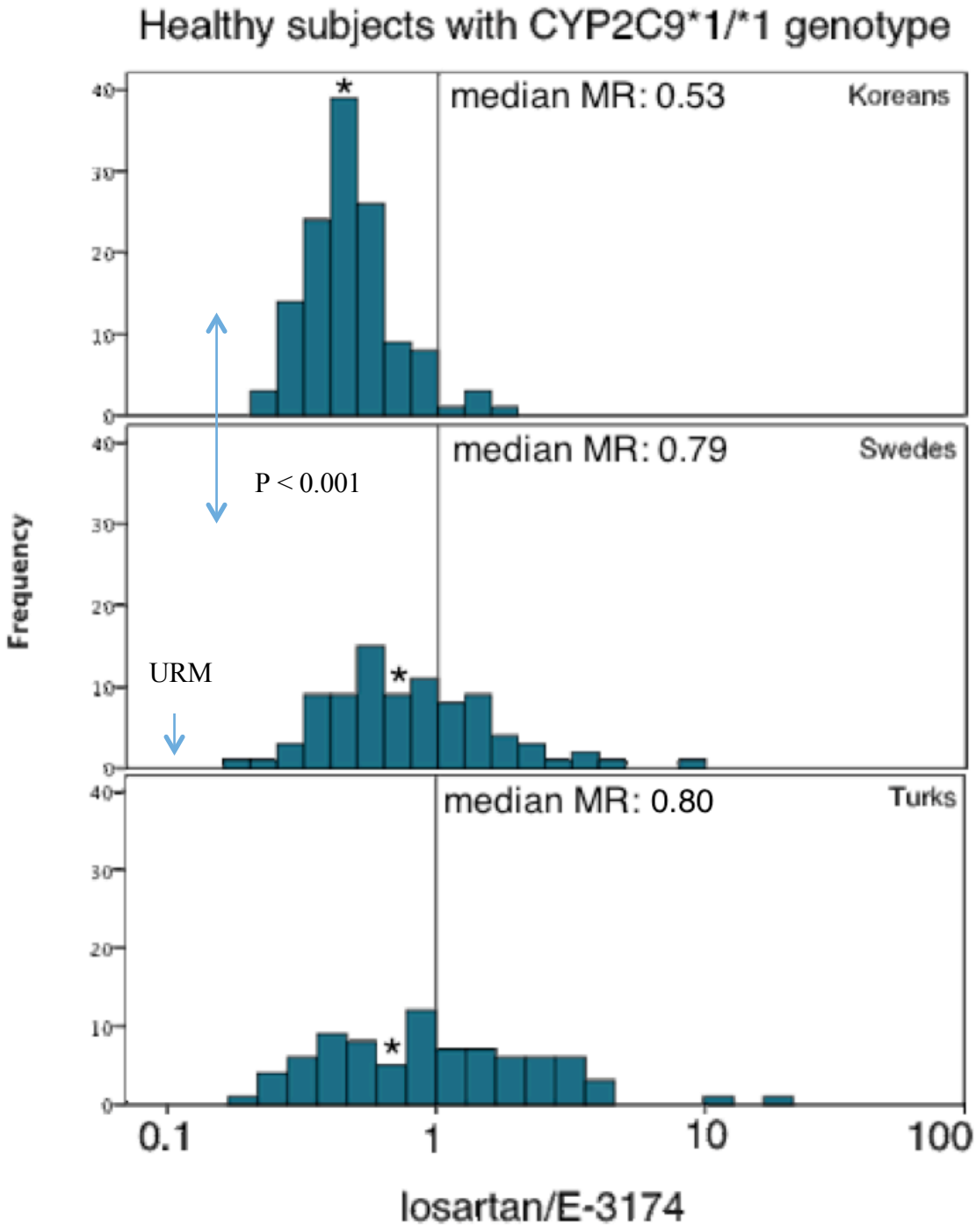

Figure 3. *Histogram showing metabolic ratio (MR) of losartan/E-3174 according to ethnicity*. The MR are log transformed, '*' indicates the median MR and 'URM' indicates the ultra-rapid metaboliser. MR=1 is indicated as an arbitral vertical line.

