The effects of diet and geographic ancestry on drug-metabolising enzyme activity in Europeans and South Asians

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BMedSc MPharm

A thesis submitted to fulfil requirements for the degree of Doctor of Philosophy

The University of Sydney

Faculty of Pharmacy

2018

Preface

This thesis is the result of original investigations carried out by Shane K. Eagles within the Faculty of Pharmacy, at the University of Sydney, under the supervision of Professor Andrew McLachlan and Adjunct Associate Professor Annette Gross. This thesis has not been submitted for award of a degree at any other university. Human research ethics approval has been obtained for the studies described in this thesis. Full acknowledgement has been made where the work of others has been used or cited. A list of conference presentations in support of this thesis is included.

Shane K. Eagles

Acknowledgements

There is a long and varied list of people to thank through whom this research was made possible. Firstly, I would like to acknowledge the Peter Coates Postgraduate Scholarship in Ethnopharmacology. Dr Peter Coates was an international leader in clinical pharmacology, global drug development and academic collaboration. This joint venture in honour of his name and work, between GlaxoSmithKline and the University of Sydney, has given me the opportunity to conduct the research presented in this thesis, for which I am extremely grateful.

I would like to thank both of my supervisors, Professor Andrew McLachlan and Adjunct Associate Professor Annette Gross for taking a chance on me, a curious Community Pharmacist with minimal research experience, and providing the resources and means to conduct this project. Your advice, time and patience throughout the years has been appreciated, and will not be forgotten.

I crossed paths with many people while based at Concord Repatriation General Hospital. Thank you to Dr Lisa Pont, Dr Alessandra Warren and Associate Professor Victoria Cogger for your advice, support, friendly smiles and company throughout those disorientating initial years of my candidature. Thank you also to the various students who moved through Building 4, ANZAC 3 over the years: Michael Dolton, Atheer Nassir, Christina Abdel Shaheed, Jade Fox and Rayan Nahas; your company and sympathetic ears helped more than you likely know. Further, I would like to thank my co-worker, Bei Lun-Lin, for her assistance with sample collection during the first phase of the clinical study, and also for helping with the initial aspects of the cocktail assay's development.

I would like to acknowledge and sincerely thank the Andrology team at Concord Hospital led by Leo Turner, who were instrumental in making the clinical study possible. Thank you very much to Leo, Sasha Savkovic, Carolyn Fennell and Glenda Fraser for assisting with participant logistics and performing sample collection on the numerous study days. Thank you also to Irene Di Pierro, Feyrouz Bacha and Ljubica Vrga for the friendly and extremely generous way in which you helped with sample processing and storage. I would like to acknowledge and thank Toni Cavalletto and Professor Fiona Blyth for organising and facilitating the movement of the clinical study to the Concord Medical Education Centre in 2016. Further, a huge thank you to Associate Professor Vasi Naganathan and his team in Geriatrics for providing the on-call clinical support and assisting with sample collection throughout this study. And of course, thank you to all of the healthy volunteers for your participation in the clinical study. I definitely could not have done this without you!

A huge thank you to Dr Sussan Ghassabian—your guidance and openness to help a struggling student was invaluable, and your advice helped me overcome many obstacles in developing my bioanalytical methods. Similarly, a big thank you to Dr Mohi Iqbal Mohammed Abdul for passing on valuable experience in bioanalytical method development. Thank you also to Padmaja Dhanvate and Dr Sarah Cui for their efforts in orientating and training me on LC-MS/MS systems. Also, I would like to acknowledge Professor Alan Boddy for his advice, mentorship and constant positive demeanour, accompanied by his rigour of the scientific method and its application to clinical pharmacology. To Dr Xiao Suo Wang—your guidance, expertise, friendship and professionalism enabled me to learn how to use UHPLC-MS/MS systems and complete my analyses, for which I am forever grateful. Thank you also to Dr Mario D'Souza for patiently and kindly teaching me the principles of mixed-

effects modelling and advanced statistics, which greatly enhanced the analysis of my clinical study data.

Thank you to my immediate family and my second family, the Légerets, for believing in me and what I am capable of. Your love and support gave me the strength to carry on.

Lastly, and most importantly, thank you Colette. From the deepest recesses of my soul, thank you. You held me when bad unexpectedly became worse. You strengthened me when nothing else would. You laughed and wept beside me and supported me through it all, whether I deserved it or not. You pushed me when nothing else would move me, and you lifted me and us up no matter how heavy this burden became. This is as much your doctoral candidature as it as mine. Thank you for your steadfast love and support over relentlessly impossible years. You are my all.

Conference presentations in support of this thesis

- Eagles SK, Gross AS and McLachlan AJ (2013). The effect of broccoli consumption on the activity of drug-metabolising enzymes in Europeans and South Asians: Study protocol. Poster presentation at ASCEPT conference, Melbourne (poster no. 559).
- Eagles SK (2013). The effect of broccoli consumption on the activity of drug-metabolising enzymes in Europeans and South Asians: Study protocol. Oral presentation at the University of Sydney Postgraduate Research Showcase.
- Eagles SK (2015). Variability in response to medicines: a focus on diet, ethnicity and drug metabolism. Invited seminar, ANZAC Institute, Concord Repatriation General Hospital.
- Invited speaker at the University of Sydney Bosch Institute Facilities User Group Meeting, 2015: Variability in response to medicines: a focus on diet, ethnicity and drug metabolism.
- Invited speaker at Agilent conference in Sydney, 2017: Eagles SK, Wang, XS, Lin B-L, Gross AS and McLachlan AJ. An updated and optimised version of the “Inje” and “Ghassabian” cocktails: a simplified and highly sensitive UHPLC-MS/MS CYP-phenotyping cocktail assay in human plasma.

List of abbreviations and symbols

Abbreviation	Definition
137X	1,3,7-trimethylxanthine (caffeine)
17U	1,7-dimethyluric acid
17X	1,7-dimethylxanthine (paraxanthine)
1U	1-methyluric acid
1X	1-methylxanthine
ADME	Absorption, distribution, metabolism and elimination
AFMU	5-acetylamino-6-formylamino-3-methyluracil
AhR	Aryl hydrocarbon receptor
ALFRED	The Allele Frequency Database
ANZCTR	Australian New Zealand Clinical Trials Registry
APAP	*N*-acetyl-*p*-aminophenol
APAPC	Paracetamol cysteine
APAPG	Paracetamol glucuronide
APAPM	Paracetamol mercapturate
APAPS	Paracetamol sulfate
AUC	Area under the concentration-time curve
BMI	Body mass index
BPA	Bisphenol A
CAF	Caffeine
CAR	Constitutive androstane receptor
CDNB	1-chloro-2,4-dinitrobenzene
CENTRAL	Cochrane Central Register of Controlled Trials
CI	Confidence interval
CL	Clearance
CNV	Copy number variation
CONSORT	Consolidated Standards of Reporting Trials
CRGH	Concord Repatriation General Hospital
CV%	Coefficient of variation; (SD/mean)*100
CYP	Cytochrome P450
D1	Study Day 1
D2	Study Day 2
D9	Study Day 9
DNA	Deoxyribonucleic acid
DXM	Dextromethorphan
DXR	Dextrorphan
EM	Extensive metaboliser
EMM	Estimated marginal means
EXP	Losartan carboxylic acid
FDA	Food and Drug Administration
GEMM	Geometric estimated marginal means
GIT	Gastrointestinal tract

GSH	Glutathione
GST	Glutathione *S*-transferases
HLB	Hydrophillic-lipophillic balance
HNF4α	Hepatocyte nuclear factor 4α
HPLC	High-performance liquid chromatography
HREC	Human Research Ethics Committee
ICH	International Council for Harmonisation
ICTRP	International Clinical Trials Registry Platform
ID	Identification
ILIS	Isotopically-labelled internal standard
IM	Intermediate metaboliser
IS	Internal standard
ITC	Isothiocyanate
LC	Liquid chromatography
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LLOQ	Lower limit of quantification
LOS	Losartan
LSD	Least-significant difference
LSM	Least-squares mean
MALDI-TOF	Matrix-assisted laser desorption ionisation-time of flight
MCR	Metabolic clearance (dose/AUC)
MD	Mean difference; the mean of the differences (Y - X)
MR	Mean ratio; the mean of the quotient (X/Y)
MRM	Multiple reaction monitoring
MRP2	Multi-drug resistance protein 2
MS	Mass spectrometry
NAT	N-acetyltransferase
NBD-Cl	7-chloro-4-nitrobenz-2-oxa-1,3-diaxole
ncRNA	Non-coding RNAs
NSW	New South Wales
OATP	Organic anion-transporting polypeptide
OCT	Organic cation transporter
OH-OME	5-hydroxyomeprazole
OME	Omeprazole
PAR	Paraxanthine
PCR	Polymerase chain reaction
PEITC	Phenethyl isothiocyanate
P-gp	P-glycoprotein
PIS	Participant Information Sheet
PM	Poor metaboliser
PXR	Pregnane X receptor
QC	Quality control
QLD	Queensland
RE	Relative error; (X/Y)*100

RNA	Ribonucleic acid
RSD	Relative standard deviation; (SD/mean)*100
SD	Standard deviation
SEM	Standard error of the mean
SLHD	Sydney Local Health District
SNP	Single nucleotide polymorphism
SPE	Solid-phase extraction
SSA	Site-specific approval
SUL	Sulforaphane
SULT	Sulfotransferase
$t_{1/2}$	Elimination half-life
TFA	Trifluoroacetic acid
TGA	Therapeutic Goods Administration
UDP	Uridine-diphosphate
UDPGA	Uridine-diphosphoglucuronic acid
UGT	Uridine-diphosphate glucuronosyltransferase
UHPLC	Ultra-high performance liquid chromatography
UM	Ultra-rapid metaboliser
US	United States of America
VIC	Victoria
WHO	World Health Organisation
XO	Xanthine oxidase

Abstract

Drug metabolism is a major determinant of variability in response to medicines. Factors affecting the activity of drug-metabolising enzymes can be classified as intrinsic, such as genetics, or extrinsic, such as diet. The effects of genetics and diet on cytochrome P450 (CYP) activity can be different between people of different geographic ancestry, and very few studies exist that explore these interactions simultaneously. Overall, this thesis examines how cruciferous vegetable-enriched diets, genetics and CYP activity interact to explain variability in drug response between Europeans and South Asians.

The aims of this thesis were to:

1. Review the relevant literature and form a theoretical framework supporting the hypotheses tested in this thesis;
2. Conduct a systematic review with meta-analyses of trials investigating the impact of cruciferous vegetable dietary intervention trials on drug metabolism;
3. Design, optimise and validate a UHPLC-MS/MS CYP-phenotyping cocktail assay in human plasma for CYP1A2, CYP2C19, CYP2C9, CYP2D6 and CYP3A4;
4. Design, optimise and validate a UHPLC-MS/MS sulforaphane assay in human plasma; and
5. On the back of hypotheses generated from the systematic review, design and conduct a controlled, 3-period crossover trial that aimed to:
    a. Investigate the short-term and medium-term effects of a broccoli-enriched diet on CYP1A2, CYP2C19, CYP2C9, CYP2D6 and CYP3A4 activities;
    b. Establish if the above effects vary between those of European and South Asian ancestry;

c. Confirm that CYP1A2 is induced in Europeans on a broccoli-enriched diet, with no or a reduced change in South Asians; and
d. Explore the contribution of diet, genetics and geographic ancestry on variability in CYP1A2, CYP2C19, CYP2C9, CYP2D6 and CYP3A4 activities.

In Chapter 2, *n* = 23 studies were identified that met the systematic inclusion criteria. Drug-metabolising enzymes represented in the literature included CYPs, UGTs, GSTs, NAT and xanthine oxidase. The type of cruciferous vegetable interventions, choice of phenotyping metrics and choice of probe drugs were highly heterogeneous, except for CYP1A2 and GST-α. Meta-analysis was possible for CYP1A2 and GST-α due to the number and nature of the studies that investigated these enzymes. Their activities were significantly increased by 15-40% after a cruciferous vegetable interaction. Dose-response relationships were established between cruciferous vegetable consumption and CYP1A2, GST-α and UGT1A1 activities. Critical analysis revealed that only 48% of the studies included a panel of high-quality characteristics in their design. Results from this review were used to formulate testable hypotheses in Chapter 5.

Chapter 3 outlines the design, optimisation and validation of a CYP-phenotyping cocktail assay that was needed to analyse samples from the crossover trial reported in Chapter 5. Samples were analysed using an Agilent 1290 infinity LC system in tandem with 6460A triple quadrupole mass spectrometers. Separation of the analytes was achieved with an Agilent Zorbax Eclipse XDB-C18 (4.6 x 50 mm, 1.8 µm) column fitted with a Zorbax Eclipse XDB-C18 (4.6 x 5.0 mm, 1.8 µm) guard column. Standard curves for all analytes were linear over wide plasma concentration ranges (0.78-3000 ng/mL) and the methods met guideline-recommended requirements for specificity, sensitivity (analyte LLOQs 0.78-23.4 ng/mL),

accuracy (intra-day RE % nominal concentration 90.7-110.2; inter-day RE % 87.0-110.5) and precision (intra-day analyte RSD % 0.46-11.4%; inter-day RSD % 1.36-11.2). Recovery and matrix effects were also investigated and concluded to be non-interfering. This improved CYP cocktail assay has been used successfully used to phenotype *n* = 21 participants of European and South Asian ancestry as reported in Chapter 5.

Similarly, a UHPLC-MS/MS assay was designed, optimised and validated to measure sulforaphane (SUL) in human plasma. Retention times for SUL and the internal standard were 3.42 min and 4.42 min, respectively. The lower-limit of quantification (LLOQ) for SUL was 0.78 ng/mL (7.8 pg on-column). All QCs had intra-day accuracy (RE) and precision (RSD) ranging between 86.4-106.7% and 2.61-10.3% respectively. Inter-day accuracy and precision ranged between 91.3-97.0% and 3.99-7.11% respectively. Recovery was low and matrix effects high, but their consistency meant that quantification of SUL was not impeded. The assay was successfully used to analyse SUL in 21 participants (> 150 plasma samples) in the above-mentioned clinical trial.

Chapter 5 reports and discusses the results from a controlled, dietary intervention crossover trial in Europeans and South Asians. A 500 g broccoli meal was consumed immediately before CYP phenotyping, and 500 g twice daily was consumed for a further six days before final CYP phenotyping. Diets high in CYP1A2 inducer foods were more prevalent in Europeans, whereas a predominantly curry diet was more common in the South Asian cohort. CYP1A2 activity was approximately 20% higher in Europeans after following a broccoli-enriched diet for six days, but this was not seen in South Asians. CYP2C19 activity was significantly related to genotype, and there was evidence of inhibition on Study Day 2 (D2) followed by a rebound in activity by the end of the study at Study Day 9 (D9). The

*CYP2C19*2* null allele showed a dose-response relationship with CYP2C19 activity. Within *CYP2C19*1C* and *CYP2C19*17* genotype groups, enzyme activity was higher in Europeans than South Asians. CYP2C9 activity increased on D2 immediately after a broccoli meal and decreased back to baseline by the end of the study on D9. Interestingly, South Asians had nearly 2-fold higher CYP2C9 activity throughout the study, even within variant genotype groups. CYP2D6 activity was variable, and SNP genotype alone was not a significant predictor of activity in the three mixed-effects models used in its analysis. Of note, enzyme activity was 2- to 3-fold higher in Europeans than South Asians within *CYP2D6*4* and *CYP2D6*10* genotypes, as well as for those that had *n* = 1 copy of the *CYP2D6* gene. No significant interactions or changes were seen in CYP3A4; however, variability was large, and *post hoc* sample size and power calculations suggest that more participants are needed when investigating this enzyme.

Chapter 5 also discusses ancestry group differences in the exposure of the cruciferous vegetable constituent SUL, which is an inducer of drug-metabolising enzymes. A predominantly curry diet, *GSTM1* and *GSTP1* genotypes were significant predictors of SUL exposure. There was no evidence of a difference in SUL exposure between the two ancestry groups when statistically controlled for genotype and diet. The 4-h SUL plasma concentration-time point had a near 1:1 correlation with the $AUC_{0\text{-}8\ h}$, with the former being recommended as an ITC exposure metric in future studies.

Overall, this thesis presents novel findings regarding how cruciferous vegetables, dietary practices, genetics and geographic ancestry interact to explain variability in drug metabolism. Future studies in this area are encouraged to simultaneously measure a variety of both intrinsic and extrinsic factors in ancestry group difference studies.

Recommendations are made for future research in this area, with specific guidance on study design and selection of high-quality characteristics.

Table of Contents

1 Introduction and background

The aim of Chapter 1 is to review and summarise the relevant literature required to construct a rationale supporting the objectives of this thesis (section 1.9). Many topics are covered in this chapter, some more relevant to the thesis objectives than others. However, all aspects of clinical pharmacology reviewed and summarised herein have been chosen because of their importance to the overall goal of this thesis: to better understand how geographic ancestry, genetics and diet contribute to variability in drug metabolism.

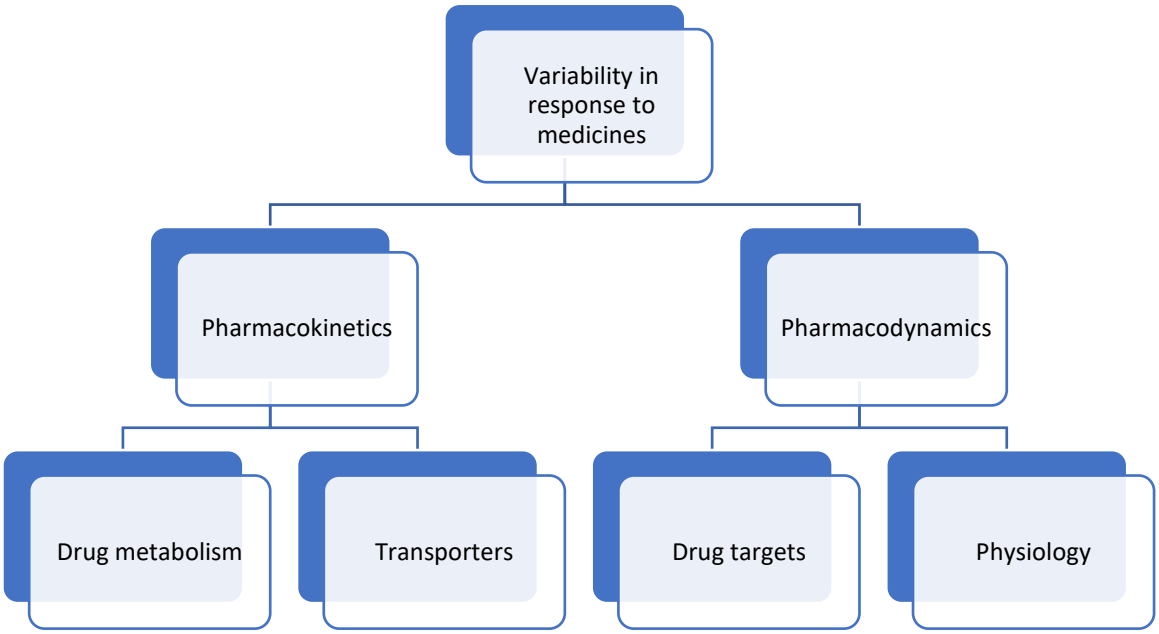
1.1 Variability in response to medicines

Medicines display significant variability in a given group of people. In fact, one of the reasons that society needs healthcare professionals and biomedical researchers is because of this variability: if everyone had the same response to a particular dose of a particular drug, then pharmacotherapy would be a simpler affair. However, variability is rampant in pharmacology—as it is in the other biomedical sciences—and understanding the nature and causes of this variability is important, as this knowledge can be translated into improved patient outcomes through the safe and efficacious use of medicines (Sorich & McKinnon, 2012).

Patient variability in response to medicines can be thought of as being made up of two over-arching subtypes of variability, namely: variability in pharmacokinetic processes, i.e. intra-/inter-subject differences in the absorption, distribution, metabolism and elimination of drugs; and variability in pharmacodynamic processes, i.e. intra-/inter-subject differences in drug targets and (patho)physiological processes. These subtypes of variability can be further subdivided again, for example, variability in drug absorption can be explained in terms of intra-/inter-person differences in gastric acidity, gastric emptying rate, intestinal transit

time, and so on (Figure 1.1). With unlimited time and resources, all of these avenues could be comprehensively explored and commented on, which is the ultimate prerequisite for functioning personalised medicine. For the purposes of this thesis, however, the scope will be limited to better understanding how various intrinsic and extrinsic factors affect variability in drug metabolism, as a subset of variability in pharmacokinetics.

Figure 1.1: Variability in response to medicines. This schematic lists some of the contributors to variability and their hierarchical sub-categories.



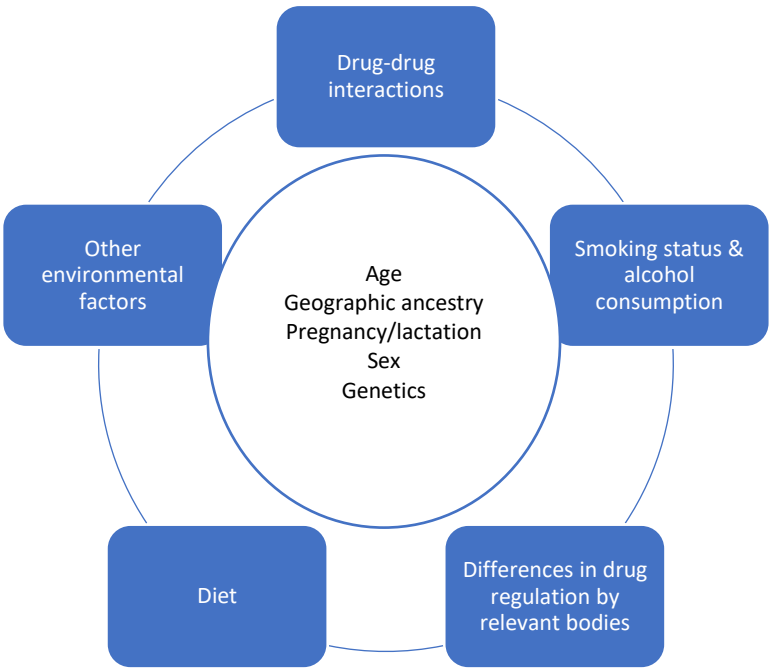
1.1.1 Intrinsic and extrinsic factors

The types of factors that affect drug metabolism in humans can be categorised as either intrinsic or extrinsic (Huang & Temple, 2008) (Figure 1.2). Intrinsic factors encompass those that are either hard to or cannot be changed, for example, age, geographic ancestry (see section 1.2), genetics and sex, whereas extrinsic factors are those that are environmental and usually modifiable, such as smoking, alcohol consumption, drug-drug/herb-drug/food-drug interactions and diet.

Thus far, in the context of understanding variability in drug metabolism, the intrinsic factor genetics has received the most attention (Bjornsson *et al.*, 2003). Specifically, variability arising from single nucleotide polymorphisms (SNPs) in genes that encode drug-metabolising enzymes has been the focus (Yang, 2015). This approach follows on from the central dogma, i.e. genes encode proteins; enzymes are proteins, therefore understanding the genes that code for drug-metabolising enzymes should explain the observed variability in their activity. However, inter-individual differences in SNPs do not explain all of the variability in drug metabolism, and far less is known about the contributions of diet and the environment.

The effect of genetics on drug-metabolising enzyme activity is discussed below in the various sub-sections of section 1.3.1. Diet, one of the most important and poorly-understood extrinsic factors, is discussed below in section 1.2.

Figure 1.2: Intrinsic and extrinsic factors that influence variability in response to medicines. Adapted from Huang and Temple (2008).



1.2 Geographic ancestry

Intrinsic and extrinsic factors that affect drug metabolism tend to 'clump' together in packages that are often inherited and shared by sociocultural groups with a common geography. This idea often appears in the pharmacological literature under the guise of 'race' or 'ethnicity'. The use of these words in human biological studies and their underlying meaning and implications have been recently discussed by Yudell *et al.* (2016) in the prestigious journal, *Science*. Yudell and colleagues describe the use of race and ethnicity as biological concepts as being "…problematic at best and harmful at worst". Their reasoning is sound: these concepts are actually social constructs as opposed to scientifically meaningful categories used to study population genetics, and cause great confusion when used in biological research. This "non-scientific misuse" of race and ethnicity makes it difficult, and sometimes impossible, to compare methodologies and data across population genetics studies. The list of issues that the use of these terms creates is growing: Yudell *et al*. mention difficulties with the interpretation of racial and ethnic effects (Kaufman & Cooper, 2001), problems with making distinctions between self-identified/assigned and assumed racial categories (Rebbeck & Sankar, 2005), and "the haphazard use and reporting of racial/ethnic variables in genetic research" (Hunt & Megyesi, 2008). The suggested solution by Yudell *et al*. is the term "ancestry", specifically "geographic ancestry". Ancestry is a term with scientific intent and purpose: it defines how we relate to others through genealogical history as a "process-based" concept, whereas race is a "pattern-based" concept that leads people awry by encouraging misinterpretation of themes and data in contemporary studies.

Therefore, throughout this thesis, when discussing collections of intrinsic and extrinsic factors between groups of people, who are linked through genealogical history, the term ‘geographic ancestry’ (‘ancestry’ for short) will be used instead of race or ethnicity.

Importantly, genetics and diet vary extensively both within and between those of different ancestries, and few studies simultaneously investigate their interaction and effects on drug metabolism. The following sections introduce drug-metabolising enzymes (section 1.3.1), the various intrinsic and extrinsic factors that affect them (sections 1.3 and 1.7), and how these factors differ between ancestry groups (section 1.8). In the literature, some ancestry groups are better represented than others. Section 1.8 describes this in detail, highlighting that knowledge gaps exists for South Asian individuals relative to other groups, such as Europeans and East Asians.

1.3 Variability in pharmacokinetics

1.3.1 Drug-metabolising enzymes

Drug-metabolising enzymes are important because of their effect on the clearance of medicines, which in turn is a significant contributor to variability in response to medicines (Zanger *et al.*, 2014; Zanger & Schwab, 2013). In fact, the first 3 sub-families of the cytochrome P450 (CYP) superfamily of drug-metabolising enzymes have been estimated to be involved in approximately 80% of oxidative drug metabolism, and almost 50% of the overall elimination of commonly used drugs (Wilkinson, 2005). For this reason, this thesis will focus on the CYPs and how diet, genetics and ancestry affect their activity. Other phase II conjugating enzymes are discussed too, as relevant to the thesis objectives set out in section 1.9.

1.3.1.1 Cytochromes P450

It has been estimated that over 90% of drugs are metabolised to some extent by five of the main CYP drug metabolising enzymes: CYP1A2, CYP2C19, CYP2C9, CYP2D6 and CYP3A4 (Rodrigues, 1999). Ulrich M. Zanger and colleagues have dedicated decades of work to better understanding how the CYPs function, and what causes variability in their activity both within and between individuals. Variability in CYP activity is well known to be exorbitant; enzyme activity can vary 100-fold and more across the various isoenzymes.

The following five sections of this thesis are dedicated to discussing these five CYP enzymes, their genetic variability and function, with reference to the two recent, comprehensive reviews published by Zanger *et al*. (Zanger *et al.*, 2014; Zanger & Schwab, 2013).

1.3.1.1.1 CYP1A2

The *CYP1A2* gene is located on chromosome 15q24.1 and is mostly abundant in the liver (Kawakami *et al.*, 2011; Nelson *et al.*, 2004; Ohtsuki *et al.*, 2012). The gene contains multiple aryl hydrocarbon receptor (AhR) response elements, therefore environmental sources of AhR ligands are strong inducers of CYP1A2 activity (Jorge-Nebert *et al.*, 2010; Nebert *et al.*, 2004; Ueda *et al.*, 2006). Clinically used substrates, inducers and inhibitors of CYP1A2 are listed in Table 1.1.

Table 1.1: Substrates, inducers and inhibitors of CYP1A2. Adapted from the Australian Medicines Handbook (AMH, 2018), Zanger *et al.* (2014); Zanger and Schwab (2013).

CYP1A2 substrates	CYP1A2 inducers	CYP1A2 inhibitors
Agomelatine	Omeprazole	Cimetidine
Amitriptyline	Phenobarbital	Ciprofloxacin (strong)
Asenapine	Phenytoin (moderate)	Combined oral contraceptives (moderate)
Axitinib	Rifampicin (moderate)	Fluvoxamine (strong)
Bendamustine	Ritonavir (moderate)	Vemurafenib
Clozapine	Tobacco smoking	Verapamil
Duloxetine		
Erlotinib		
Fluvoxamine		
Imipramine		
Lidocaine		
Olanzapine		
Ondansetron		
Paracetamol		
Pirfenidone		
Pomalidomide		
Propranolol		
Rasagiline		
Ropinirole		
Ropivacaine		
Theophylline		
Warfarin (R-isomer)		
Zolmitriptan		

Two genetic variants have been flagged as being of clinical significance: rs2069514 (*CYP1A2*1C*) and rs762551 (*CYP1A2*1F*). *CYP1A2*1C* is a −3860G>A SNP in the promotor region of the gene leading to decreased inducibility, and *CYP1A2*1F* is a −163C>A SNP in the intron 1 region of the gene leading to increased inducibility (Pharmacogene Variation Consortium at www.pharmvar.org). However, variations in these genotypes explain a low amount of the variability seen in CYP1A2 activity within and between individuals (Klein *et al.*, 2010; Perera *et al.*, 2012a), suggesting that environmental factors such as diet play a larger role in determining its activity than genetics. In fact, this is supported and confirmed by the large effect that diet has on CYP1A2 activity (Chapter 2).

1.3.1.1.2 CYP2C19 and CYP2C9

The *CYP2C19* and *CYP2C9* genes are located on chromosome 10q23.3. CYP2C9 is expressed in the liver approximately 10-fold higher than CYP2C19, which has been attributed to difficulties with the CYP2C19 promotor region interacting with hepatocyte nuclear factor 4α (HNF4α) (Coller *et al.*, 2002; Koukouritaki *et al.*, 2004; Ohtsuki *et al.*, 2012; Rettie & Jones, 2005). Clinically used substrates, inducers and inhibitors of CYP2C19 and CYP2C9 are listed in Table 1.2 and Table 1.3, respectively.

Three *CYP2C19* genetic variants are of particular clinical importance: rs4244285 (*CYP2C19*2*), rs4986893 (*CYP2C19*3*) and rs12248560 (*CYP2C19*17*). *CYP2C19**2 is a 19154G>A splicing defect leading to a null allele and significantly reduced activity; *CYP2C19*3* is a 17948G>A SNP also causing a null allele; and *CYP2C19*17* is a −806C>T SNP in the promotor region of the gene causing increased expression and activity (Pharmacogene Variation Consortium at www.pharmvar.org). Because of these relatively prevalent null alleles, genotype-phenotype correlations are observed for CYP2C19.

Importantly, the interaction between diet, genetics and geographic ancestry and their combined effects on CYP2C19 activity have not been assessed.

There are two *CYP2C9* genetic variants that are of significance to variability in response to medicines: rs1799853 (*CYP2C9*2*) and rs1057910 (*CYP2C9*3*). *CYP2C9**2 is a 3608C>T SNP leading to decreased activity and *CYP2C9*3* is a 42614A>C SNP also causing decreased activity (Pharmacogene Variation Consortium at www.pharmvar.org). CYP2C9 is expressed, to a lesser extent, in extra-hepatic tissues such as the intestines and cardiovascular system, and therefore environmental agents that reach these tissues in high concentrations could affect activity (DeLozier *et al.*, 2007). As with CYP2C19, the effects of diet, genetics and geographic ancestry have not been simultaneously investigated for CYP2C9.

Table 1.2: Substrates, inducers and inhibitors of CYP2C19. Adapted from the Australian Medicines Handbook (AMH, 2018) and Zanger *et al.* (2014); Zanger and Schwab (2013).

CYP2C19 substrates	CYP2C19 inducers	CYP2C19 inhibitors
Amitriptyline	Efavirenz (moderate)	Cimetidine
Axitinib	Enzalutamide (moderate)	Clarithromycin
Bortezomib	Rifampicin (strong)	Efavirenz
Brivaracetam	Ritonavir (strong)	Esomeprazole
Citalopram	St John's wort	Etravirine
Clopidogrel		Fluconazole (strong)
Cyclophosphamide		Fluoxetine (strong)
Diazepam		Fluvoxamine (strong)
Escitalopram		Ketoconazole
Esomeprazole		Omeprazole
Etravirine		Oxcarbazepine
Imipramine		Topiramate
Lansoprazole		Voriconazole (moderate)
Omeprazole		
Pantoprazole		
Phenobarbital		
Phenytoin		
Propranolol		
Voriconazole		
Warfarin (R-isomer)		
Zolmitriptan		

Table 1.3: Substrates, inducers and inhibitors of CYP2C9. Adapted from the Australian Medicines Handbook (AMH, 2018) and Zanger *et al.* (2014); Zanger and Schwab (2013).

CYP2C9 substrates	CYP2C9 inducers	CYP2C9 inhibitors
Amitriptyline	Aprepitant (moderate)	Amiodarone (moderate)
Bosentan	Bosentan	Benzbromarone (moderate)
Celecoxib	Carbamazepine (moderate)	Efavirenz
Cyclophosphamide	Dabrafenib	Etravirine
Etravirine	Enzalutamide (moderate)	Fluconazole (moderate)
Fluoxetine	Rifampicin (moderate)	Fluoxetine
Fluvastatin	Ritonavir (moderate)	Fluvoxamine
Glibenclamide	St John's wort	Miconazole
Gliclazide		Ritonavir
Glimepiride		Voriconazole
Glipizide		
Ibuprofen		
Phenytoin		
Rosiglitazone		
Rosuvastatin		
Ruxolitinib		
Tamoxifen		
Voriconazole		
Warfarin (S-isomer)		

1.3.1.1.3 CYP2D6

The *CYP2D6* gene is located on chromosome 22q13.1. As with some of the other CYP enzymes, CYP2D6 expression is, to some extent, regulated by HNF4α (Corchero *et al.*, 2001; Hara & Adachi, 2002). Clinically used substrates and inhibitors of CYP2D6 are listed in Table 1.5.

The determinants of CYP2D6 activity are mostly due to SNPs in various splice variants (Zanger *et al.*, 2001). In fact, CYP2D6 is almost exclusively under genetic control, with little evidence of environmental induction (Bock *et al.*, 1994; Glaeser *et al.*, 2005), apart from one isolated study reporting phenotypic increases in activity following rifampicin administration (Caraco *et al.*, 1997) (this is why Table 1.5 lists substrates and inhibitors of CYP2D6 but no inducers). However, this long-held view has been challenged in recent years, with new *in*

vivo evidence demonstrating induction of *CYP2D6* after administration of inducers of other CYPs such as corticosteroids (Farooq *et al.*, 2016). CYP2D6 has the largest list of allelic variants known to affect its activity; the most significant of these are listed in Table 1.4. Frequencies of null alleles between geographic ancestry groups are discussed in section 1.8, however it is worth mentioning here that for CYP2D6, a quad-modal frequency distribution of population activity phenotypes is achieved by genotype. This distribution is made up of: ultra-rapid metabolisers (UMs), who have multiple copy variants of the *CYP2D6* gene; extensive metabolisers (EMs), who are homozygous for the normal allele; intermediate metabolisers (IMs), who are either homozygous or heterozygous for reduced-activity alleles; and poor metabolisers (PMs), who are homozygous for at least one null allele of the *CYP2D6* gene (Raimundo *et al.*, 2004; Zanger & Hofmann, 2008).

Table 1.4: Clinically significant CYP2D6 SNPs. Adapted from Zanger and Schwab (2013).

CYP allele (PharmVar)	Accession no. (rs no.)	SNP	Effect
CYP2D6*3	rs35742686	2549delA	Null allele
CYP2D6*4	rs3892097	1846G>A	Null allele
CYP2D6*5	Recombination	n/a	Null allele
CYP2D6*6	rs5030655	1707delT	Null allele
CYP2D6*10	rs1065852	100C>T	Reduced expression and activity
CYP2D6*17	rs28371706 rs16947	1023C>T 2850C>T	Reduced expression and activity
CYP2D6*41	rs28371725	2988G>A	Reduced expression and activity
***CYP2D6* copy no.**	Recombination	Copy no. variations	Increased expression and activity

Table 1.5: Substrates and inhibitors of CYP2D6. Adapted from the Australian Medicines Handbook (AMH, 2018) and Zanger *et al.* (2014); Zanger and Schwab (2013).

CYP2D6 substrates	CYP2D6 inhibitors
Amitriptyline	Abiraterone
Aripiprazole	Amiodarone
Atomoxetine	Bupropion (strong)
Bortezomib	Celecoxib
Brexpiprazole	Cimetidine
Carvedilol	Cinacalcet (moderate)
Chlorpromazine	Cobicistat
Cinacalcet	Duloxetine (moderate)
Clozapine	Fluoxetine (strong)
Codeine	Methadone
Dapoxetine	Mirabegron (moderate)
Darifenacin	Paroxetine (strong)
Dextromethorphan	Terbinafine (strong)
Donepezil	
Duloxetine	
Flecainide	
Fluoxetine	
Fluvoxamine	
Galantamine	
Gefitinib	
Haloperidol	
Imipramine	
Lidocaine	
Metoclopramide	
Metoprolol	
Nebivolol	
Nortriptyline	
Olanzapine	
Ondansetron	
Oxycodone	
Paroxetine	
Perhexiline	
Propranolol	
Risperidone	
Tamoxifen	
Tolterodine	
Tramadol	
Venlafaxine	
Vortioxetine	

1.3.1.1.4 CYP3A4

The *CYP3A4* gene is located on chromosome 7q22.1. By liver microsomal weight, it alone constitutes 14-24% of the CYPs (Lin *et al.*, 2002; Ohtsuki *et al.*, 2012; Shimada *et al.*, 1994; Wolbold *et al.*, 2003), emphasising its important contribution to oxidative drug metabolism in humans. It also has an important role in intestinally-mediated first-pass metabolism due to its relative abundance in enterocytes (Daly, 2006; Ding & Kaminsky, 2003; Von Richter *et al.*, 2004). Upstream pregnane X receptor (PXR) and constitutive androstane receptor (CAR) binding sites make this gene readily inducible, as seen across a wide variety of endogenous, exogenous and xenobiotic substrates (Jover *et al.*, 2009; Matsumura *et al.*, 2004; Qiu *et al.*, 2010). Clinically used inducers and inhibitors of CYP3A4 are listed in Table 1.6; substrate lists are vast, and are therefore not displayed.