4.7 THE EFFECT OF BEHÇET'S DISEASE ON CYP2C9 CATALYSED METABOLISM

The key differences between the Turkish healthy subjects and the BD patients were the medication they took and the lack pathophysiology of the disease in the healthy subjects. The colchicine dosages received by the BD patients ranged from 1 to 4 mg/day, with 29 patients receiving 3 mg/day, 11 receiving 2 mg/day, 4 receiving 1 mg/day and 2 patients receiving 4 mg/day. The list of their concurrent medications with median dosage is shown in Appendix 1, in **Paper III**. Among these drugs, one subject was found to be on CYP2C9 inducer carbamazepine at 600 mg daily (with genotype *CYP2C9*1/*2* and losartan MR 2.0), and several were on CYP2C9 substrate/inhibitors: warfarin with median dosage of 5 mg daily (eight individuals), omeprazole 20 mg daily (three individuals) and sertraline 50 mg daily (one individual). The patient who was on warfarin was genotyped as *CYP2C9*1/*2* and the patient with sertraline had the MR of 1.92, which was close to the median MR of the BD patients groups (1.74). The three patients on omeprazole showed no significant impact on their CYP2C9 activity (MR= 0.91-1.64). Among the eight individuals on warfarin, three were *CYP2C9*1/*1* and they had the MR of 0.98 to 1.26, which are lower than the median for the group but not lower than the healthy subjects' (MR=0.80).

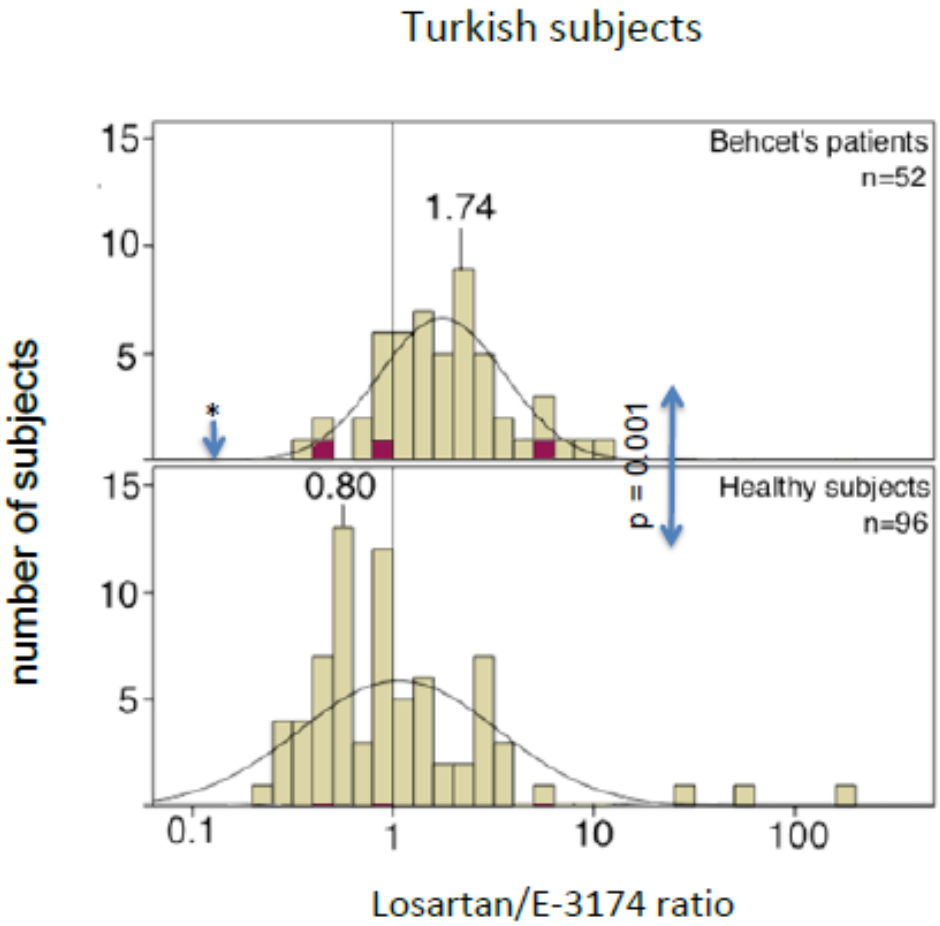

Figure 4. *Comparison of losartan/E-3174 ratio between the Behçet's disease patients and Turkish healthy subjects.* The three patients in red represent patients who were not on colchicine treatment. MR: metabolic ratio. MR=1 is indicated as an arbitral vertical line. Numbers represents the median MR of each group. *;Swedish ultra-rapid metaboliser with Behçet's disease. MR < 0.13 [12]. Figure reproduced with permission.