Two *CYP3A4* genetic variants of significance have been highlighted: rs2740574 (*CYP3A4*1B*) and rs35599367 (*CYP3A4*22*). *CYP3A4*1B* is a −392A>G polymorphism in the upstream promotor region of the gene that potentially leads to decreased activity, and *CYP3A4*22* is a 15389 C>T SNP in intron 6 causing decreased expression and activity (Pharmacogene Variation Consortium at www.pharmvar.org). While the interaction between CYP3A4 substrates such as statins have been investigated for effects on activity by genotype, the effect of diet by geographic ancestry is less-represented in the literature.

Overall, these five main CYP enzymes play a major role in the metabolism of commonly used medicines in humans. Importantly, their activity is differentially affected by SNPs in select variants and various environmental exposures. As a collective, they represent an ideal panel of important enzymes to study the effects of diet, genetics and geographic ancestry on drug metabolism in humans.

Table 1.6: Substrates, inducers and inhibitors of CYP3A4. Adapted from the Australian Medicines Handbook (AMH, 2018) and Zanger *et al.* (2014); Zanger and Schwab (2013).

CYP3A4 inducers	CYP3A4 inhibitors
Aprepitant	Aprepitant (moderate)
Bosentan (moderate)	Atazanavir (moderate)
Carbamazepine (strong)	Ciclosporin (moderate)
Corticosteroids	Cimetidine (moderate)
Dabrafenib	Clarithromycin (strong)
Efavirenz (moderate)	Cobicistat (strong)
Enzalutamide (strong)	Crizotinib (moderate)
Etravirine (moderate)	Darunavir
Lumacaftor (strong)	Diltiazem (strong)
Modafinil (moderate)	Erythromycin (moderate)
Nevirapine	Fluconazole (moderate)
Phenobarbital	Fluvoxamine (moderate)
Phenytoin (strong)	Fosamprenavir (moderate)
Rifabutin	Grapefruit juice (moderate)
Rifampicin (strong)	Idelalisib (strong)
Ritonavir	Imatinib (moderate)
St John's wort (strong)	Indinavir (strong)
Tipranavir	Isavuconazole (moderate)
Vemurafenib	Itraconazole (strong)
	Ketoconazole (strong)
	Lopinavir
	Miconazole
	Netupitant
	Palbociclib
	Posaconazole (strong)
	Ritonavir (strong)
	Saquinavir (strong)
	Ticagrelor
	Tipranavir
	Verapamil (moderate)
	Voriconazole (strong)

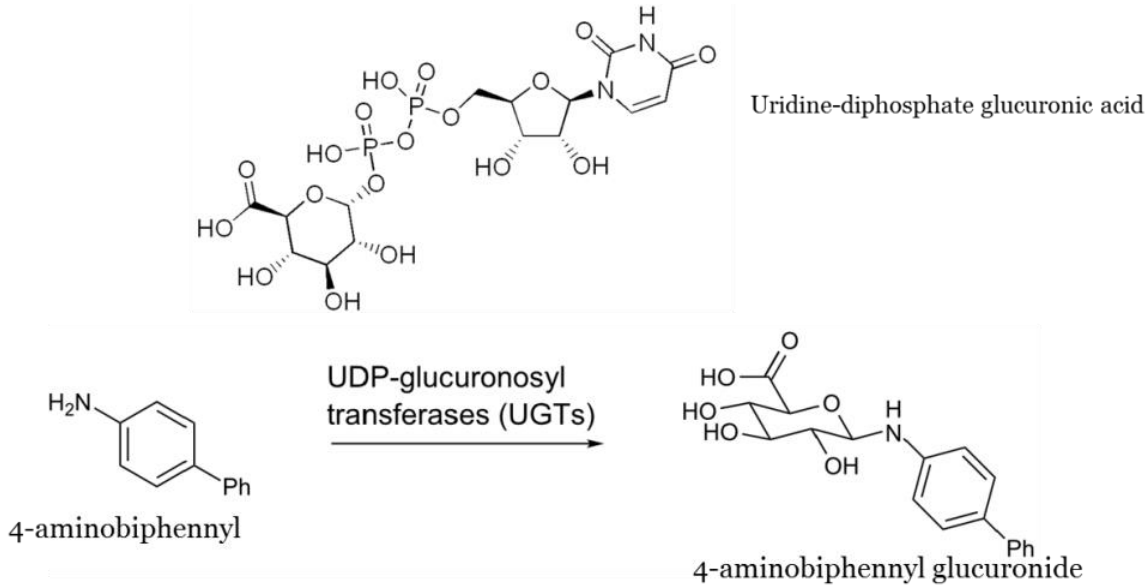
1.3.1.2 Uridine 5'-diphospho-glucuronosyltransferases

As discussed above, much of the drug metabolism literature focusses on the CYP superfamily of drug metabolising enzymes due to familiarity with their molecular genetic mechanisms and well-characterised substrate profiles (Daly, 1995). However, the depth of knowledge encompassing other enzyme superfamilies, such as the uridine-diphosphate glucuronosyltransferases (UGTs), has grown as a result of increased overall knowledge of drug metabolism and an evolving appreciation of how these superfamilies' substrate specificities overlap and interact (Ginsberg *et al.*, 2010; Yang *et al.*, 2017).

UGTs are responsible for the glucuronidation and elimination of a wide range of endogenous substances, xenobiotics, environmental pollutants, carcinogens and their phase I metabolites (Miners *et al.*, 2002). UGTs are type I transmembrane proteins found in the smooth endoplasmic reticulum within cells and are expressed in high concentrations in the liver, but also expressed in extrahepatic tissues such as the lungs, kidney and gastrointestinal tract (GIT) (Cappiello *et al.*, 1991). The primary function of UGTs is to catalyse the transfer of a sugar moiety from the cofactor uridine-diphosphoglucuronic acid (UDPGA) to hydroxyl, carboxylic, amino or sulphur constituents on the substrate (Ginsberg *et al.*, 2010).

The outcome of this chemical biotransformation is an increase in the substrate's molecular weight and hydrophilicity, facilitating excretion in bile and/or urine via the liver and/or kidney.

Figure 1.3: An example of glucuronidation of 4-aminobiphenyl, adapted from Al-Zoughool and Talaska (2006).



The UGT superfamily is divided into three broad groups based on homology sequencing: *UGT1A*, found on chromosome 2; and *UGT2A* and *2B*, found on chromosome 4 (Nagar & Remmel, 2006). *UGT1* members have exons 2-5 in common and variations in exon 1 determine the enzyme's subtype, whereas *UGT2* members have six exons—all of which are variable—that have no overlap with *UGT1* exons (Maruo *et al.*, 2005). At least 13 isoforms are encoded by the *UGT1* locus, with nine of these being functional enzymes: UGT1A1, 1A3, 1A4, 1A5, 1A6, 1A7, 1A8, 1A9 and 1A10 (Miners *et al.*, 2002). Functional UGT2 subtypes include UGT2A1, 2B4, 2B7, 2B10, 2B11, 2B15, 2B17 and 2B28 (Levesque *et al.*, 2001). Despite these subfamilies having differing amino acid sequences there is substantial overlap in their substrate specificity. While this redundancy is beneficial for the organism as it provides alternate glucuronidation pathways in the presence of inefficient variants or absent enzymes, the lack of substrate specificity between UGT subtypes creates difficulty in designing studies that assess single glucuronidation pathways. Although this overlap in specificity is prominent, the subfamilies do differ in their general affinity for endogenous sex

steroids. UGT1s appear to have greater activity against oestrogens and their catechol metabolites (Lepine *et al.*, 2004), while UGT2s tend to better glucuronidate androgens (Belanger *et al.*, 2003). A summary of known substrates by UGT subtype is displayed in Table 1.7.

Table 1.7: Known substrates of UGT isoforms, adapted from Ginsberg et al. (2010) and Levesque et al. (2001). Note: References for substrates mentioned outside of these papers are listed in the far-right column.

UGT Isoform	Substrates	References
UGT1A1	Bilirubin, estrogens (β-estradiol, hydroxyestradiols, hydroxyestrones), buprenorphine, flavonoids, anthraquinone, 4-nitrophenol, 2-amino-5-nitro-4-fluoromethylphenol, raloxifene[1]	[1]Trontelj *et al.* (2009)
UGT1A3	Estrone, hydroxyestrone, hydroxyestradiol, amines (cyproheptadine, losartan), anthraquinones, femoprofen, ibuprofen, umbelleferone, flavonoids	
UGT1A4	Amines (clozapine, aminobiphenyl, naphthylamine, benzidine, aminofluorene, imipramine), 4-hydroxytamoxifen, 5α-pregnene-3α,20β-diol	
UGT1A5	Unknown	
UGT1A6	Phenols (eugenol, β-naphthol, 4-nitrophenol), paracetamol, serotonin, amines, 2-amino-5-nitro-4-fluoromethylphenol, salicylic acids, deferiprone[2]	[2]Benoit-Biancamano *et al.* (2009)
UGT1A7	Phenols (α-naphthol), acetaminophen, 4-methlumbelliferone, octyl gallate, propyl gallate, benzo(a)pyrene metabolites	
UGT1A8	Estrogens (hydroxyestrone, hydroxyestradiol, 17α-ethinylestradiol), naltrexone, phenols, flavonoids, anthraquinones, phenolphthalein, mycophenolic acid, 4-aminobiphenyl	
UGT1A9	Estrogens, retinoic acid, thyroid hormones, paracetamol, SN-38 (active metabolite of irinotecan), phenols, 4-methylumbelliferone, propofol, flavonoids, anthraquinones, mycophenolic acid	
UGT1A10	β-estradiol, mycophenolic acid, phenols, flavonoids	
UGT2A1	Phenols (vanillin, 3-hydroxybiphenol, 4-hydroxybiphenol), scopoletin, aliphatic compounds (citronellol), steroids	
UGT2B4	Eugenol, catechol estrogens, hyodeoxycholic acid, 1-napthol, 4-methylumbelliferone, 5β-pregnane-3α,20β-one	
UGT2B7	Estrogens (4-hydroxyestrone, 4-hydroxyestradiol), androsterone, morphine, dihydromorphine, codeine, oxycodone, naloxone, naltrexone, valproic acid, serotonin, hyodeoxycholic acid, losartan, flurbiprofen[3], gemfibrozil[4], haloperidol[5], 6α-hydroxyprogesterone[6], 21-hydroxyprogesterone[6], lorazepam[7], *trans*-4-hydroxytamoxifen[8], endoxifen[8],	[3]Wang *et al.* (2011), [4]Mano *et al.* (2007), [5]Kato *et al.* (2012), [6]Bowalgaha *et al.* (2007), [7]Chung *et al.* (2008), [8]Blevins-Primeau *et al.* (2009)
UGT2B10	Unknown	
UGT2B11	Unknown	
UGT2B15	Dienestrol, phenols, flavonoids (naringenin, apigenin), anthraquinones, estrogens, (2-hydroxyestrone, 4-hydroxyestrone), bisphenol A, testosterone, dihydroxytestosterone, oxazepam[9]	[9]He *et al.* (2009)
UGT2B17	Androgens (androgen, testosterone, dihydrotestosterone, androstenediols)	
UGT2B28	Eugenol, etiocholanolone, androstane-3α,20β-diol, 4-methylumbelliferone, 1-napthol, estradiol, androsterone, hyodeoxycholic acid, lithocholic acid, testosterone	

1.3.1.3 Glutathione *S*-transferases

The glutathione *S*-transferases (GSTs) are another type of important phase II drug-metabolising enzyme, recently reviewed by Allocati *et al.* (2018). The GSTs catalyse the attachment of glutathione (GSH) to an electrophilic substrate, with the resulting conjugate being more water soluble than before, facilitating excretion in the urine (Pool-Zobel *et al.*, 2005). GSTs are localised within the cell into three main sub-categories: cytosolic, mitochondrial and microsomal GSTs (Hayes *et al.*, 2005; Oakley, 2011; Sheehan *et al.*, 2001). Important cytosolic GSTs in humans include the alpha, mu, pi and theta subtypes. The co-substrate binding sites in these enzymes, termed the 'H-site', displays marked variability, leading to a vast array of substrate binding affinities. GSTs have numerous roles in endogenous cellular biology, including, but not limited to protecting the cell against oxidative stress, and biotransformation of leukotrienes and prostaglandins. However, this thesis is concerned with their role in the detoxification of drugs and their metabolites, and factors that contribute to variability in their activity.

The GSTs are polymorphic like most of the other drug-metabolising enzymes presented in this chapter. Gene deletion can create a null allele for *GSTM1* and *GSTT1*, and the *GSTP1* rs1695 A>G SNP is associated with reduced activity. GSTM1, GSTP1 and GSTT1 have an important role to play in the metabolism of numerous antineoplastic drugs, paracetamol and the isothiocyanates (section 1.7.1) (Whirl-Carrillo *et al.*, 2012). In fact, isothiocyanate exposure (and subsequent changes in CYP1A2 activity) are modulated by the presence of the *GSTM1* and *GSTT1* null alleles (Peterson *et al.*, 2009) (Chapter 2). Importantly, the effects of genetics, diet and geographic ancestry on GSTs have not been simultaneously assessed in the context of variability in response to medicines.

1.4 Transporters

While outside the specific scope of this thesis, which focuses on drug metabolism, it is important to acknowledge the impact drug-transporting proteins have on variability in pharmacokinetics. As with drug-metabolising enzymes, polymorphisms in the genes that encode these transporters, and extrinsic factors such as drug interactions, can lead to a variable response, and hence differences in the systemic exposure of drugs between individuals. König *et al.* (2013) and Koo *et al.* (2015) provide excellent and comprehensive coverage of this topic. This section provides a brief overview of transporters, their genetic variability and effects on drug disposition.

Transporters either efflux substrates back into the lumen on the apical side of the cell or uptake substrates into the cell through the basolateral membrane (König *et al.*, 2013). Two well-studied examples of efflux transporters are P-glycoprotein (P-gp; encoded by the *ABCB1* gene) and multi-drug resistance protein 2 (MRP2; encoded by the *ABCC2* gene); well-known uptake transporters include organic anion-transporting polypeptide (OATP)1B1 (encoded by the *SLCO1B1* gene) and organic cation transporter (OCT)1 (encoded by the *SLC22A1* gene).

Transporter genes are regulated by mechanisms similar to drug-metabolising enzymes, for example, induction can occur through interactions between the gene and the PXR, CAR and the vitamin D receptor (Tirona, 2011). Several studies have shown transporter induction following exposure to rifampicin and St John's wort (Dürr *et al.*, 2000; Greiner *et al.*, 1999). Polymorphisms in transporter genes have been shown to contribute to variability in drug exposure: in one study, the *SLC22A2* 808G>T SNP significantly affected metformin renal clearance in the presence of cimetidine (Wang *et al.*, 2008). Another well-studied

polymorphism, the *SLCO1B1* 521T>C SNP (*OATP1B1*5*), has been shown to greatly reduce the activity of the OATP1B1 transporter. Assessing the relative frequencies of transporter SNPs in different geographic ancestries is of interest, as is the effect of diet on drug transporters, but both topics lie outside the scope of this thesis.

1.5 Epigenetics

Epigenetics encompasses heritable gene expression patterns that cannot be explained in terms of the DNA sequence itself; it can be thought of as the processes that occur 'on-top' of the DNA ('epi', meaning 'upon', in Greek), rather than what is coded in DNA itself. Such epigenetic mechanisms include DNA methylation, post-transcriptional modification of histones and gene expression changes by non-coding RNAs (ncRNAs) (Ivanov *et al.*, 2012). The ways in which epigenetic mechanisms affect drug-metabolising enzymes and transporters are largely unknown, with this field still being in its infancy. This topic has been comprehensively reviewed by Zanger and Schwab (2013), Zanger *et al.* (2014), Kacevska *et al.* (2011) and Ivanov *et al.* (2012). Epigenetic mechanisms have an inhibitory effect on the expression of drug-metabolising enzyme genes: for a comprehensive list, the reader is directed to Table 1 in Kacevska *et al.* (2011)'s review in *Clinical Pharmacology and Therapeutics*.

This territory remains largely uncharted, with epigenetic targets and mechanisms being referred to as "genetic dark matter" (Stefanska & MacEwan, 2015; Zanger *et al.*, 2014). Further, extremely few studies have investigated how diet, genetics and geographic ancestry interact with epigenetics to cause variability in drug response. These themes lie outside the scope of this thesis, but as epigenetic methodologies evolve and improve, these avenues should be explored for the major drug-metabolising enzymes and transporters.

1.6 Measuring variability in drug-metabolising enzyme activity

Appropriate *in vivo* bioanalytical methods are required before one can study the effects of intrinsic and extrinsic factors on drug metabolism in humans. In particular, estimation of CYP activity in humans requires *in vivo* measurement of selective substrates specific for the CYP enzyme(s) in question, along with a suitable, validated pharmacokinetic metric (Tucker *et al.*, 1998). These probe drugs should ideally be easy to administer (preferably via the oral route), have no interactions amongst themselves and be well-tolerated by the patient at low doses (Ghassabian *et al.*, 2009). Therefore, the design, validation and optimisation of these analytical methods are often complex, time consuming and resource-intensive. A significant investment is made to get these tools 'right', so that accurate and precise measurement of CYP activity can be made before and after interventions that test hypotheses in this area of clinical pharmacology.

1.6.1 Simultaneous phenotyping of multiple CYP-isoenzymes: the 'cocktail' approach

CYP-phenotyping cocktails aim to achieve the above simultaneously for multiple CYP450 enzymes, namely CYP1A2, CYP2D6, CYP2C19, CYP2C9 and CYP3A4, by dosing individuals with enzyme-specific probe drugs, and using the resulting concentration-time data to calculate metrics that characterise CYP drug-metabolising activity. This approach has been colloquially called the CYP-phenotyping 'cocktail', and has recently been extensively reviewed by de Andrés and Llerena (2016).

Multiple CYP-phenotyping methods using liquid chromatography-tandem mass spectrometry (LC-MS/MS) have been developed with a range of probe drugs, internal standards, sampling strategies, biological matrices of interest and methods of analyte extraction (de Andrés & Llerena, 2016). To have utility as a diagnostic or investigative tool in

clinical and research settings, the phenotyping approach should be minimally invasive with simplified sample processing, and reduced costs wherever possible. Of note, complex, multiple-step analytical protocols have served as a barrier to the routine inclusion of CYP-phenotyping in these contexts, with some methods requiring: different extraction techniques for their various analytes; specialised automated systems; lengthy incubations with reagents before extraction can occur; and different chromatographic conditions across analytes, which necessitate repeat sample processing and multiple injections (De Andrés *et al.*, 2014; Ghassabian *et al.*, 2009; Grangeon *et al.*, 2017; Lammers *et al.*, 2016). Lack of assay sensitivity also means that larger volumes (0.5 – 10 mL) of plasma are needed. Further, some methods employ probes such as flurbiprofen (Bosilkovska *et al.*, 2014a) and tolbutamide (Yin *et al.*, 2004), which are not widely available in appropriate dosage forms. Other issues arise with the use of foods containing the probe phenotyping compounds as opposed to standardised medicines (e.g. coffee or carbonated cola beverages as a caffeine source), which likely contain additional compounds that could affect drug-metabolising enzymes in humans (Bosilkovska *et al.*, 2014a; Bosilkovska *et al.*, 2016; Bosilkovska *et al.*, 2014b). Consequently, there exists a need for bioanalytical methods that address the above issues, which can be readily established for clinical studies requiring simultaneous CYP-phenotyping.

The design, validation and optimisation of one such CYP-phenotyping cocktail is covered in Chapter 3, aligning with the thesis objectives outlined in section 1.9.

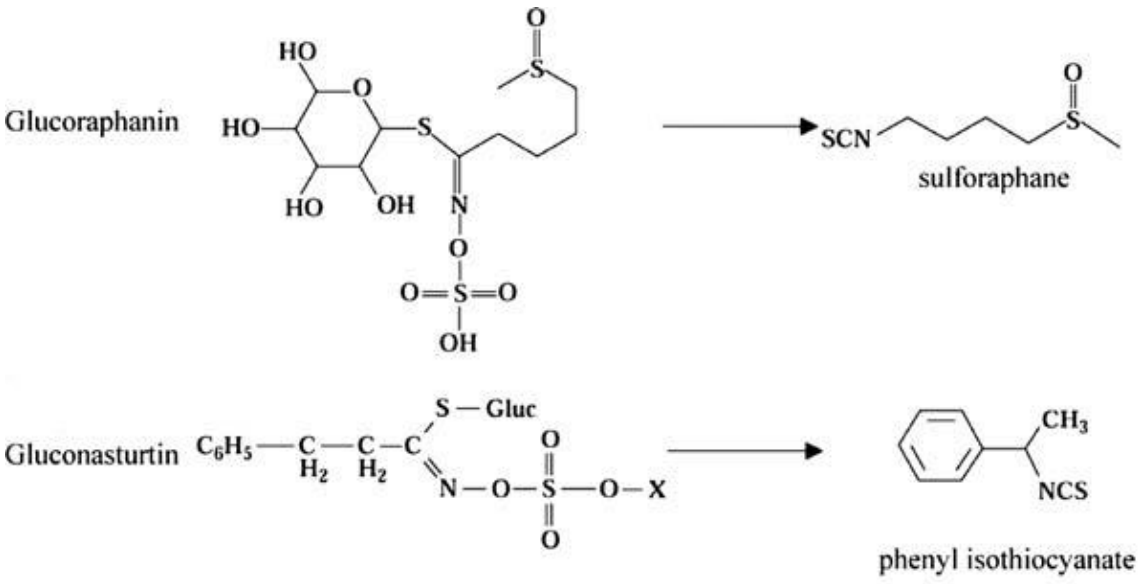
1.7 Diet as an extrinsic source of variability in drug metabolism

The old adage "you are what you eat" has been shown to be ostensibly true throughout decades of research across many disciplines. In the context of clinical pharmacology, a recent review by Yang (2015) pays tribute to the work of the late Allan Conney and colleagues, who pioneered work in this area. Conney *et al*. identified that polycyclic aromatic hydrocarbons, famously found in char-grilled meat, induce the metabolic activity of CYP1A1 and CYP1A2 (Conney, 1982), opening the door to diet and drug metabolism research in humans (Conney, 2003). This group of researchers is well-known for their work that identified ingested cruciferous vegetables as inducers of CYP1A2 (Pantuck *et al.*, 1979), and a comprehensive review of such trials is discussed in Chapter 2 of this thesis. Because *Cruciferae* are found in diets throughout the world (Li *et al.*, 2015), they were chosen as a candidate food to study in the context of variability in drug metabolism, and differences in this variability across geographic ancestries.

1.7.1 Cruciferous vegetables and their constituents

Due to their apparent anti-cancer properties, the isothiocyanates (ITCs) have been of interest to the scientific community over the past two decades (Gupta *et al.*, 2014). These compounds are found in high concentrations in cruciferous vegetables such as broccoli, cabbage and watercress, which feature almost ubiquitously in many diets across the globe (Steinkellner *et al.*, 2001). These compounds are present in plants as thioglycoside conjugates called glucosinolates, which are hydrolysed by myrosinase (released when plant cells are damaged by cutting, chewing, etc.) to isothiocyanates (Grubb & Abel, 2006) (Figure 1.17).

Figure 1.17: The two most represented glucosinolates and their isothiocyanate hydrolysis products. Adapted from Cartea and Velasco (2008).



One of the mechanisms by which these compounds elicit their cancer-protecting effects is through the induction of phase I and II drug-metabolising enzymes, which significantly contribute to the metabolism, and therefore clearance, of carcinogenic compounds and other xenobiotics (Cheung & Kong, 2010; Thornalley, 2002; Zhang, 2004). Most studies investigating these effects are designed to test hypotheses in a cancer-focussed context, with the two most represented ITCs being sulforaphane (SUL) and phenethyl isothiocyanate (PEITC) (Lamy *et al.*, 2011) (Figure 1.17). However, few studies approach the interaction between *Cruciferae* and their constituents and drug-metabolising enzymes in a clinical pharmacology context.

The majority of published studies investigating these effects focus on the CYP1A2 isoform (section 1.3.1.1.1). Generally, data show that the constituents in *Crucifeae* induce or increase the activity of this enzyme *in vivo* but have mixed effects *in vitro* (Chapter 2). Contrary to *in vivo* data, PEITC has been shown to inhibit CYP1A2, CYP2C19, CYP2C9, CYP2D6 and CYP3A4 *in vitro* (Nakajima *et al.*, 2001). Its glucosinolate precursor, gluconasturtiin, has

been detected in concentrations ranging from 0.1-0.9 µmol per gram of dry weight broccoli across 50 different accessions of broccoli subspecies (Kushad *et al.*, 1999). Therefore, consumption of 500 g broccoli daily could lead to ingestion of up to 450 µmol of gluconasturtiin, and hence a corresponding amount of phenethyl isothiocyanate, in a 24-hour period. A related isothiocyanate compound, sulforaphane, is present in high concentrations within broccoli as the thioglycoside conjugate glucoraphanin, and it has been estimated that consumption of 100 g of broccoli may release 40 µmol of this particular isothiocyanate (Hecht, 1995). As with PEITC, there is also evidence that sulforaphane can inhibit CYP1A2. Importantly, both PEITC and SUL have been detected in significant concentrations in plasma following cruciferous vegetable consumption (Fahey *et al.*, 1997; Ji & Morris, 2003). These data suggest that it is reasonable to hypothesise that a clinically meaningful concentration of these bioactive phytochemicals may be achieved in hepatic tissues and other sites of drug metabolism, potentially leading to altered activity of important CYP-mediated xenobiotic elimination.

The manner in which cruciferous vegetables are prepared in a culinary context for human consumption can affect the quantity of phytochemicals absorbed after their ingestion. A comprehensive review in this area outlines evidence for altered glucosinolate concentrations in cruciferous vegetables following “domestic cooking” (boiling), steaming, microwaving and stir-fry cooking (Verkerk *et al.*, 2009). Verkerk *et al.* identified that some glucosinolates and their metabolites are water-soluble, and cooking methods involving submerging vegetables in water, or with high surface area contact with water, could facilitate leeching and a reduced concentration of these phytochemicals within plant tissues. This is supported by a study that showed a 77% decrease in glucosinolate content within broccoli that was boiled for 30 minutes (Song & Thornalley, 2007).

Studies that involve microwaving cruciferous vegetables display mixed effects on glucosinolate content. Vallejo *et al.* (2002) showed that microwaving broccoli for five minutes at 1000 W power reduced glucosinolate levels by 74%. In contrast, a similar study found no significant decrease in glucosinolate content when microwaving for three minutes at 900 W power (Song & Thornalley, 2007). It remains unclear if microwaving can truly affect phytochemical levels within cruciferous vegetables, however it is worth noting that *in vivo* broccoli diet studies with a drug-metabolising enzyme endpoint report significant changes in enzyme activity even if the broccoli was allowed to be microwaved (Hakooz & Hamdan, 2007; Kall *et al.*, 1997).

Importantly, ITCs are predominantly metabolised by GSTs (section 1.3.1.3), therefore factors affecting GST activity are also likely to contribute to variability in ITC exposure. The contributions of GST genotype and cruciferous vegetable preparation and cooking methods to variability in ITC exposure should not be ignored, and trials that measure their concentration should have appropriate measures in place to control for this variability.

1.8 Differences in CYP activity between Europeans and South Asians

Chapter 1 has introduced the reader to the theme of variability in response to medicines (section 1.1), with a focus on intrinsic and extrinsic factors affecting this variability (section 1.1.1), geographic ancestry (section 1.2), drug-metabolising enzymes (section 1.3.1), and the effects of *Cruciferae*-enriched diets on drug metabolism (section 1.7.1). Often, these factors interact and co-vary within geographic ancestry groups, which can be useful when attempting to explain variability in response to medicines in these populations.

Ultimately, ancestry is a 'cluster' of various intrinsic and extrinsic factors that vary or co-vary within a particular geographic ancestry group, such as frequencies of SNPs in drug-metabolising genes, or dietary practices. Therefore, any study that aims to address hypotheses in this area should measure the effect of individual intrinsic and extrinsic factors and how they themselves vary between ancestry groups. Further, doing so allows for appropriate statistical control of particular factors, which is useful in determining whether there remains residual, unexplained variability in a given endpoint. Several good examples of this are demonstrated in Chapter 5 of this thesis, such as CYP2C19 activity being higher in Europeans than South Asians within genotype groups, i.e. despite having the same alleles in the variants tested, one ancestry group still had higher activity than the other, some of which could be explained by relative differences in dietary practices between the groups. This approach allows for identification of new avenues of research to explore and a better understanding of the relative contribution of factors affecting variability in response to medicines.

Of all geographic ancestries, the most is known about Europeans, as much of the modern clinical pharmacology movement originated in either Europe or America (in the early to mid-

1900s, most Americans were European immigrants or their descendants) (Dollery, 2006). In fact, because most drugs have traditionally been developed in European patient cohorts, they are also the reference group for comparison when registering a drug for use in non-European populations. In the modern pharmaceutical industry context, global clinical trials aim to address the pharmacological differences between ancestry groups, and the International Council for Harmonisation (ICH) sets out guidelines to streamline and improve this process (Ichimaru *et al.*, 2010). Compared to Europeans, fewer trials are conducted in those people of South Asian ancestry: less than 1% of global clinical trials are conducted in populations hailing from the Indian sub-continent (Thiers *et al.*, 2008). However, this region of the world has one of the highest population growth rates and high annual growth in pharmaceutical infrastructure and demand. Further, the effects of diet and genetics on drug-metabolising enzyme activity in South Asians are under-represented in pharmacogenomic databases such as PharmGKB (https://www.pharmgkb.org) (Whirl-Carrillo *et al.*, 2012) and The Allele Frequency Database (ALFRED) (https://alfred.med.yale.edu) (Rajeevan *et al.*, 2003). Therefore, better understanding the variability in response to medicines between Europeans and South Asians is of growing importance, hence choosing to focus on these two geographic ancestries in this thesis.

The remainder of this section will discuss the currently known differences in CYP genetics between Europeans and South Asians, and finally comment on known differential effects of diet practices on CYP activity between these groups.

1.8.1 Differences in CYP SNP frequencies between Europeans and South Asians

The 1000 Genome Project (http://www.internationalgenome.org) was a vast collaborative undertaking that aimed to identify and catalogue as many gene variants as possible in select

ancestry groups across the globe (Auton *et al.*, 2015). Table 1.8 summarises some of the data from this project, namely frequencies of important CYP SNPs in both Europeans and South Asians. It can be seen that for many of these important variants, frequencies of activity change alleles are higher in one group over the other. For example, with regards to CYP2C19, Europeans have a higher proportion of the increased activity genotypes (TT, CT) for *CYP2C19*17*, and South Asians have a higher frequency of the *CYP2C19*2* null allele genotypes (AA, AG), suggesting that with all else being equal, Europeans would have higher CYP2C19 activity than South Asians. Similar allelic patterns are seen for CYP1A2, with Europeans having higher frequencies of the increased inducibility genotypes for *CYP1A2*1F* (AA, AC), with South Asians having higher frequencies of the decreased inducibility genotypes for *CYP1A2*1C* (AA, AG). For CYP2C9 and CYP3A4, the ancestry group differences in high or low activity genotype frequencies are similar. CYP2D6 is highly polymorphic (section 1.3.1.1.3), and variants that cause either reduced or no activity are numerous. Frequencies of the null or reduced activity CYP2D6 variant genotypes are higher in Europeans for *CYP2D6*3*, *CYP2D6*4*, *CYP2D6*6* and *CYP2D6*10*, but more prevalent in South Asians for *CYP2D6*42* (Auton *et al.*, 2015).

Ultimately, though, it is the enzyme activity *in vivo* (i.e. the phenotype) rather than the genotype that dictates whether a particular CYP-substrate is effectively metabolised or not. Therefore, environmental factors known to affect CYP activity, such as diet, should also be investigated when aiming to explore variability in response to medicines between geographic ancestries.

Table 1.8: Important CYP SNPs, their details, effects and frequencies in European and South Asian populations. Data sourced from the 1000 Genome Project (http://www.internationalgenome.org) (Auton *et al.*, 2015).

CYP allele designation (rs no.) [SNP] | function	Genotype frequency (count)		
CYP1A2*1C **(rs2069514) [−3860G>A] | ↓ inducibility**	**GG**	**AA**	**AG**
European	0.960 (483)	Nil	0.040 (20)
South Asian	0.847 (414)	0.006 (3)	0.147 (72)
CYP1A2*1F **(rs762551) [−163C>A] | ↑ inducibility**	**CC**	**AA**	**AC**
European	0.115 (58)	0.475 (239)	0.410 (206)
South Asian	0.227 (111)	0.297 (145)	0.476 (233)
CYP2C19*1C **(rs3758581) [80161A>G] | undetermined**	**GG**	**AA**	**AG**
European	0.865 (435)	0.002 (1)	0.133 (67)
South Asian	0.787 (385)	0.006 (3)	0.207 (101)
CYP2C19*2 **(rs4244285) [19154G>A] | null allele**	**GG**	**AA**	**AG**
European	0.722 (363)	0.012 (6)	0.266 (134)
South Asian	0.436 (213)	0.151 (74)	0.413 (202)
CYP2C19*3 **(rs4986893) [17948G>A] | null allele**	**GG**	**AA**	**AG**
European	1.000 (503)	Nil	Nil
South Asian	0.975 (477)	Nil	0.025 (12)
CYP2C19*17 **(rs12248560) [−806C>T] | ↑ activity**	**CC**	**TT**	**CT**
European	0.596 (300)	0.044 (22)	0.360 (181)
South Asian	0.753 (368)	0.025 (12)	0.223 (109)
CYP2C9*2 **(rs1799853) [3608C>T] | ↓ activity**	**CC**	**TT**	**CT**
European	0.773 (389)	0.022 (11)	0.205 (103)
South Asian	0.933 (456)	0.002 (1)	0.065 (32)
CYP2C9*3 **(rs1057910) [42614A>C] | ↓↓ activity**	**AA**	**CC**	**AC**
European	0.857 (431)	0.002 (1)	0.141 (71)
South Asian	0.787 (385)	0.006 (3)	0.207 (101)
CYP2D6*3 **(rs35742686) [2549delA] | null allele**	**TT**	**-T**	**--**
European	0.968 (487)	0.026 (13)	0.006 (3)
South Asian	0.996 (487)	0.004 (2)	Nil
CYP2D6*4 **(rs3892097) [100C>T] | null allele**	**CC**	**TT**	**CT**
European	0.674 (339)	0.046 (23)	0.280 (141)
South Asian	0.806 (394)	0.025 (12)	0.170 (83)
CYP2D6*6 **(rs5030655) [1707delT] | null allele**	**AA**	**-A**	**--**
European	0.960 (483)	0.040 (20)	Nil
South Asian	0.998 (488)	0.002 (1)	Nil
CYP2D6*10 **(rs1065852) [4180G>C] | ↓ activity**	**GG**	**AA**	**AG**
European	0.646 (325)	0.050 (25)	0.304 (153)
South Asian	0.710 (347)	0.039 (19	0.252 (123)
CYP2D6*17 **(rs28371706) [1023C>T]; (rs16947) [2850C>T] | ↓ activity**	**rs28371706**		
	GG	**AA**	**AG**
European	0.996 (501)	Nil	0.004 (2)
South Asian	1.000	Nil	Nil
	rs16947		
	GG	**AA**	**AG**
European	0.461 (232)	0.147 (74)	0.392 (197)
South Asian	0.427 (209)	0.151 (74)	0.421 (206)

CYP allele designation (rs no.) [SNP] | function	Genotype frequency (count)		
CYP2D6*41 **(rs28371725) [2988G>A] | ↓ activity**	**CC**	**TT**	**CT**
European	0.827 (416)	0.014 (7)	0.159 (80)
South Asian	0.779 (381)	0.022 (11)	0.198 (97)
CYP3A4*1B **(rs2740574) [−392A>G] | undetermined**	**TT**	**CC**	**CT**
European	0.946 (476)	0.002 (1)	0.052 (26)
South Asian	0.922 (451)	0.002 (1)	0.076 (37)
CYP3A4*22 **(rs35599367) [15389 C>T] | ↓ activity**	**GG**	**AA**	**AG**
European	0.903 (454)	0.002 (1)	0.095 (48)
South Asian	0.988 (483)	Nil	0.012 (6)

1.8.2 Differences in diet and CYP activity between Europeans and South Asians

One of the largest cross-sectional observational studies to investigate how diet, genetics and CYP1A2 activity vary between Europeans and South Asians was conducted by Perera *et al.* (2012a). In this study, median CYP1A2 activity was 29% higher in the European cohort compared to the South Asians. This was attributed to different frequencies of diets known to affect CYP1A2 activity, namely those high in foods that induce or inhibit the CYPs (see section 1.7). Heavy consumption of CYP1A2 inducer foods was relatively higher in the Europeans, whereas heavy inhibitor consumption was more prevalent in the South Asians. Further, a predominantly curry diet was more frequent in the South Asian ancestry group. Indian curries contain foods known to inhibit CYP1A2, such as turmeric, celery, cumin and dill (Lampe *et al.*, 2000b; Peterson *et al.*, 2006; Peterson *et al.*, 2009), which could explain some of this variability in enzyme activity between the ancestry groups.

Studies like this are rare, with this one being unique in the sense that it was the first observational study to simultaneously investigate genetics, diet and CYP activity in Europeans and South Asians. Importantly, these observational results have not yet been confirmed in a follow-up, controlled, crossover trial, nor have the other four main CYP enzymes been investigated in this context. Because the CYPs share so many overlapping molecular regulatory mechanisms, it is likely that factors affecting CYP1A2 also affect the activities of CYP2C19, CYP2C9, CYP2D6 and CYP3A4 (Zanger & Schwab, 2013). These potential avenues of enquiry were used to form the thesis objectives in the following section of this thesis.