4.8 THE EFFECT OF COLCHICINE TREATMENT ON CYP2C9 CATALYSED METABOLISM

In nine patients about to be prescribed with colchicine, losartan MR was measured before starting treatment and again after 2 weeks of colchicine treatment. The dosages of colchicine given to the nine patients (four patients received 3 mg/day, three patients received 2 mg/day and two patients received 1 mg/day) were very similar to those given to our large group of BD patients. There was no difference in geometric mean MR before (3.00) compared to 2.80 during colchicine (P=0.89, 90 % CI: 0.38–3.02) (Figure 5). Three patients did not show any change in their MR, three had lower MR during colchicine treatment and three had higher MR.

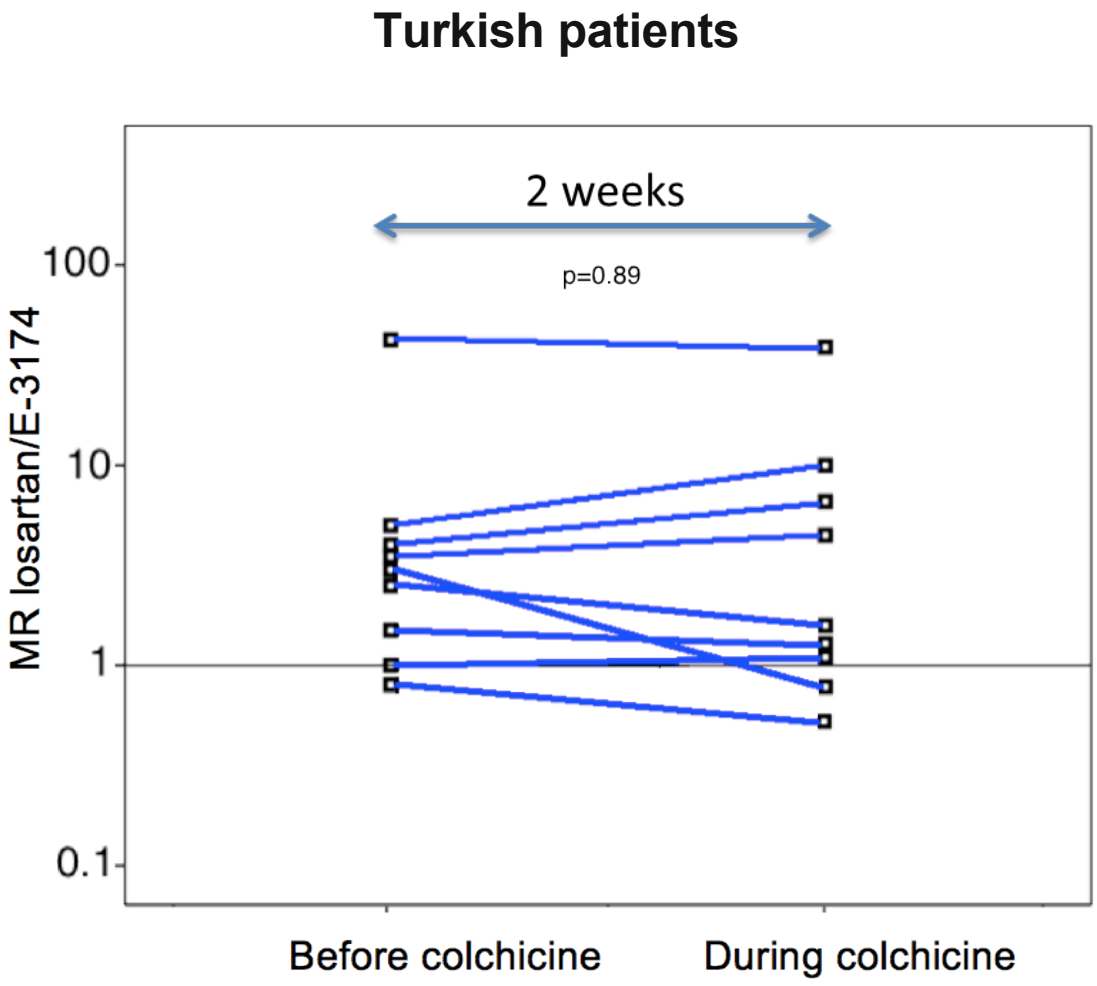

Figure 5. *Metabolic ratio before and during colchicine in inflammation- related diseased patients.* MR; metabolic ratio and MR=1 is indicated as an arbitral vertical line

4.9 THE EFFECT OF ENVIRONMENT ON CYP2C9 CATALYSED METABOLISM

In addition to ethnicity, *CYP2C9* genotype is a strong determinant for CYP2C9 metabolic activity in this study group. There is however, a fraction of external factors that also plays an important role in determining CYP2C9 metabolic activity. In **Paper II**, the effects of external factors such as age, sex, weight, ethnicity, and smoking were evaluated together with CYP2C9 genotype to determine their significance on CYP2C9 metabolism. Univariate analysis showed that ethnicity ($P<0.0001$), *CYP2C9* genotype ($P<0.0001$), and smoking ($P=0.009$) are the main predictors of the differences in CYP2C9 MR. Stepwise multiple regression analysis shows that 50% of the CYP2C9 MR variation is due to the three mentioned factors. Among the three, *CYP2C9* genotype is, however, the most important predictor accounting for 40% of the variation factor.

After grouping the subjects according to their ethnicity, univariate analysis indicated *CYP2C9* genotype as the main contributor for variations in CYP2C9 metabolic activity in both ethnic groups ($P<0.0001$ in both groups). Interestingly, smoking was a second significant predictor to influence CYP2C9 metabolic activity in Koreans ($P=0.003$). Multivariate regression analysis showed that CYP2C9 is the main factor contributing to variation in CYP2C9 MR in both populations (26.6% in Koreans and 39.6% in Swedes). Adding smoking to the CYP2C9 genotype effect explaines 31% between-subject variability in CYP2C9 MR among Koreans. None of the tested potential non-genetic predictors including body weight and age had a significant impact on the CYP2C9 phenotype in Swedes.

5 DISCUSSION

This thesis was initially formulated with the aim of understanding the effect of several biological and molecular factors that affect the human CYP2C9 enzyme metabolic activity. These variants are genetics, environmental factors such as age, weight, sex, and lifestyle (smoking), as well as geographical factors (ethnic and dietary).

The true birth of this study was in 2010, when an ultra-rapid metaboliser of phenytoin and losartan, both CYP2C9 substrates was discovered. She also suffered from Behçet's disease [12]. The patient was an extreme outlier compared to the 148 healthy Swedish subjects (URM in Figure 1). This group of Swedish healthy subjects was already shown to portray some interesting features. First; although after they were classified according to their genotype, there was still a wide inter-individual difference in their CYP2C9 MR. Second; the median CYP2C9 MR of healthy Swedes was significantly different from the median CYP2C9 MR of the Korean healthy subjects. The Koreans had a lower CYP2C9 MR compared to Swedes. These observations led to the hypothesis that there might be an unknown molecular or environmental factor contributing to a lower CYP2C9 MR (higher CYP2C9 metabolic activity).

To test the hypothesis, direct capillary sequencing on the *CYP2C9* gene of the ultra-rapid metaboliser and three other Swedish "control" normal metabolisers was carried out. As discussed in **Paper I**, the three controls were found to carry 4 synonymous SNPs but no allelic variation was found of the ultra-rapid metaboliser. This discovery led to a new hypothesis that the SNPs detected might be causing the controls and the rest of the Swedish population to have a lower CYP2C9 metabolism. Factors that might contribute to CYP2C9 metabolic variabilities were observed in **Study II**. The possibility of Behçet's disease being an inducer to a lower MR was investigated in **Study III**. Finally, in **Study IV**, the external factor that plays a role in the redox of CYP2C9, the *POR* gene variant **28*, and its effect on CYP2C9 metabolic activity was studied.

5.1 THE EFFECT OF GENETICS ON CYP2C9 CATALYSED METABOLISM

In general, Swedes were observed to have a lower CYP2C9 activity compared to Koreans. *CYP2C9* genotyping showed that there is a relation between alleles *2 and *3 in Swedes and **3* and **13* in Koreans to lower CYP2C9 metabolic activity. However, comparing Koreans and Swedes with *CYP2C9*1/*3* genotypes, Koreans are still observed to display a significantly higher CYP2C9 activity. To date, it has been well known that almost all known functioning *CYP2C9* allelic variations are responsible for defective enzymes. Having just one of the functional variant alleles will result in a lower CYP2C9 activity. Population studies have reported that some of these *CYP2C9* allelic variations are ethnicity-specific, for example, in this study, no *CYP2C9*2* was detected in the Koreans and **2* and **3* were not detected in the Canadian Inuit population [60]. The Inuits are thought to be of Asian ancestry and showed a geographical aspect of genetic variability in not carrying the *CYP2C9*3* allele. *CYP2C9*2* and **3* alleles occur at almost the same frequencies in

Swedish and Turkish subjects in this study. *CYP2C9*5* and *CYP2C9*6*, for example, were found to be inherited by a small group of African-Americans [61]. *CYP2C9*13* was detected in one of the Korean subjects in **Study II** and the allele was also detected in low frequency in Japanese [62] and in Koreans [58] but it occurred up to 2% in the Chinese population [43].