1.9 Thesis objectives and outline

Chapter 1 has summarised and discussed the published literature surrounding variability in response to medicines and the tools required to measure and explore this variability. Ultimately, a growing understanding of this observed variability is needed to produce better patient outcomes by providing new ways to account for intra- and inter-patient differences in the efficacy and safety of drugs.

In this context, the importance of the CYP superfamily of drug-metabolising enzymes was discussed in detail, alongside the importance of several key phase II drug-metabolising enzymes. Further, the way in which diet affects these enzymes was discussed, with a focus on how cruciferous vegetables and their constituents induce and inhibit drug-metabolising enzymes. The concept of geographic ancestry as a collection of known (and unknown) intrinsic and extrinsic factors affecting response to medicines was introduced, and the relevant background for European and South Asian ancestries was covered. It was identified that relatively few studies have investigated how diet and genetics interact between and within ancestry groups such as Europeans and South Asians, and a need for more studies in this area was commented on.

Before hypotheses can be constructed that examine ancestral differences in CYP enzyme activity, the literature must be searched systematically to identify what has already been studied, how well these studies were designed and where gaps in the literature exist. Therefore, apart from the objectives of Chapter 1 (above), the first main objective of this thesis was, for the first time, to conduct a systematic review of all published cruciferous vegetable intervention trials with drug metabolism endpoints. The sub-objectives of this review are stated in the introduction of Chapter 2, but ultimately, results from this review

were used to generate hypotheses that aimed to investigate the relationships between diet, genetics, geographic ancestry and drug metabolism.

Further, before such hypotheses can be tested, appropriate bioanalytical methods must be designed, validated and optimised to ensure timely analysis of participant samples. The CYP phenotyping cocktail approach was introduced in section 1.6.1 as a technique with increasing popularity in pharmacokinetic phenotyping studies. However, these assays are complex and resource intensive, often with cumbersome, time-consuming methods. Therefore, the second main objective of this thesis was to design, validate and optimise a UHPLC-MS/MS CYP-phenotyping cocktail assay that was relatively simple, sensitive and high-throughput compared to similar assays. This process is described, reported and discussed in Chapter 3.

Similarly, a bioanalytical method was required to measure sulforaphane, a candidate isothiocyanate affecting changes in CYP activity, in a biological matrix. Previously published methods have long chromatographic run times, require large sample volumes and have complicated sample extraction procedures (discussed in section 4.4). Therefore, the third main objective of this thesis was to design, validate and optimise a UHPLC-MS/MS assay to measure sulforaphane in human plasma that was relatively simple, sensitive and high-throughput compared to similar assays.

Lastly, the hypotheses generated in Chapter 2 were tested through designing and conducting a dietary intervention trial in Europeans and South Asians. The sub-objectives of this trial are outlined in section 5.1, however in short, for the first time, this trial aimed to explore the effects of cruciferous vegetable consumption, other dietary components, genetics and geographic ancestry on the activities of CYP1A2, CYP2C19, CYP2C9, CYP2D6

and CYP3A4. Because these five CYP enzymes contribute to the metabolism of the majority of medicines used in humans, factors that affect their activity significantly contribute to variability in response to medicines. Therefore, exploring the way in which diet, genetics, and European and South Asian ancestries interact with these drug-metabolising enzymes will aid in better understanding variability in response to medicines, and in turn, contribute to the safer and more efficacious use of drugs in these populations.

2 The effects of cruciferous vegetable-enriched diets on drug metabolism: a systematic review and meta-analysis of dietary intervention trials in humans

2.1 Introduction

As discussed in section 1.1, variability in response to medicines has a significant impact on the clinical outcomes of drug therapy. Ultimately, a complex interplay of intrinsic and extrinsic factors explains why individuals respond differently when given the same dose of the same medicine (section 1.1.1). Studies in this area aim to explore differences in the expression and activity of drug-metabolising or drug-transporter proteins, as these proteins significantly contribute to the clearance of a drug, and therefore influence its systemic exposure (Yasuda *et al.*, 2008). Variability in the genes that encode these enzymes and transporters has been identified as a major source of inter-individual differences in systemic drug exposure (Bjornsson *et al.*, 2003).

Section 1.7.1 outlined the effects of cruciferous vegetables or their constituents, which are an important portion of many people's diets, on drug-metabolising activity in humans. Cruciferous vegetables contain isothiocyanates (ITCs), which are phytochemicals considered to cause the enzyme induction and inhibition observed after eating *Cruciferae*-enriched diets (Steinkellner *et al.*, 2001). Phenethyl isothiocyanate (PEITC) and sulforaphane (SFN) are the two most widely studied ITCs (Lamy *et al.*, 2011). In general, the ingestion of cruciferous vegetables is associated with induction of cytochrome P450 (CYP) 1A2 activity *in vivo* (Perera *et al.*, 2012a), however there is contention between findings, and the clinical significance of the potential diet-drug interactions remains uncertain.

Chapter 2 outlines the methods, results, discussion and conclusions of a systematic review which aimed to 1) systematically review dietary intervention trials in humans that investigated the effects of cruciferous vegetables on drug-metabolising enzymes; 2) critically analyse the design of these studies; 3) interpret the findings in the context of study quality; 4) where possible, conduct meta-analyses on the collected data to determine the size and significance of any *Cruciferae*-induced changes in drug metabolism; and 5) use this information to generate hypotheses which examine the interactions between diet, geographic ancestry, genetics and drug metabolism.

2.2 Methods

2.2.1 Databases and searches

The Cochrane Handbook for Systematic Reviews of Interventions, Version 5.1.0 (Higgins & Green, 2011) was used to guide the design of the methodology of this review. Medline, Embase and the Cochrane Central Register of Controlled Trials (CENTRAL) were searched from their earliest entries through to July 2017 via the Ovid platform (Wolters Kluwer, 2017). Search terms and the strategies used for each database are listed in Appendix 8.1. The reference lists from potentially relevant studies were hand-searched to identify additional dietary intervention trials. The search was limited to studies published in English. Study abstracts were screened for potential relevance by one author and two authors independently assessed the published papers for eligibility as per Section 2.2.2. Any differences were discussed until agreement was reached.

2.2.2 Assessment of study eligibility

Studies were deemed eligible if they included healthy volunteers or patients, implemented a dietary intervention which involved cruciferous vegetables and had a pharmacokinetic phenotyping metric as an endpoint. A phenotyping metric was defined as an index derived from the administration of an enzyme-specific probe drug or substrate followed by measurement of the concentration of the parent compound and/or metabolite(s) in a biological matrix (plasma, urine, saliva, etc.). Studies using endogenous markers (e.g. conjugated/unconjugated bilirubin as a probe for UDP-glucuronosyltransferase (UGT) activity) were also included. Interventions involving cruciferous vegetable extracts and/or their phytochemical isolates were excluded. Observational, cross-sectional studies were not included in this review.

2.2.3 Data extraction and study quality assessment

Data that were gathered included: number of subjects/place of study participant demographics; the drug-metabolising enzymes studied; the size and nature of any changes in enzyme activity following cruciferous vegetable consumption; duration and details of the dietary intervention; number of dietary intervention periods tested; and the type of study design (randomised crossover, non-randomised crossover or parallel). For reported pharmacokinetic metrics, point estimates and descriptive statistics were extracted using a uniform data extraction sheet (Appendix 8.2) and checked by a second author. In the event that numeric point estimates were not available, data were extracted from figures and graphs using WebPlotDigitizer (http://arohatgi.info/WebPlotDigitizer/app). Where data were available and computation/imputation was possible, the size, variability and significance of enzyme activity changes following dietary interventions involving cruciferous vegetable between studies were calculated.

Several methodological characteristics were taken into consideration when assessing the quality of each study. Selected guidelines from the Consolidated Standards of Reporting Trials (CONSORT) statement (Schulz *et al.*, 2010), the CONSORT Explanation and Elaboration document (Altman *et al.*, 2001) and criteria outlined by Mills *et al.* (2004) were used to guide the choice of study characteristics included in the critical analysis. Study characteristics indicating higher quality included: study design (with randomised crossover being the highest quality followed by non-randomised crossover, then parallel group study design); similarity between groups (for designs using more than one group of participants); sample size ≥ 10 participants (rationale by Kakuda *et al.* (2014)); inclusion of a basal control diet; kJ- and/or participant weight-standardised diets; appropriate choice of statistical analyses, previous/concurrent validation of assay methods; and measures to ensure

participant adherence to study protocols, including diet diaries and supervised consumption of the intervention diet.

2.2.4 Statistical analyses

The methods outlined in the Cochrane Handbook (Higgins & Green, 2011) and those of Elbourne *et al*. (Elbourne *et al.*, 2002) were used to calculate mean differences and their standard errors of study endpoints. The upper limits of *P*-value inequalities were used when calculating standard errors of mean differences to ensure that any estimates were conservative (e.g. $P < 0.05$ taken as $P = 0.05$). Due to most studies recruiting < 60 participants, appropriate values from the Student's *t*-distribution were used in the calculation of 95% confidence intervals (CI) for the mean difference between experimental and control periods (Higgins & Green, 2011).

As all but one study used a crossover or pre-test, post-test design, within-subject correlation coefficients for endpoints across study periods were necessary to estimate the standard deviation of the mean difference between experimental and control periods. Correlation coefficients were directly calculated for studies when the individual participant data were available (Higgins & Green, 2011), however the majority of studies did not allow for this. For CYP1A2, correlation coefficients from the data presented in Chapter 5 were used. Because repeated-measures pharmacokinetic data generally display high within-subject correlation across study periods in crossover trials (Shen *et al.*, 2006), an average value of 0.8 (estimated using within-subject correlation data presented in Chapter 5) was used in the estimation of the standard deviations of the mean difference for enzymes where calculation or imputation of correlation coefficients was not possible.

For studies reporting natural log-back-transformed geometric mean ratios of point estimates, the method of Higgins *et al.* (2008) was used to estimate the mean difference and its standard error on the non-logarithmic scale.

Meta-analysis was deemed appropriate for the studies that investigated the effects of cruciferous vegetables on metrics of CYP1A2 activity and GST-α. As the studies used a variety of pharmacokinetic metrics to represent the activity of these two enzymes, the mean differences of endpoints were divided by their pooled standard deviations to allow for comparisons across measurement scales (Higgins & Green, 2011). For both enzymes, studies were meta-analysed using a random-effects model under the generic inverse-variance method in RevMan (Version 5.3). Heterogeneity across meta-analysed trials was formally assessed using the Chi^2 method as outlined in the Cochrane Handbook (Higgins & Green, 2011).

2.3 Results

2.3.1 Database search results

The database searches revealed 3,118 publications that were potentially relevant; this was reduced to 2,285 studies after removing duplicates between the databases (Figure 2.1). Ninety-one studies were confirmed to be clinical/*in vivo* after scanning titles and abstracts. The full-text of the 91 studies was read, and a further four studies for assessment were identified from the publication citations, which were also assessed for eligibility. In total, 23 cruciferous vegetable dietary intervention trials investigating an impact on drug metabolism in humans were included in this systematic review (Table 2.1).

Figure 2.1: Flow diagram of study identification and selection processes with subtotals.

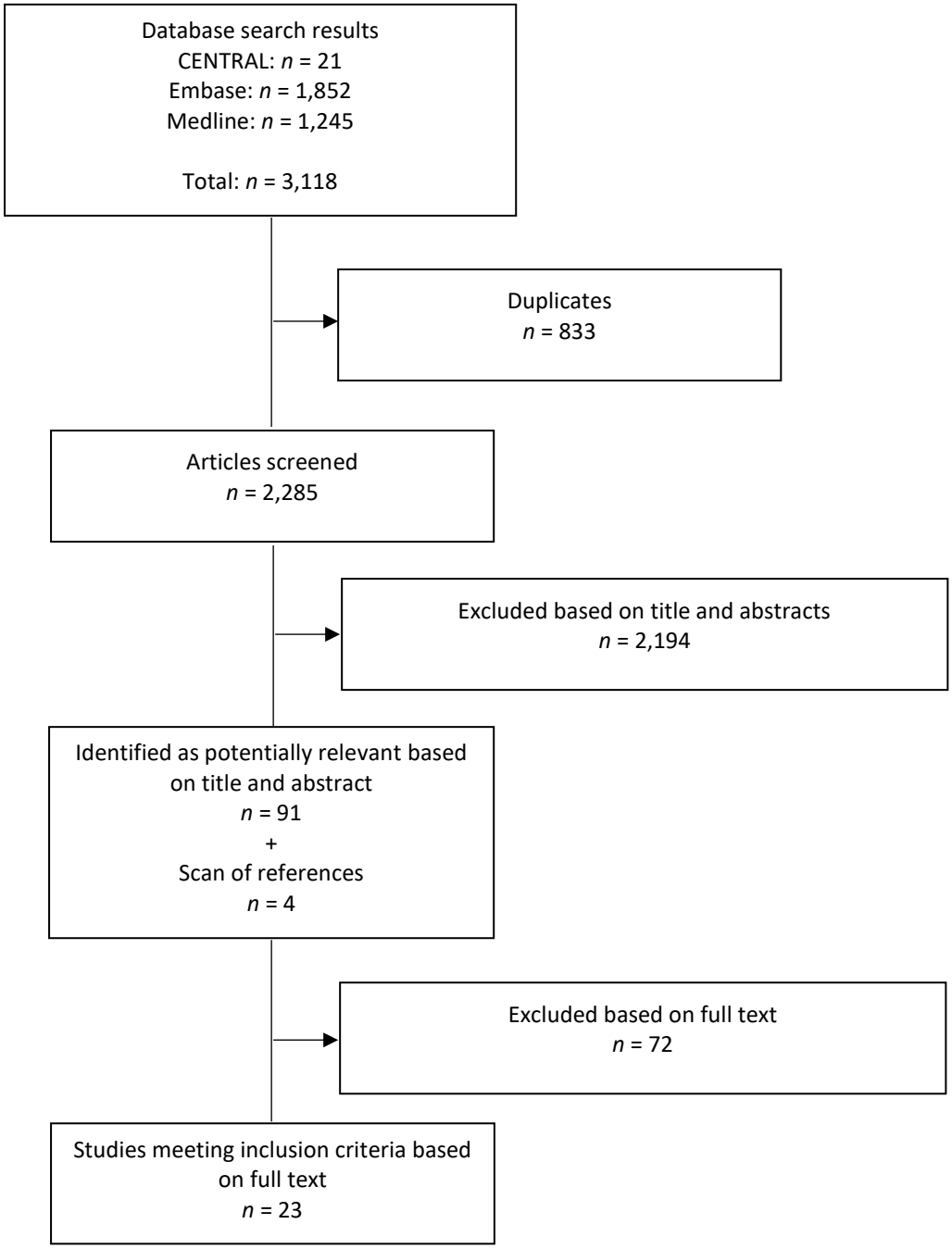


Table 2.1: Drug-metabolising enzymes, probe drugs and phenotyping metrics represented in the literature by publication

Study	Sample size (*n*)[1]	Enzymes	Probe drugs (dose)	Phenotyping metric[2]	Mean difference in metric (95% CI)[3]
de Waard *et al.* (2008)	6	CYP1A2 CYP2A6 NAT2 XO	Caffeine (2 cups of tea or 1 cup of coffee)	CYP1A2: (AFMU + 1X + 1U)/17U CYP2A6: 17U/(17U + 17X + 1U+ 1X+ AFMU) NAT2: AMFU/1X XO: 1U/(1U + 1X)	0.99 (0.78, 1.19)[a] 1.07 (0.93, 1.20)[a] 1.01 (0.66, 1.36)[a] 0.96 (0.90, 0.99)[a]
Hakooz and Hamdan (2007)	10	CYP1A2 CYP2A6	Caffeine (100 mg)	CYP1A2: (17U + 17X)/137X CYP2A6: 17U/(17U + 17X + 1U+ 1X+ AFMU)	20.2 (10.4, 30.0) 0.10 (0.06, 0.14)
Kall *et al.* (1996)	16	CYP1A2 CYP2E1	Caffeine (100 mg) Chlorzoxazone (500 mg)	(AFMU + 1X + 1U)/17U 2-h hydroxychlorzoxazone/chlorzoxazone plasma concentration	1.19 (0.65, 1.73) 0.07 (-0.03, 0.18)
Lampe *et al.* (2000b)	36	CYP1A2 NAT XO	Caffeine (200 mg)	CYP1A2: (AFMU + 1X + 1U)/17U CYP1A2: (AFMU + 1X + 1U)/17X CYP1A2: (17U + 17X)/137X CYP1A2:17X/137X NAT: AFMU/(1X + 1U + AFMU) XO: 1U/(1U + 1X)	0.97 (0.82, 1.12) 0.006 (0.00, 0.01) 1.00 (0.75, 1.25) 0.99 (0.76, 1.22) 0.00 (-0.02, 0.02) 0.00 (-0.01, 0.01)
McDanell *et al.* (1992)	6	CYP1A2	Study 1: Caffeine (2 g Nescafe® in 200 mL water) Study 2: Caffeine (1 g Nescafe® in 100 mL water)	Study 1: Caffeine AUC Caffeine MCR (dose/AUC) Study 2: Caffeine AUC Caffeine MCR (dose/AUC)	55.0 (26.3, 83.7) -0.03 (-0.05, -0.01) 53.0 (-42.7, 149) -0.02 (-0.05, 0.01)
Murray *et al.* (2001)	20	CYP1A2	Caffeine (2 mg/kg dissolved in decaffeinated coffee)	Caffeine CL $AUC_{0-\infty}$	6.10 (2.81, 9.39) -0.15 (-0.07, 0.09)
Pantuck *et al.* (1984)	10	CYP1A2 UGT1A9 UGT1A6	Antipyrine (1.8 mg/kg) Phenacetin (900 mg)	Antipyrine CL Phenacetin AUC 4-h conjugated/unconjugated APAP	0.35 (0.17, 0.53) -2,565 (-3,514, -1,617) 0.52 (0.16, 0.88)
Peterson *et al.* (2009)	73	CYP1A2	Caffeine (200 mg)	(AFMU + 1X + 1U)/17U	0.39 (0.18, 0.61)
Vistisen *et al.* (1992)	9	CYP1A2 NAT XO	Caffeine (1-4 cups of coffee)	CYP1A2: (AFMU + 1X + 1U)/17U NAT: AFMU/1X or AFMU/(1U + 1X + 17U) XO: 1U/(1U + 1X)	0.35 (-0.45, 1.14) 0.06 (-0.24, 0.36) 0.01 (-0.07, 0.09)
Murphy *et al.* (2001)	15	CYP2A6	Coumarin (5 mg)	Urine 7-hydroxycoumarin concentration	0.00 (-0.28, 0.28)
Desager *et al.* (2002)	9	CYP2E1	Ethanol (0.5 g/kg in cold water)	Ethanol $AUC_{0-3.33}$ Acetaldehyde $AUC_{0-3.33}$	-2.45 (-6.27, 1.37) 2.94 (0.30, 5.58)