A higher frequency of *CYP2C9* defective variant alleles occurred in the Swedes and Turks compared to the Koreans (Table 2). Several other studies have also reported that a higher number of defective *CYP2C9* alleles in Caucasian individuals [63, 64]. This finding could explain the overall lower CYP2C9 activity seen in the Caucasian population compared to Asians. Referring to Figure 1, the Swedes and Turks have a broader distribution curve, suggesting that there are possibly more contributing genetic factors to the metabolic rate diversity. Further genotype and phenotype cross-match studies should be done to completely understand the actual effect of these allelic variations.

Although copy number variation (CNV) has been extensively reported for *CYP2D6*, there might not have been any significant finding on any clinically important CNV on *CYP2C9* and *CYP2C19*. A pre-screening done on the genome of the ultra-rapid metaboliser has shown that she did not carry any CNV in and around her *CYP2C9* and *CYP2C19* genetic region. Therefore, CNV is not the explanation of the ultra-rapid metabolism.

Papers I and III highlighted that there is a high number of Swedish and Turkish *IVS1+83T>C, IVS2+73T>C*, and *IVS6+95A>G* allele carriers respectively. The Swedish and Turkish healthy subjects showed that there is no linkage observed among the studied SNPs. In the Koreans, however, *IVS1+83T>C, IVS2+73T>C,* and *IVS6+95A>G* are linked to each other. However, there are only two individuals who carry the polymorphisms. A Japanese population based study also reported the three SNPs as haplotype **1h* that is also linked to *IVS1+83T>C* [65]. In this population, 4 individuals out of 263 subjects carried the haplotype and 2 other individuals had an additional SNP in the haplotypes. Allele *IVS8 +109 T>A* on the other hand occurs in a homogeneously high frequency in all populations. The observed effect of *IVS8 +109 T>A* on the Swedish healthy subjects was probably due to its linkage to another important polymorphism that is not yet determined. This SNP also does not have any effect on the CYP2C9 activity of the Turkish healthy subjects and Behçet's disease patients (**Paper III**).

*POR*28* is known to increase CYP2C9 catalysed drug metabolism *in vitro* [39]. **Study IV** shows that the Swedish individuals carrying the mutated allele have a significantly lower CYP2C9 MR mean compared to the Swedish individuals without. Interestingly, the ultra-rapid metaboliser was also genotyped as a *POR*28* carrier. Of course, this is probably just a small contributing factor to her extreme CYP2C9 metabolic rate. This finding is also in accordance to the earlier *in vitro* report showing that POR*28 causes an increase of CYP2C9 activity [39]. Interestingly, in Koreans, where there are more individuals carrying the *POR*28* allele, the statistical analysis showed no effect of the **28* allele on CYP2C9 MR in this population. Looking from another perspective, the overall CYP2C9 MR of

Koreans is significantly lower compared to Swedes, which supports that the SNP is somehow linked to a lower CYP2C9 MR. There are probably more than one allelic variation in the POR gene that might be responsible to this difference. For example, SNPs *366+89C>T* and *1248+20G>A* in *POR* were found to be occurring at a frequency of 30% in Caucasian liver samples [66]. The combinations of these allelic variations and their frequencies in each ethnic population would have an influence on the metabolic activity of an enzyme. Lack of *POR*5* and **13* alleles in the healthy subjects is expected as the two alleles are very common in individuals with congenital malformations [39].

Gene-gene interaction, in this case the combination of *CYP2C9* and *POR* genotypes affecting CYP2C9 activity is very important in understanding drug metabolism. A very classic example is the effect of *CYP2C9* interaction with *VKORC1* that affects warfarin dosing. In warfarin metabolism, CYP2C9 plays a role in the metabolism of the enantiomer S-warfarin and the pharmacodynamics of warfarin is controlled by its target, vitamin K epoxide reductase (VKORC1). A large cohort study has found that *CYP2C9, VKORC1*, age, sex, and drug interactions explained 59% of warfarin dose variability in European subjects [67]. Meanwhile a study in Malaysia found that a combination of age and *CYP2C9*3* and *VKORC1* genotypes accounts for 37% of the variability [35].

5.2 THE EFFECT OF BEHÇET'S DISEASE ON CYP2C9 CATALYSED METABOLISM

The ultra-rapid metaboliser was on phenytoin for her epilepsy, but she also was being treated for Bechet's disease. The possibility of the disease being a factor that causes an inhibition or induction is tested in **Study III.** The significant difference of geometric mean of MR between the Turkish healthy subjects and the Behçet's disease patiets showed that the disease does not increase CYP2C9 metabolic activity. In fact, the disease seems to be causing a lower CYP2C9 activity (higher MR). The three factors that might be causing this down-regulation of CYP2C9 are: genetics, medications and the disease itself.

Genetics was ruled out in **Study III** because no significant difference was found in the frequency of any of the *CYP2C9* allele between the two Turkish groups. The two remaining factors are very likely to have an inhibiting effect on CYP2C9 metabolism. Behçet's disease is an inflammatory syndrome. Recent studies have shown that some inflammation response agents were responsible to down-regulate CYP2C9. For example, inflammation response agents IL-6 and TGF were found to be responsible to down regulate CYP2C9 [48]. Additionally, very recently, a group in Germany found that microRNA miR-130 which is associated to inflammation was reported to directly affect CYP2C9 regulation. The study was done by inserting the 3'-UTR of *CYP2C9* gene to the firefly gene of a plasmid. Luciferase activity in HuH7 cells was measured and the binding of miR-130 to the UTR putative binding sites inhibited the expression of CYP2C9 [47]. This study directly supports the observation of CYP2C9 having lower activity in the Behçet's disease group.

5.3 THE EFFECT OF DRUGS ON CYP2C9 CATALYSED METABOLISM

Studies have shown that drugs not only are metabolised by CYP enzymes, they also influence the induction and inhibition of these catalysts. In the example of the ultra-rapid metaboliser, fluconazole that was prescribed to her inhibited CYP2C9 metabolic activity and almost completely stopped the metabolism of phenytoin. In **Study III**, the effect of colchicine on CYP2C9 was discussed. All except three of the Behçet's disease patients were on colchicine, which led us to test nine inflammation-related disease patients with losartan before and again during colchicine treatment. Results from the nine patients showed that colchicine given over two weeks does not have any significant effect on CYP2C9 metabolism.

Some of the studied Behçet's diseased patients were on medications known to induce CYP2C9 activity, for example carbamazepine, and CYP2C9 substrate/inhibitors: warfarin, omeprazole, and sertraline. However, overall, they are within the normal distribution curve of the Behçet's disease group and still portray a lower CYP2C9 metabolic activity compared to the healthy Turkish subjects.

5.4 THE EFFECT OF OTHER ENVIRONMENTAL FACTORS ON CYP2C9 CATALYSED METABOLISM

Paper II explores the effect of other factors that might have an effect on CYP2C9 metabolic activity along with genetics in Koreans and Swedes. Estimation by regression analysis shows *CYP2C9* genotype to have different impact on determining the metabolism by CYP2C9 enzyme. Another factor that is known to have an effect on the metabolic activity is oral contraceptives [26]. Therefore, in all of our study, individuals on OC were excluded.

In **Study II,** besides genetics, we tested the effects of ethnicity, age, sex, weight and smoking habits on CYP2C9 enzyme activity. Preliminary univariate analysis pointed out that genetics, ethnicity, and smoking are the most likely factors determining inter-individual CYP2C9 MR differences. The effect of ethnicity was already seen and reported in **Study I** and by comparing the geometric mean of the CYP2C9 MR between both populations. Separating the subjects according to their ethnicity, the effect of *CYP2C9* genotypes was reported in **Paper II** by comparing the subjects' MR (Paper II, Figure 2).

The effect of smoking was not significant in the Swedes but observed in the Koreans. Smoking has been known to affect CYP1A2 activity through *aryl hydrocarbon receptor (AhR)* activation by polycyclic aromatic hydrocarbon (PAH) [68, 69]. However, the effect of smoking on CYP2C9 should not be excluded. For example, the effect of smoking on CYP3A4 was found in a study to be due to the binding of PAH from cigarette smoke to common *CYP2C9* receptor, *the pregnane-X-receptor (PXR)* [70]. However, there are a few inconsistent findings regarding the effects of smoking on the CYP2C9 enzymes. A study found significantly induced CYP2C9 activity in bronchial biopsies of smokers compared to

non-smokers [56]. In contrast, the metabolism of warfarin by CYP2C9 of cigarette smokers is not significantly different from non-smokers [55].