Study	Sample size (*n*)[1]	Enzymes	Probe drugs (dose)	Phenotyping metric[2]	Mean difference in metric (95% CI)[3]
Leclercq *et al.* (1998)	10	CYP2E1	Chlorzoxazone (500 mg)	Chlorzoxazone $AUC_{0-\infty}$ Chlorzoxazone CL Hydroxychlorzoxazone $AUC_{0-\infty}$	24.2 (12.8, 35.7) -0.91 (-1.34, -0.48) 1.10 (-1.92, 4.12)
Chen *et al.* (1996)	10	CYP2E1 (indirect) UGT1A9 UGT1A6 SULT1A1 SULT2A1	Paracetamol (1 g)	APAP CL APAP AUC APAPG AUC APAPS AUC APAPC AUC APAPM AUC	-0.50 (-1.14, 0.14) 0.10 (-1.62, 1.82) 14.7 (3.76, 25.6) 2.20 (-4.52, 8.92) -0.70 (-1.13, -0.27) -0.18 (-0.22, -0.14)
Bogaards *et al.* (1994)	10	GST-α	n/a	Plasma concentration of enzyme	212 (100, 324)
Lampe *et al.* (2000a)	43	GST-α	NBD-Cl (200 µM) CDNB (1.22 µM)	Serum concentration of enzyme NBD-Cl extinction coefficient CDNB extinction coefficient	386 (-35.8, 807.35) 2.21, (-0.78, 5.21) 0.22 (-0.20, 0.64)
Navarro *et al.* (2009a)	67	GST-α	n/a	Serum concentration of enzyme	327 (0.59, 653)
Nijhoff *et al.* (1995)	10	GST-α GST-π	n/a	GST-α: Plasma concentration of enzyme GST-α: Urine concentration of enzyme GST-π: Plasma concentration of enzyme GST-π: Urine concentration of enzyme	0.30 (0.04, 0.56) (males) -0.04 (-0.14, 0.06) (females) 0.07 (0.01, 0.13) (males) -0.03 (-0.08, 0.02) (females) 0.42 (-0.83, 1.67) (males) 0.21 (-0.67, 1.09) (females) 0.11 (0.05, 0.17) (males) 0.03 (-0.09, 0.15) (females)
Riso *et al.* (2009)	20	GST-α	CDNB (20 mM)	Formation of NBD-Cl	-4.00 (-10.4, 2.39)
Riso *et al.* (2014)	10	GST-α	CDNB (20 mM)	Formation of NBD-Cl	8.80 (-4.51, 22.1)
Chang *et al.* (2007)	*UGT1A1*6/**6: 26 *UGT1A1*6/**7: 23 *UGT1A1*7/**7: 14	UGT1A1	Bilirubin (endogenous)	*UGT1A1*6/**6: serum total bilirubin *UGT1A1*6/**6: serum indirect bilirubin *UGT1A1*6/**6: serum direct bilirubin *UGT1A1*6/**7: serum total bilirubin *UGT1A1*6/**7: serum indirect bilirubin *UGT1A1*6/**7: serum direct bilirubin *UGT1A1*7/**7: serum total bilirubin *UGT1A1*7/**7: serum indirect bilirubin *UGT1A1*7/**7: serum direct bilirubin	0.72 (-0.27, 1.71 0.65 (-0.25, 1.55) 0.80 (-0.05, 0.21) -0.76 (-1.97, 0.45) -0.72 (-1.82, 0.38) -0.04 (-0.20, 0.12) -3.49 (-7.10, 0.12) -3.34 (-6.70, 0.02) 0.19 (-0.15, 0.53)

Study	Sample size (*n*)[1]	Enzymes	Probe drugs (dose)	Phenotyping metric[2]	Mean difference in metric (95% CI)[3]
Navarro *et al.* (2009b)	*UGT1A1*1/*1*: 29 *UGT1A1*1/*28*: 36 *UGT1A1*28/*28*: 5	UGT1A1	Bilirubin (endogenous)	*UGT1A1*1/*1*: serum total bilirubin *UGT1A1*1/*28*: serum total bilirubin *UGT1A1*28/*28*: serum total bilirubin	-1.03 (-2.06, -0.01) -0.17 (-2.15, 1.81) -3.42 (-6.84, -0.01)
Navarro *et al.* (2011)	Overall: 65 *UGT1A6*1/*1*: 27 *UGT1A6*1/*2*: 25 *UGT1A6*2/*2*: 13 *UGT2B15*1/*1*: 16 *UGT2B15*1/*2*: 33 *UGT2B15*2/*2*: 17 Overall: 65 *UGT1A6*1/*1*: 27 *UGT1A6*1/*2*: 25 *UGT1A6*2/*2*: 13 *UGT2B15*1/*1*: 16 *UGT2B15*1/*2*: 33 *UGT2B15*2/*2*: 17	UGT1A6 UGT2B15 SULT1A1 SULT2A1	Paracetamol (1 g)	Overall: APAPG ratio *UGT1A6*1/*1*: APAPG ratio *UGT1A6*1/*2*: APAPG ratio *UGT1A6*2/*2*: APAPG ratio *UGT2B15*1/*1*: APAPG ratio *UGT2B15*1/*2*: APAPG ratio *UGT2B15*2/*2*: APAPG ratio Overall: APAPS/APAP *UGT1A6*1/*1*: APAPS/APAP *UGT1A6*1/*2*: APAPS/APAP *UGT1A6*2/*2*: APAPS/APAP *UGT2B15*1/*1*: APAPS/APAP *UGT2B15*1/*2*: APAPS/APAP *UGT2B15*2/*2*: APAPS/APAP	2.70 (1.18, 4.22) 3.20 (0.01, 6.40) 2.60 (0.01, 5.20) 2.30 (0.01, 4.60) 4.60 (0.01, 9.20) 2.10 (0.01, 4.20) 2.20 (0.01, 4.40) -1.10 (-1.48, -0.52) -0.90 (-1.80, -0.01) -0.80 (-1.60, -0.01) -1.10 (-2.20, -0.01) -0.80 (-1.70, 0.10) -1.20 (-1.81, -0.59) -0.70 (-1.64, 0.24)
Pantuck *et al.* (1984)	10	UGT1A9 UGT1A6 UGT2B7 UGT2B15 SULT1A1 SULT2A1	Paracetamol (1.5 g) Oxazepam (45 mg)	APAP plasma AUC APAP CL APAPG plasma AUC APAPS plasma AUC Oxazepam plasma AUC Oxazepam CL Oxazepam glucuronide plasma AUC	-14.8 (-29.6, -0.01) 2.90 (0.01, 5.80) -13.2 (-20.1, -6.35) -20.5 (-41.0, -0.01) -1,438 (-2,876, -0.01) 1.10 (0.01, 2.20) -260 (-613, 92.9)

1 Total sample size as reported in publication or by subgroup where appropriate.

2 Abbreviations (as listed in column from top): AFMU, 5-acetylamino-6-formylamino-3-methyluracil; 1X, 1-methylxanthine; 1U, 1-methyluric acid; 17U, 1,7-dimethyluric acid; 17X, 1,7-dimethylxanthine (paraxanthine); 137X, 1,3,7-trimethylxanthine (caffeine); $t_{1/2}$, elimination half-life; CL, clearance; AUC, area under concentration-time curve; 4-h, 4-hour post-dose; APAP, *N*-acetyl-*p*-aminophenol (paracetamol); $AUC_{0\text{-}\infty}$, AUC from time 0 extrapolated to infinity; MCR, metabolic clearance (dose/AUC); APAPG, paracetamol glucuronide; APAPS, paracetamol sulfate; APAPC, paracetamol cysteine; APAPM, paracetamol mercapturate; NBD-Cl, 7-chloro-4-nitrobenz-2-oxa-1,3-diaxole; CDNB, 1-chloro-2,4-dinitrobenzene; APAPG ratio, [APAPG/(APAP + APAPG + APAPS)] x 100.

3 The 95% confidence interval (CI) of the mean difference between experimental and control measures of enzyme activity ($metric_{experimental}$ – $metric_{control}$) was estimated using the standard error of the mean difference and relevant values from the Student's *t*-distribution. Mean difference and standard error are as reported in the literature, extracted from data using a web-based digitiser programme ((http://arohatgi.info/WebPlotDigitizer/app) or calculated/imputed as outlined in the Methods section, unless otherwise stated.

a Data are ratio of $metric_{experimental}$/$metric_{control}$ with 95% CI as reported in the manuscript.

2.3.2 Nature of cruciferous vegetable dietary interventions

Cruciferous vegetables used in the eligible dietary intervention studies reported included broccoli, Brussels sprout, cabbage, cauliflower, radish and watercress. All studies reported standardised preparation and weighing of any cruciferous vegetables consumed. Methods of preparing these vegetables included steaming, boiling, stir-frying and raw consumption, however, not all studies instructed participants to cook the vegetables in the same manner, with some leaving this decision to the participants (Hakooz & Hamdan, 2007; Kall *et al.*, 1996), and other studies not reporting this information (Chang *et al.*, 2007; Lampe *et al.*, 2000b; Nijhoff *et al.*, 1995; Peterson *et al.*, 2009). Studies with controlled crossover designs included a washout period of at least 7 days between diets or between phenotyping sessions, with most allowing at least a 2- or 4-week washout. Table 2.2 summarises the types of dietary interventions and the cruciferous vegetables studied, alongside details of their preparation, consumption and any steps to standardise preparation.

2.3.3 Drug-metabolising enzymes and probe drugs assessed

The drug-metabolising enzymes investigated were CYP1A2 (*n* = 9) (de Waard *et al.*, 2008; Hakooz & Hamdan, 2007; Kall *et al.*, 1996; Lampe *et al.*, 2000b; McDanell *et al.*, 1992; Murray *et al.*, 2001; Pantuck *et al.*, 1979; Peterson *et al.*, 2009; Vistisen *et al.*, 1992), CYP2A6 (*n* = 3) (de Waard *et al.*, 2008; Hakooz & Hamdan, 2007; Murphy *et al.*, 2001), CYP2E1 (*n* = 3) (Chen *et al.*, 1996; Desager *et al.*, 2002; Leclercq *et al.*, 1998), glutathione *S*-transferase (GST)-α (*n* = 6) (Bogaards *et al.*, 1994; Lampe *et al.*, 2000a; Navarro *et al.*, 2009a; Nijhoff *et al.*, 1995; Riso *et al.*, 2009; Riso *et al.*, 2014), GST-π (*n* = 1) (Nijhoff *et al.*, 1995), UDP-glucuronosyltransferase (UGT)1A1 (*n* = 2) (Chang *et al.*, 2007; Navarro *et al.*, 2009b), UGT1A6 (*n* = 4) (Chen *et al.*, 1996; Navarro *et al.*, 2011; Pantuck *et al.*, 1984; Pantuck *et al.*, 1979), UGT1A9 (*n* = 3) (Chen *et al.*, 1996; Pantuck *et al.*, 1984; Pantuck *et al.*, 1979),

UGT2B15 (*n* = 2) (Navarro *et al.*, 2011; Pantuck *et al.*, 1984), UGT2B7 (*n* = 1) (Pantuck *et al.*, 1984), *N*-acetyl transferase (NAT)2 (*n* = 3) (de Waard *et al.*, 2008; Lampe *et al.*, 2000b; Vistisen *et al.*, 1992), sulfotransferase (SULT)1A1 (*n* = 3) (Chen *et al.*, 1996; Navarro *et al.*, 2011; Pantuck *et al.*, 1984), SULT2A1 (*n* = 3) (Chen *et al.*, 1996; Navarro *et al.*, 2011; Pantuck *et al.*, 1984) and xanthine oxidase (XO) (*n* = 3) (de Waard *et al.*, 2008; Lampe *et al.*, 2000b; Vistisen *et al.*, 1992) (Figure 2.2). The number of investigations (*n* = 46) was greater than the number of studies included in the review (*n* = 23) as some studies simultaneously investigated multiple drug-metabolising enzymes.

The phenotyping probe drugs and metrics studied for each enzyme are listed in Table 2.1. Probe substrates used to investigate the enzymes were: caffeine for CYP1A2, CYP2A6, NAT2 and XO activity; ethanol or chlorzoxazone for CYP2E1 activity; paracetamol for UGT1A6, UGT1A9, UGT2B15, SULT1A1 and SULT2A1 activity; oxazepam for UGT2B7 and UGT2B15 activity; 1-chloro-2,4-dinitrobenzene or amount of enzyme for GST-α activity; and endogenous bilirubin for UGT1A1 activity. The phenotyping metrics used varied between studies, including metabolite-parent substrate ratios of relevant pharmacokinetic parameters (e.g. AUC or concentration at a particular time post-dose), changes in clearance, AUC or half-life of substrates.

Table 2.2: Details of the various Cruciferous vegetable dietary interventions by publication.

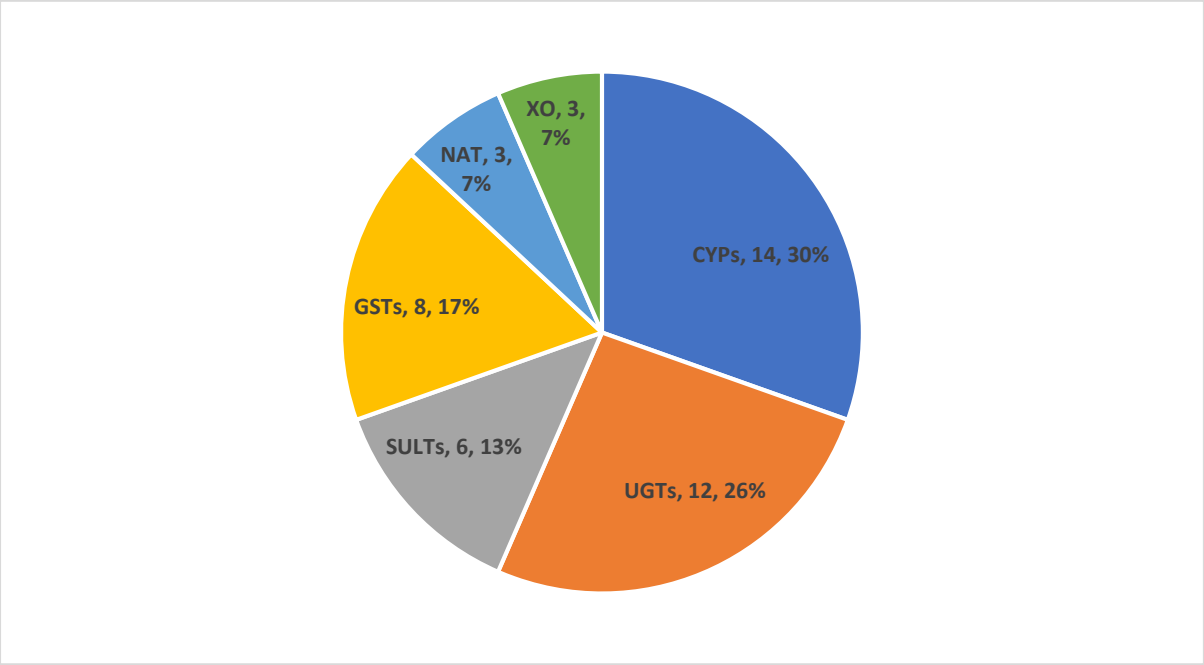
Study	Cruciferous vegetables consumed (amount/day))	Intervention details	Preparation of diet	Notes
Bogaards *et al.* (1994)	Brussels sprouts (300 g)	Two diets: basal (control) and Brussels sprouts. Diets consumed daily for 7 days each with no washout period before crossover.	Not stated.	Basal diet was "glucosinolate free" but further details not provided.
Chang *et al.* (2007)	Broccoli (100 g) Cabbage (35 g) Daikon radish sprouts (16 g) Dose adjusted per 55 kg body weight	Two diets: basal (control) and fruit and vegetable diet. Diets consumed daily for 2 weeks each with 2-week washout period before crossover.	Not stated.	Amount of cruciferous vegetables given standardised to a 55-kg body weight then adjusted for each participant to nearest 5 kg increment in body weight. Diets designed to contain 56% carbohydrate, 16% protein and 28% fat overall. Study diets contained other fruit and vegetables alongside crucifers.
Chen *et al.* (1996)	Watercress (50 g)	Two diets: habitual diet and watercress. Habitual diet followed throughout, with watercress consumed either at 10 pm night before phenotyping or not at all with a 2-week washout period before crossover (randomised crossover).	Watercress consumed as a homogenate made by blending with 50 mL water for 2-3 minutes.	
de Waard *et al.* (2008)	Broccoli (150 g) Brussels sprouts (300 g)	Two diets: grapefruit juice then cruciferous (sequential). Diets consumed daily for 3 days each with 3-week washout period.	Broccoli prepared as a soup and Brussels sprouts served as part of a meal.	Cruciferous vegetables and citrus fruits were avoided during the washout period.
Desager *et al.* (2002)	Watercress (50 g)	Two diets: habitual diet and watercress. Habitual diet followed throughout with a standardised breakfast before each round of phenotyping. Watercress consumed either at 10 pm night before phenotyping, 7:30 am morning of phenotyping or not at all (randomised crossover).	Watercress consumed as a homogenate made by blending with 250 mL water for 2 minutes.	Standardised breakfast included 100 g bread, chocolate paste and 150 mL coffee.
Hakooz and Hamdan (2007)	Broccoli (500 g)	One diet: broccoli (sequential) Broccoli consumed daily for 6 days. No details of washout period	Broccoli eaten raw with salad and dressing, steamed, microwaved or boiled (participant's preference).	Broccoli added to participant's normal diet.
Kall *et al.* (1996)	Broccoli (500 g)	Three diets: basal (control), cruciferous-devoid then broccoli (sequential). Diets consumed daily for: 2 days (basal), 6 days (cruciferous-devoid) and 12 days (broccoli) No washout period.	Broccoli distributed evenly between lunch and dinner; lunch-broccoli eaten raw with bread and a pasta salad and dinner-broccoli either steamed, microwaved or boiled (participant's choice).	Basal diet based on bread, potatoes, rice and boiled meat.
Lampe *et al.* (2000a)	Radish sprouts (16 g) Cauliflower (150 g) Broccoli (200 g) Cabbage (70 g)	Four diets: basal (control), cruciferous, allium and apiaceous. Diets consumed daily for 6 days 2-week washout period.	Not stated.	Diets designed to deliver 2,000 kcal as 60% carbohydrate, 12% protein and 28% fat overall. 'Unit foods' added to basal diet to maintain body weights of participants based on daily kJ requirements.

Study	Cruciferous vegetables consumed (amount/day))	Intervention details	Preparation of diet	Notes
Lampe *et al.* (2000b)	Radish sprouts (16 g) Cauliflower (150 g) Broccoli (200 g) Cabbage (70 g)	Four diets: basal (control), cruciferous, allium and apiaceous. Diets consumed daily for 6 days with 2-week washout period	Not stated.	Diets designed to deliver 2,000 kcal as 60% carbohydrate, 12% protein and 28% fat overall. 'Unit foods' added to basal diet to maintain body weights of participants based on daily kJ requirements.
Leclercq *et al.* (1998)	Watercress (50 g)	Two diets: habitual diet and watercress. Habitual diet followed throughout with a standardised breakfast before each round of phenotyping. Watercress consumed either at 10 pm night before phenotyping, 7:30 am morning of phenotyping or not at all (randomised crossover).	Watercress consumed as a homogenate made by blending with 250 mL water for 2 minutes.	Standardised breakfast included 100 g bread, chocolate paste and 150 mL coffee.
McDanell *et al.* (1992)	Brussels sprouts (400 g) Cabbage (800 g)	Two studies: Study 1—basal (control) diet plus 200 g cabbage eaten for 3 meals in 24 hours; next day at 8:00 am basal diet plus 200 g cabbage for breakfast before phenotyping (sequential). Study 2—basal (control) diet plus 200 g Brussels sprouts eaten for 2 meals in 24 hours; phenotyping next morning while fasting (sequential).	Vegetables lightly steamed.	
Murphy *et al.* (2001)	Watercress (170.4 g)	Two diets: habitual diet and watercress (sequential). Habitual diet followed for two weeks with three days of watercress consumption followed by phenotyping (56.8 g three times a day on two occasions and one 56.8 g serving immediately before phenotyping on one occasion).	Watercress consumed fresh and uncooked.	
Murray *et al.* (2001)	Broccoli (250 g) Brussels sprouts (250 g)	Three diets: habitual diet, cruciferous then habitual again (sequential). Diet consumed daily for 12 days No washout period.	Brussels sprouts peeled and broccoli stalks removed. Broccoli or Brussels sprouts prepared as a soup for breakfast or dinner as part of a 6-day menu plan which was repeated during the 12-day cruciferous vegetable diet.	Both soups were consumed each day; the vegetable not consumed at breakfast was eaten at dinner.
Navarro *et al.* (2009a)	Broccoli (203 g) Cauliflower (152 g) Red cabbage (36 g) Green cabbage (36 g) Radish sprouts (16 g) Dose adjusted per 70 kg body weight	Four diets: basal (control), cruciferous, double-cruciferous and cruciferous plus apiaceous. Diets consumed daily for 14 days Each with 3-week washout period.	Not stated.	Diets designed to deliver either 7 g/kg (single-dose cruciferous and cruciferous plus apiaceous) or 14 g/kg (double-dose cruciferous) cruciferous vegetables. Amount of cruciferous vegetables given standardised to a 70-kg body weight then adjusted for each participant to nearest 5 kg increment in body weight.
Navarro *et al.* (2009b)	Broccoli (203 g) Cauliflower (152 g) Red cabbage (36 g) Green cabbage (36 g) Radish sprouts (16 g)	Four diets: basal (control), cruciferous, double-cruciferous and cruciferous plus apiaceous. Diets consumed daily for 14 days Each with 3-week washout period.	Not stated.	Diets designed to deliver either 7 g/kg (single-dose cruciferous and cruciferous plus apiaceous) or 14 g/kg (double-dose cruciferous) cruciferous vegetables.

Study	Cruciferous vegetables consumed (amount/day))	Intervention details	Preparation of diet	Notes
	Dose adjusted per 70 kg body weight			Amount of cruciferous vegetables given standardised to a 70-kg body weight then adjusted for each participant to nearest 5 kg increment in body weight.
Navarro *et al.* (2011)	Broccoli (203 g) Cauliflower (152 g) Red cabbage (36 g) Green cabbage (36 g) Radish sprouts (16 g) Dose adjusted per 70 kg body weight	Four diets: basal (control), cruciferous, double-cruciferous and cruciferous plus apiaceous. Diets consumed daily for 14 days Each with 3-week washout period.	Not stated.	Diets designed to deliver either 7 g/kg (single-dose cruciferous and cruciferous plus apiaceous) or 14 g/kg (double-dose cruciferous) cruciferous vegetables. Amount of cruciferous vegetables given standardised to a 70-kg body weight then adjusted for each participant to nearest 5 kg increment in body weight.
Nijhoff *et al.* (1995)	Brussels sprouts (300 g)	Two diets: basal (control) and Brussels sprouts. Diets consumed daily for 7 days each with no washout period before crossover.	Not stated.	Basal diet was "glucosinolate free" but further details not provided.
Pantuck *et al.* (1979)	Brussels sprouts (300 g) Cabbage (200 g)	Three diets: basal (control), cruciferous then basal again (sequential). Diets consumed daily for 10 days No washout period.	Vegetables lightly steamed and distributed evenly between lunch and dinner.	Diets designed to deliver 2,500-2,600 kcal with 60% carbohydrate, 12% protein and 28% fat overall.
Pantuck *et al.* (1984)	Brussels sprouts (300 g) Cabbage (200 g)	Three diets: basal (control), cruciferous then basal again (sequential). Diets consumed daily for 10 days No washout period.	Vegetables lightly steamed and distributed evenly between lunch and dinner.	Diets designed to deliver 2,500-2,600 kcal with 60% carbohydrate, 12% protein and 28% fat overall.
Peterson *et al.* (2009)	Broccoli (203 g) Cauliflower (152 g) Red cabbage (36 g) Green cabbage (36 g) Radish sprouts (16 g) Dose adjusted per 70 kg body weight	Four diets: basal (control), cruciferous, double-cruciferous and cruciferous plus apiaceous. Diets consumed daily for 14 days with 3-week washout period.	Not stated.	Diets designed to deliver either 7 g/kg (single-dose cruciferous and cruciferous plus apiaceous) or 14 g/kg (double-dose cruciferous) cruciferous vegetables. Amount of cruciferous vegetables given standardised to a 70-kg body weight then adjusted for each participant to nearest 5 kg increment in body weight.
Riso *et al.* (2009)	Broccoli (200 g)	Two diets: basal (control) and broccoli. Diets consumed daily for 10 days each with 20-day washout period before crossover.	Broccoli steamed before consumption.	Basal diet was habitual diet devoid of cruciferous vegetables.
Riso *et al.* (2014)	Broccoli (200 g)	Two diets: basal (control) and broccoli (sequential). Basal diet consumed once the day before phenotyping and broccoli meal consumed immediately before phenotyping.	Broccoli consumed steamed with cooked pasta, olive oil and salt.	Basal diet consisted of three standardised meals 1 day before phenotyping: Breakfast—milk and shortbread biscuits; Lunch—two sandwiches (cooked ham and cheese and raw ham); Dinner—Steak with potatoes, pasta or rice with butter and Parmesan cheese and two slices of wheat bread.

Study	Cruciferous vegetables consumed (amount/day))	Intervention details	Preparation of diet	Notes
Vistisen *et al.* (1992)	Broccoli (500 g)	Three diets: habitual diet, broccoli and cruciferous-devoid. Diets consumed daily for 10 days 4-week washout period.	Broccoli lightly steamed and distributed evenly between lunch and dinner.	No controlled basal diet; habitual diet involved following usual dietary consumption patterns.

Figure 2.2: Drug-metabolising enzymes represented in the dietary intervention literature by type of enzyme (name, *n*, % total) (total *n* = 46).



CYP—Cytochrome P450s
GST—Glutathione *S*-transferases
NAT—*N*-acetyltransferase
SULTs—Sulfotransferases
UGTs— Uridine 5'-diphospho-glucuronosyltransferases
XO—Xanthine oxidase

2.3.4 Changes in drug-metabolising enzyme activity

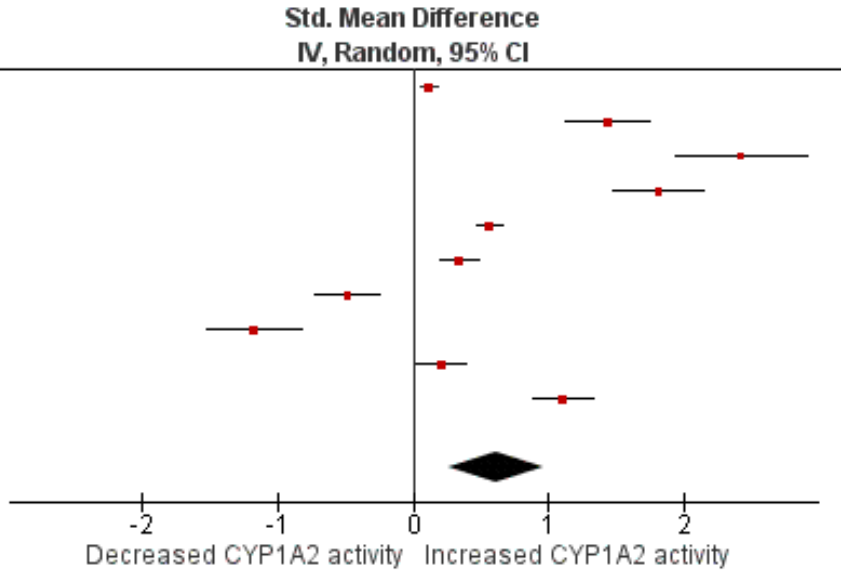
The extent of changes in drug-metabolising enzyme activity ranged from -20% to 450% following the various cruciferous vegetable dietary interventions (Table 2.1). The most consistent and significant increases were for CYP1A2, ranging from 11% to 249% (de Waard *et al.*, 2008; Hakooz & Hamdan, 2007; Kall *et al.*, 1996; Lampe *et al.*, 2000b; Murray *et al.*, 2001; Pantuck *et al.*, 1979; Peterson *et al.*, 2009; Vistisen *et al.*, 1992). Meta-analysis of the 10 experiments investigating CYP1A2 showed a significant increase of 0.61 standardised units (95% CI = 0.26, 0.97; $P = 0.0007$) (Figure 2.3) following *Cruciferae*-enriched diets, which approximates to a 20-40% increase in CYP1A2 activity, depending on the metric of choice. These studies were highly heterogenous ($Chi^2 = 421.13$ with 9 degrees of freedom; $P < 0.00001$), likely caused by variability in intervention diets and study design across the CYP1A2 trials.

Figure 2.3: Forest plot showing pooled estimate of standardised mean difference in CYP1A2 activity during cruciferous vegetable versus basal/control diet.

Study or Subgroup	Std. Mean Difference	SE	Weight	Std. Mean Difference IV, Random, 95% CI	Year
Peterson 2009	0.118	0.033	10.6%	0.12 [0.05, 0.18]	2009
de Waard 2008	1.439	0.163	9.8%	1.44 [1.12, 1.76]	2008
Hakooz 2007	2.426	0.248	8.8%	2.43 [1.94, 2.91]	2007
Murray 2001	1.816	0.17	9.7%	1.82 [1.48, 2.15]	2001
Lampe 2000a	0.564	0.05	10.5%	0.56 [0.47, 0.66]	2000
Kall 1996	0.341	0.072	10.4%	0.34 [0.20, 0.48]	1996
McDanell 1992b	-0.485	0.121	10.1%	-0.48 [-0.72, -0.25]	1992
McDanell 1992a	-1.177	0.182	9.6%	-1.18 [-1.53, -0.82]	1992
Vistisen 1992	0.213	0.094	10.3%	0.21 [0.03, 0.40]	1992
Pantuck 1979	1.111	0.112	10.2%	1.11 [0.89, 1.33]	1979
Total (95% CI)			**100.0%**	**0.61 [0.26, 0.97]**	

Heterogeneity: $Tau^2 = 0.31$; $Chi^2 = 421.13$, df = 9 ($P < 0.00001$); $I^2 = 98\%$

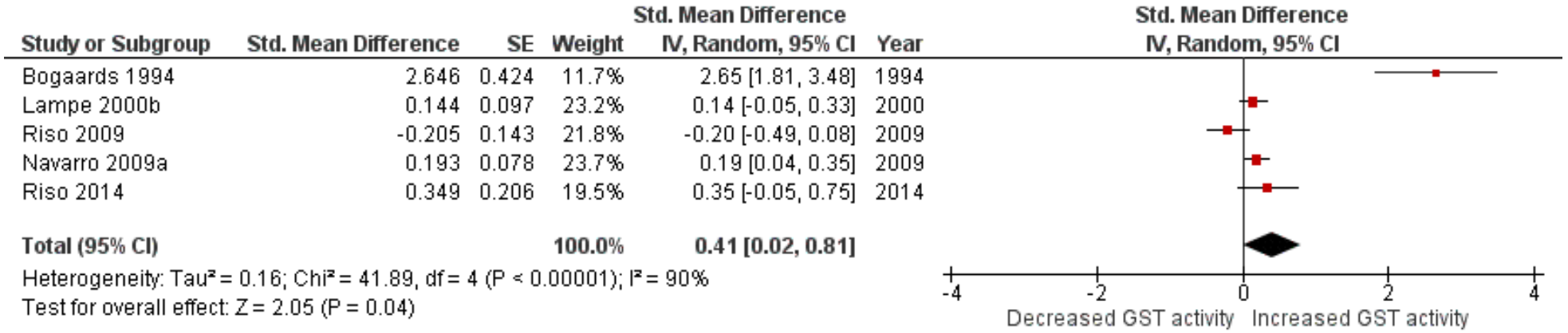
Test for overall effect: $Z = 3.37$ ($P = 0.0007$)



For CYP2A6 only one study reported a significant increase in CYP2A6 activity (MD 95% CI = 0.10 [0.06, 0.14]; *P* = 0.002) (Hakooz & Hamdan, 2007) with the remaining two studies reporting no significant change in activity. One of the CYP2E1 studies reported a significant decrease in activity (inhibition) following a watercress-enriched diet intervention (MD 95% CI = -0.91 [-1.34, -0.48]; *P* < 0.05) (Leclercq *et al.*, 1998).

With regards to GST-α, four studies reported an increase in enzyme activity or plasma concentration of the enzyme ranging from 10-61% (Bogaards *et al.*, 1994; Lampe *et al.*, 2000a; Navarro *et al.*, 2009a; Nijhoff *et al.*, 1995). Meta-analysis of the five GST-α trials showed a significant increase in activity of 0.41 standardised units (95% CI = 0.02, 0.81; *P* = 0.04) (Figure 2.4) after the various cruciferous vegetable interventions, corresponding to an estimated 15-35% increase in GST-α activity. The GST-α trials were also highly heterogenous (Chi^2 = 41.89 with 4 degrees of freedom; *P* < 0.00001). Only one study investigated GST-π and reported no significant effects of cruciferous vegetable consumption (MD 95% CI = 0.42 [-0.83, 1.67]; *P* > 0.05) (Nijhoff *et al.*, 1995).

Figure 2.4: Forest plot showing pooled estimate of standardised mean difference in GST-α activity during cruciferous vegetable versus basal/control diet.



For NAT2, one study reported a small increase in activity (MR 95% CI 1.01 [0.66, 1.36]; $P < 0.05$) (de Waard *et al.*, 2008), however this study had a small sample size ($n = 6$) and only two quality characteristics. Heterogeneity and poor study design prevented detailed analysis of the results of XO activity studies, although one study (de Waard *et al.*, 2008) demonstrated a decrease in activity after cruciferous vegetable consumption (MR 95% CI = 0.96 [0.90, 0.99]; $P < 0.05$).

UGT enzyme activity could not be compared across studies as the metrics chosen varied and all of the probe drugs administered to participants were non-selective substrates for the UGT enzymes (Chen *et al.*, 1996; Pantuck *et al.*, 1984). Nevertheless, one well-designed study examining UGT1A6 and UGT2B15 activity found evidence of a 4-5% increase in enzyme activity across different UGT genotype groups (MD 95% CI = 2.70 [1.18, 4.22]; $P < 0.0001$) (Navarro *et al.*, 2011). Of interest, in this same study, a corresponding 12% decrease in SULT1A1 and SULT2A1 activity was observed (MD 95% CI = -1.10 [-1.48, -0.52]; $P < 0.0001$) following the cruciferous vegetable-enriched diet intervention. A similar result was reported in one of the other studies investigating sulfotransferases (Pantuck *et al.*, 1984).

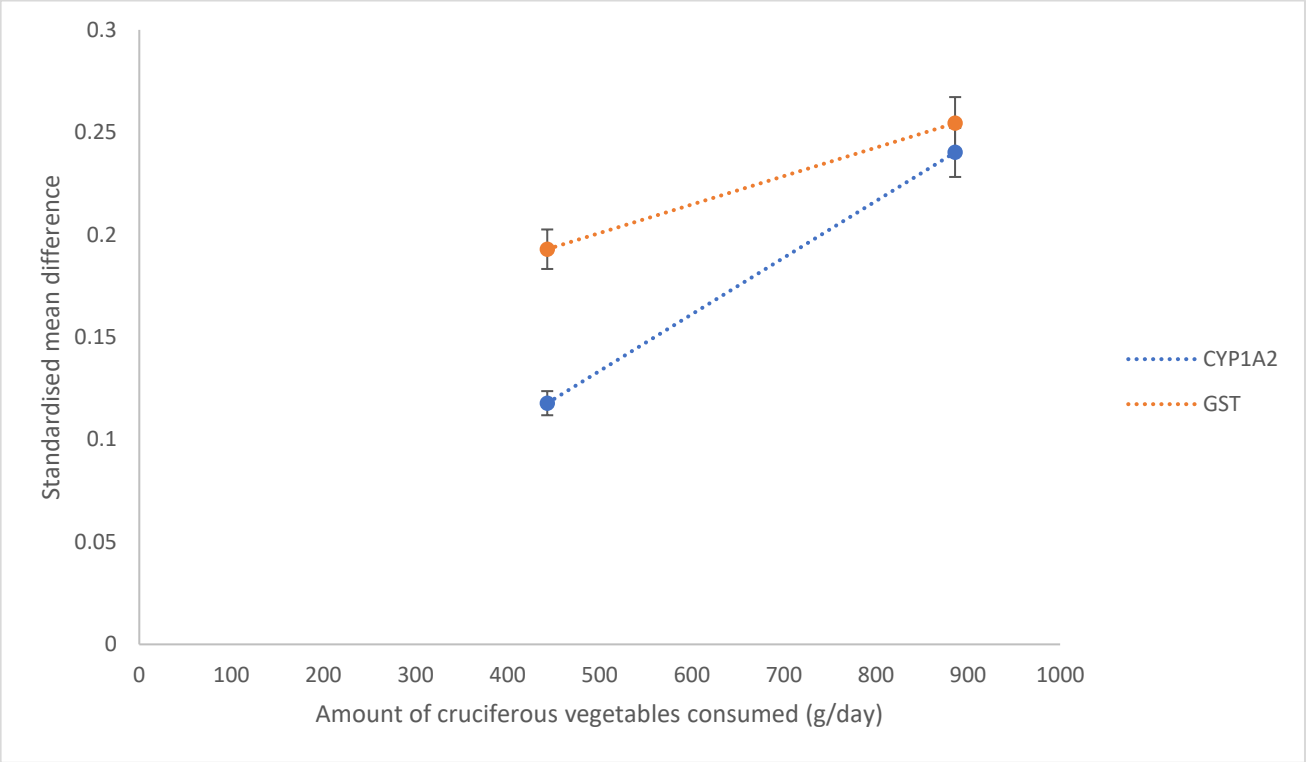
Of note, three studies reported evidence of dose-response relationships between the amount of cruciferous vegetables consumed and the changes in CYP1A2 (Peterson *et al.*, 2009), UGT1A1 (Navarro *et al.*, 2009b) and GST-α (Navarro *et al.*, 2009a) activity. Consuming double the amount of cruciferous vegetables relative to a standard *Cruciferae*-enriched diet increased CYP1A2 activity in a dose-dependent manner ($MD_{\text{Double-dose – Single-dose}}$ 95% CI = 0.35 [0.17, 0.54]; $P < 0.05$) (Figure 2.5). Similar dose-response trends were seen for the UGT1A1 (Navarro *et al.*, 2009b) and GST-α (Navarro *et al.*, 2009a) studies.