The difference in the CYP2C9 MR between the Koreans and Swedes is mostly due to their differences in *CYP2C9* genotypes (40% in Swedes and 27% in Koreans). The evidence of *POR*28* allele having an effect on the activity of this enzyme was seen in **Study IV**. But there are other un-identified genetic, epigenetic, or environmental factors that contribute to the variability of CYP2C9 activity. The effect of the interactions between these known and yet-to-be-identified factors on CYP2C9 activity would be intersting to observe in the future.

Diet for example, is an interesting factor because it is not only directly linked to genetic build-up, but it is a social-behavioral issue and it is very subjective to an individual's culture background and life-style. Koreans, for example have a different diet than Swedes. The Koreans' staple food contains soy products, meat, and rice. They are also more likely to eat spicy food that may contribute to a different metabolic capacity than the Swedes who mainly consumes dairy, eggs and wheat products. Diet is unique to every individual and it consists of mixtures of compounds that would interact with the hepatic cytochromes just like drugs. cranberries, garlic, ginkgo, and St. John's wort were found to have inhibition properties towards CYP2C9 activity [71]. Additionally, Korean researchers reported an induced CYP3A4 expression *in vitro* and *in vivo* by human PXR and CCAAT-enhancer-binding proteins beta (C/EBPb) from capsaicin, a type of principal pungent ingredient in hot red and chili peppers [54].

The contributor to the variation of CYP2C9 metabolic activity is mainly genetics. However, other factors such as oral contraceptives and anti-fungal medications, and comorbidities are very important to account for. This study has found that the genotypes of *CYP2C9* and *POR* genes are important to determine the initial metabolic activity of the CYP2C9 enzyme. Disease, such as inflammation-related illness may significantly reduce the activity of this enzyme and therefore might interfere with the effectiveness of treatments. Other factors such as diet, alcohol consumption and smoking although the mechanism of interactions is complex and difficult to understand would also be considered as a variation factor.

5.5 FUTURE WORK

Although there are maybe more questions raised at the moment on the impact of pharmacogenetics in shaping the future of personalised medicine, there are numerous studies being done currently in order to answer them. An interesting question raised from this study is the size of impact *POR* genotype has on the CYP2C9 activity, and whether the ultra-rapid metaboliser had a unique mutation on her *POR* gene that caused the high CYP2C9 activity. It would be worthwhile to fully understand the molecular reason to the high CYP2C9 activity, and if it could be controlled. Therefore, it would be of a great advantage to be able to sequence the ultra-rapid metaboliser's *POR* gene and test the functionallity of any non-synonymous mutation found. Additionally, some *POR* genetic variations were also reported to be affecting other CYP 450 enzymes; looking into the effect of *POR* allelic variation onto other metabolic enzymes while also cross-checking the effects among different ethnicities would be a natural path to follow.

6 SUMMARY

Based on the observation of ultra-rapid metaboliser of phenytoin, we designed a study that investigated for any molecular or other potential contributing factors behind the observed inter-individual variations in CYP2C9 metabolism using losartan as a probe. Realizing that there is also an inter-ethnic difference in CYP2C9 metabolism, we further investigated for any contributing factor for the observed CYP2C9 enzyme activity variability between Koreans and Swedes. Turkish subjects were recruited in one of our sub-studies to investigate the effect of Behçet's disease on CYP2C9 metabolic activity.

The conclusions of this thesis are:

- Apart from *CYP2C9*2* and **3*, other genetic or environmental factors may contribute for inter-individuals or inter-ethnic variation in CYP2C9 metabolism. We found that *IVS8-109A>T* does have a lowering effect on losartan metabolism. We also found that there are polymorphisms of *CYP2C9* haplotypes between the Koreans and Swedes.
- Interestingly, we found that genetics have different levels of impact on the enzyme activity of Koreans than on Swedes. Swedes are more affected by *CYP2C9* genotype (**2* and **3*) than Koreans (40% vs 27% predicted impact, respectively). Smoking is seen as a predictor in Koreans and the reason is unknown.
- There was a significant different in CYP2C9 activity between the healthy Turkish subjects and the Behçet's patients. In the Behçet's patients group, we identified genetics, the disease and concomitant drugs as predictors to lower CYP2C9 metabolic activity.
- We found that the inflammatory reaction agents actually cause an inhibition towards CYP2C9 regulation after observing that there is no difference in the *CYP2C9* allele frequencies between the two groups and the drugs used by a majority of the patients have no conclusive effect after two weeks.
- The impact of the common P450 oxidoreductase variant *A503V* (*POR*28*) on CYP2C9-losartan metabolism was investigated. An interesting link between *POR*28* allele and a 1.4-fold increase of activity in CYP2C9-losartan metabolism in Swedes was found. No effect was observed in individuals with *POR*28* on the metabolic activity of CYP2C9 in Koreans.

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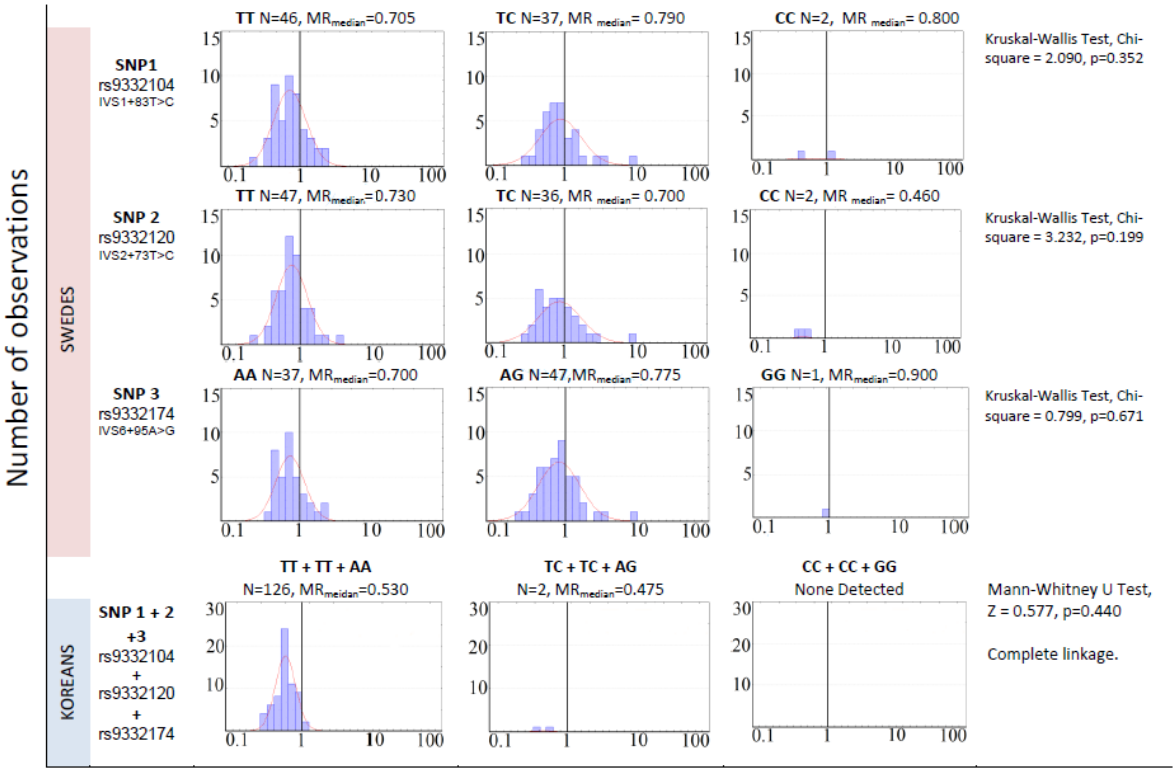
ERRATA: THIS PAGE LISTS ERRORS FOUND IN THE SUBMITTED VERSION OF PAPER I, TOGETHER WITH CORRECTIONS WHERE APPLICABLE.

Location: Fig 1A, Paper I ; the genotype change and median MR added to SNP 2 and SNP3 for Swedes and SNP 1+ 2 + 3 in Koreans

Fig 1A: Distribution of the Log metabolic ratio (MR) in individuals genotyped for **SNP 1- 3** in Swedes and Koreans (MR=1 is indicated as a line in the histogram)



Location: Fig 1B, Paper I; rs number changed for SNP 4

Fig 1B: Distribution of the Log metabolic ratio (MR) in individuals genotyped for **SNP 4** in Swedes and Koreans (MR=1 is indicated as a line in the histogram)