Table 2.3: Critical analysis of quality characteristics across the 23 studies.

Study	Design	Randomisation[1]	*n* ≥ 10[2]	Group similarity[3]	Basal diet[4]	kJ/weight standardization[5]	Adherence[6]	Statistical analyses[7]	Analytical technique[8]	Score[9]
Chang, 2007	Crossover	+	+	+	+	+	+	+	+	8
Lampe, 2000a	Crossover	+	+	+	+	+	+	+	+	8
Lampe, 2000b	Crossover	+	+	+	+	+	+	+	+	8
Navarro, 2009a	Crossover	+	+	+	+	+	+	+	+	8
Navarro, 2009b	Crossover	+	+	+	+	+	+	+	+	8
Navarro, 2011	Crossover	+	+	+	+	+	+	+	+	8
Peterson, 2009	Crossover	+	+	+	+	+	+	+	+	8
Nijhoff, 1995	Crossover	+	-	+	+	-	+	+	+	6
Pantuck, 1979	Crossover	-	+	-	+	+	+	+	+	6
Pantuck, 1984	Crossover	-	+	-	+	+	+	+	+	6
Riso, 2009	Crossover	+	+	-	+	-	+	+	+	6
Bogaards, 1994	Parallel	-	+	+	+	-	-	+	+	5
Kall, 1996	Crossover	-	+	-	+	-	+	+	+	5
Murray, 2001	Crossover	-	+	-	+	-	+	+	+	5
Riso, 2014	Crossover	-	+	-	+	-	+	+	+	5
Chen, 1996	Crossover	+	+	-	-	-	-	+	+	4
Desager, 2002	Crossover	+	-	+	-	-	-	+	+	4
Vistisen, 1992	Crossover	+	-	-	+	-	-	+	+	4
Leclercq, 1998	Crossover	-	+	-	-	-	-	+	+	3
Murphy, 2001	Crossover	-	+	-	-	-	-	+	+	3
De Waard, 2008	Crossover	-	-	-	+	-	-	-	+	2
Hakooz, 2007	Crossover	-	+	-	-	-	-	-	+	2
McDanell, 1992	Crossover	-	-	-	-	-	-	+	-	1

1 Studies when participants were randomised to dietary intervention groups are (+) (pre-test, post-test designs marked as '-').
2 Number of participants that completed the study. $N \geq 10$ was chosen as per the rationale discussed by Kakuda *et al.* (2014).
3 Indicates whether participant groups were similar at baseline before randomisation (one-group designs, considering activity pre-diet modification post modification, post-test designs, marked as '-').
4 Indicates whether design incorporated a control diet period (either standardised or *Cruciferae*-free?).
5 Presence (+) or absence (-) of controlling kJ intake in participants throughout study or diet standardization based on initial participant weight in kg.
6 Studies with at least two adherence measures (+), e.g. food diary, supervised meal consumption, other studies (-).
7 Choice of statistical test was appropriate for design (+); failure to report marked as '-'.
8 Previous or concurrent validation of any analytical techniques used to analyse participant samples.
9 Sum of number of '+' attributes for the 10 quality characteristics recorded.

Figure 2.5: Dose-response relationship between increase in CYP1A2 activity and daily



2.3.5 Study design, quality and critical analysis

Studies were first ranked based on their design (randomised crossover > non-randomised crossover > parallel group design) and then by other quality characteristics. Of the 23 studies, 12 employed a randomised, controlled, crossover design (Chang *et al.*, 2007; Chen *et al.*, 1996; Desager *et al.*, 2002; Lampe *et al.*, 2000a; Lampe *et al.*, 2000b; Navarro *et al.*, 2009a; Navarro *et al.*, 2011; Navarro *et al.*, 2009b; Nijhoff *et al.*, 1995; Peterson *et al.*, 2009; Riso *et al.*, 2009; Vistisen *et al.*, 1992) meeting four to eight of the quality characteristics; 10 followed a non-randomised crossover (pre-test, post-test) design (de Waard *et al.*, 2008; Hakooz & Hamdan, 2007; Kall *et al.*, 1996; Leclercq *et al.*, 1998; McDanell *et al.*, 1992; Murphy *et al.*, 2001; Murray *et al.*, 2001; Pantuck *et al.*, 1984; Pantuck *et al.*, 1979; Riso *et al.*, 2014) meeting one to six quality characteristics; and one study had a parallel design with two cohorts of participants (Bogaards *et al.*, 1994), although it contained more quality

characteristics (five) than half of the lower-ranked non-randomised crossover studies. With regards to sample size, 78% of the studies had ≥ 10 participants (overall range: *n* = 6 to 73), which has been suggested to be a sufficient number in quasi-experimental crossover pharmacokinetic studies (Kakuda *et al.*, 2014). The majority (74%) of study designs included a basal control diet. Participant adherence to study protocols was addressed in 61% of the studies, with the vast majority of these being randomised controlled trials. Adherence measures included diet diaries for the participants, supervised consumption of dietary intervention meals and housing participants in a research/clinical facility for the duration of the study. Two studies failed to report details of the statistical analyses used in significance testing (de Waard *et al.*, 2008; Hakooz & Hamdan, 2007). All but one study (McDanell *et al.*, 1992) previously or concurrently validated the quantitative assays used to measure substrate and metabolite concentrations or enzyme levels

2.3.6 Discussion

This systematic review investigated the effects of cruciferous vegetable dietary interventions on drug-metabolising enzymes in humans. While there was marked variability in the nature of cruciferous vegetable interventions implemented across the studies, the largest changes in enzyme activity were seen after dietary interventions containing broccoli, cabbage, cauliflower and Brussels sprouts. These cruciferous vegetables also demonstrated a dose-response relationship with CYP1A2 (Peterson *et al.*, 2009), GST-α (Navarro *et al.*, 2009a) and UGT1A1 (Navarro *et al.*, 2009b) activity, in that the increase in enzyme activity roughly doubled the when doubling the amount of these vegetables consumed. The dietary interventions studied affected enzyme activity after at least one week of exposure, with most dietary interventions being consumed for two weeks, giving time for enzyme induction to occur. Conversely, studies with enzyme inhibition hypotheses, such as those investigating CYP2E1, administered their dietary interventions within 12 hours of or immediately before phenotyping to ensure any effect on activity was observed (Chen *et al.*, 1996; Leclercq *et al.*, 1998). Methods of preparing the cruciferous vegetables for the dietary intervention were different across the studies. It has been shown that there are significant differences in the ITC content of cruciferous vegetables depending on whether they are boiled, steamed, stir-fried or microwaved (Verkerk *et al.*, 2009). Therefore, it is difficult to meaningfully compare results between the different trials, especially those that did not use standardised dietary interventions.

The investigated drug-metabolising enzymes included representative CYPs, UGTs and SULTs alongside GST-α, GST-π, NAT2 and xanthine oxidase. The most frequently studied enzymes across the 23 studies were CYP1A2, GST-α, UGT1A6 and UGT1A9 (Table 2.1), and most of

these studies had multiple high-quality characteristics. A wide variety of pharmacokinetic metrics were used especially for CYP1A2. Caffeine-derived composite metrics involving multiple metabolites, such as (AFMU + 1X + 1U)/17X, were used to quantify CYP1A2 activity. Simpler and less resource-intensive indices (by virtue of requiring the analysis of fewer metabolites, therefore allowing for simpler assays) have since been validated, such as the 4-h paraxanthine/caffeine concentration ratios in plasma or saliva (Perera *et al.*, 2012b; Perera *et al.*, 2011). In general, the CYPs have more validated *in vivo* phenotyping probes than the UGTs (Argikar *et al.*, 2008; Miners *et al.*, 2006). While some relatively enzyme-specific UGT probes have shown promise in human studies (Court, 2005; Court *et al.*, 2002), substrate redundancy means that most drugs used for phenotyping UGTs are not specific for the one UGT (Miners *et al.*, 2006). The UGT studies included in this assessment used substrates that were metabolised by more than one enzyme, i.e. paracetamol, racemic oxazepam and endogenous bilirubin. Therefore, it is not possible to determine the specific UGT enzymes induced following cruciferous vegetable consumption, as is also the case for the SULT studies reviewed. However, because it is the overall clearance of a drug that affects systemic concentrations, these studies provide information regarding the potential for diet-drug interactions, even if they cannot identify the specific enzymes involved. It is worth noting that the included studies investigating UGT enzyme activity all reported a link between UGT genotype and ITC exposure—something that has not been formally addressed in the context of how ITC exposure related to changes in CYP activity.

With regards to changes in enzyme activity, of particular note are the studies that investigated CYP1A2 and GST-α. Nearly all of these studies scored highly with regards to their quality characteristics. Further, findings were consistent across these studies, with CYP1A2 and GST-α activity being increased by cruciferous vegetable diets. Individual studies

reported increases in enzyme activity ranging from 15-40% (Table 2.1). The meta-analyses performed demonstrated a significant effect on CYP1A2 and GST-α, with consumption of *Cruciferae* increasing the activities of these enzymes by 20-40% and 15-35%, respectively. Changes in the pharmacokinetics of a medicine as measured by changes in AUC, clearance or phenotyping metrics in the order of 20-30% (Macaluso *et al.*, 2015) can be considered to be of potential clinical relevance, warranting further investigation. This suggests that diets high in cruciferous vegetables could affect the efficacy of drugs (or toxicity of prodrugs) which are substrates for these enzymes. Importantly, all studies included in this review enrolled healthy volunteers; the effect of cruciferous vegetable diets on drug-metabolising enzymes in specific patient groups remains unknown. Therefore, future controlled crossover studies with pharmacokinetic and pharmacodynamic endpoints would be of benefit to ascertain whether specific dietary recommendations are needed for patients undergoing drug therapy with CYP1A2 and GST-α substrates.

Overall, the quality of the literature in this area was considered below average, with only 48% of the 23 included studies found to have adequate sample sizes for their intended purpose, employ a controlled, crossover design, and have multiple high-quality characteristics. These 'gold-standard' studies all implemented resource-intensive adherence measures, such as housing participants for the duration of the study and supervising consumption of dietary intervention meals (Chang *et al.*, 2007; Lampe *et al.*, 2000a; Lampe *et al.*, 2000b; Navarro *et al.*, 2009a; Navarro *et al.*, 2011; Navarro *et al.*, 2009b; Nijhoff *et al.*, 1995; Peterson *et al.*, 2009; Riso *et al.*, 2009). Importantly, nearly all of these studies were framed in the context of a cancer research, in order to understand their contribution to carcinogen clearance as a proposed mechanism of anti-cancer properties (Peterson *et al.*, 2009). This review provides a new commentary and perspective on these data in a clinical

pharmacology context, highlighting how these effects might affect drug therapy patient outcomes.

One limitation of this systematic review was that any database-searchable studies published in languages other than English would not be included. However, the studies included using these methods did not find any papers in other languages during the title and abstract scanning stages of the search process. Meta-analysis was not possible for all enzymes in this review due to the heterogeneous nature or limited number of studies, which was a direct result of deliberately including all drug-metabolising enzymes represented in this literature. While this review has achieved its aims as set out above, it is important to note that the search strategy identified at least 2,000 *in vitro* and other *in vivo* studies that didn't meet the inclusion criteria, and these studies could also provide valuable insight into the mechanisms by which phytochemicals in cruciferous vegetables bring about the observed effects on drug-metabolising enzyme activity reported here. Lastly, the choice to exclude studies that used cruciferous vegetable or ITC isolates in their interventions greatly limited the number of studies included in the review. This decision was made while designing the review's methodology such that any literature included represented dietary interventions which were as 'real-world' as possible, i.e. whole-food or food-homogenate dietary interventions similar to those consumed in the community. Conversely, the strengths of this review lie in its design, with methods adapted from guidelines such as The Cochrane Handbook for Systematic Reviews of Interventions (2011), increasing confidence that all published literature in this area has been included in these findings.

Despite several *in vitro* reports regarding ITCs inhibiting detoxification enzymes (Hamilton & Teel, 1996; Nakajima *et al.*, 2001; Skupinska *et al.*, 2009a; Skupinska *et al.*, 2009b), the

findings of this review are that cruciferous vegetable-enriched diets induce drug metabolism across multiple phase I and II enzymes rather than inhibit it. One explanation for these observations could be that ITCs affect drug-metabolising enzymes in a similar fashion to isoniazid, i.e. short-term inhibition followed by eventual induction of detoxification enzymes (O'Shea *et al.*, 1997; Zand *et al.*, 1993), which is in-line with the above *in vitro*/animal and human studies. Therefore, future in-human studies in this area should include a cruciferous vegetable intervention immediately before phenotyping as well as after 1-2 weeks of consumption to assess potential short-term inhibition and longer-term induction of drug-metabolising enzymes. The proposed mechanisms for ITC-induction of drug metabolism are comprehensively discussed and reviewed elsewhere (Cheung & Kong, 2010; Thornalley, 2002; Zhang, 2004).

2.3.7 Conclusions

In summary, diets high in cruciferous vegetables increase the activity of CYP1A2 and GST-α in healthy volunteers by between 15-40%, with these findings being supported by meta-analysis of multiple studies exhibiting high-quality characteristics. Therefore, people regularly eating large amounts of cruciferous vegetables and concomitantly taking medicines, which are substrates for these enzymes could have altered drug-exposure profiles, contributing to changes in the efficacy and toxicity of affected medicines. It follows that further prospective, controlled, dietary intervention trials involving different substrates of CYP1A2 and GST-α are needed to assess the clinical relevance of cruciferous vegetable food-drug interactions in their relevant disease-state contexts and patient populations.

The quality of the evidence covering the other enzymes included in this review is below average, and it remains unclear if these and other important drug-metabolising enzymes are affected to a clinically significant extent. This statement is especially pertinent for the remaining members of the five main CYP enzymes, namely CYP2D6, 2C19, 2C9 and 3A4, for which there are no published studies that analyse their activity following a cruciferous vegetable intervention.

These data suggest that any future trials investigating the interaction between CYP1A2 activity and *Cruciferae*-enriched diets should show subsequent induction of CYP1A2 enzyme activity. It is important to note that none of the studies included in this review were designed to detect any differences in response to cruciferous vegetables between various geographic ancestries; South Asians were not represented in the data. Further, evidence has been presented that UGT and GST genotypes, especially the null-alleles of these genes, attenuate the response to ITC exposure.

Therefore, the findings of this review generate a rationale to explore how geographic ancestry, genetics and *Cruciferae*-enriched diets interact to affect CYP enzyme activity, which is of interest in Europeans and South Asians for the reasons laid out in Chapter 1. Hypotheses based on this rationale are presented and tested in a prospective, 3-period, controlled trial in Chapter 5 of this thesis.

However, before conducting such a trial, appropriate bioanalytical methods that allow for the effective estimation of CYP activity *in vivo* and measurement of ITC systemic exposure are required. The design, validation and optimisation of two such assays are presented over the next two chapters, and their successful application in a clinical trial is reported in Chapter 5.

3 An improved and optimised version of the 'Inje' and 'Ghassabian' cytochrome P450-phenotyping cocktails: a simplified and highly sensitive UHPLC-MS/MS cocktail assay in human plasma

3.1 Introduction

The importance of studying CYP enzyme activity has been reviewed and discussed in section 1.3.1.1. Further, the utility of the CYP-phenotyping cocktail approach and its relevant background to this thesis was reviewed and discussed in sections 1.6.

Chapter 3 therefore covers the design, validation and optimisation of a simplified UHPLC-MS/MS CYP-phenotyping assay in human plasma, that can be used to simultaneously phenotype CYP1A2, CYP2C19, CYP2C9, CYP2D6 and CYP3A4 across a variety of clinical and research settings. The starting points for this study were the Inje (Ryu *et al.*, 2007) and Ghassabian (Ghassabian *et al.*, 2009) cocktails because of the wide global availability and previous internal and external validation of their CYP450 enzyme-specific probe drugs: caffeine (CYP1A2), omeprazole (CYP2C19), losartan (CYP2C9), dextromethorphan (CYP2D6) and midazolam (CYP3A4).

3.2 Methods

3.2.1 Chemicals and reagents

Caffeine, losartan potassium, omeprazole, paraxanthine and phenacetin were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia); dextromethorphan, dextrorphan tartrate, midazolam and α-hydroxymidazolam were purchased from Kinesis (Redland Bay, QLD, Australia); 5-hydroxyomeprazole and losartan carboxylic acid (EXP-3174) were purchased from Ramidus AB (Lund, Sweden). Acetonitrile, methanol, water, ammonium formate and formic acid were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia); all solvents and reagents were HPLC-grade or above. Oasis HLB solid-phase extraction (SPE) cartridges (3 mL, 60 mg) were purchased from Waters (Dundas, NSW, Australia).

3.2.2 Stock and working solutions

Stock solutions (1 mg/mL) of analytes and the internal standard (IS) phenacetin were prepared by weighing and dissolving compounds in acetonitrile (phenacetin), water (caffeine and paraxanthine) or methanol (all other analytes); dextromethorphan, dextrorphan tartrate, midazolam and α-hydroxymidazolam were supplied as certified standard solutions in sealed amber vials (1 mg/mL in methanol). All stock solutions were stored at -20 °C in silanised vials (Shimadzu, Rydalmere, NSW, Australia). Working solutions for preparation of plasma calibrators and quality control (QC) samples were made by mixing microlitre aliquots of stock solutions and serially diluting with water:methanol:acetonitrile (2:1:1, v/v) to construct the standard curves (10 µL working solution added to 100 µL plasma). Working solutions were prepared with each batch of samples analysed and stored at -20 °C in 2 mL Eppendorf tubes (POCD, Artarmon, NSW, Australia).

3.2.3 Chromatographic conditions

Separation of the analytes was achieved by using an Agilent Zorbax Eclipse XDB-C18 column (4.6 x 50 mm, 1.8 µm) fitted with a Zorbax Eclipse XDB-C18 (4.6 x 5 mm, 1.8 µm) guard column on an Agilent 1290 infinity LC system. Mobile phase A contained 0.1% formic acid (v/v) and 5 mM ammonium formate in water, with mobile phase B consisting of 0.1% formic acid (v/v) and 5 mM ammonium formate in methanol and acetonitrile (50:50, v/v). A gradient was started at 50% B and maintained isocratically for 13 min, then increased to 95% B over 30 s and maintained for 1 min, returning to 50% B at 15 min for 1 min of equilibration. The injection volume was 10 µL with a total run time of 16 min at a flow rate of 0.5 mL/min. Column temperature was set at 30 °C and the autosampler was kept at 4 °C.

3.2.4 Mass spectrometer settings

The samples were analysed on an Agilent 1290 Infinity LC system in tandem with 6460A triple quadrupole mass spectrometers (Agilent Technologies, Santa Clara, CA, USA). Tandem MS was performed using electrospray ionisation equipped with jet stream technology in positive ion mode. The gas temperature was optimised at 350 °C with a flow rate of 12 L/min, while the sheath gas was 375 °C with a flow rate of 11 L/min. Capillary voltage was 3500 °C and the nebuliser pressure was 25 psi. All analytes were detected in multiple reaction monitoring (MRM) mode with fragment voltage set at 135 V using nitrogen as the collision gas. For each analyte and the IS, one quantitative and one to three qualitative ion transitions were monitored. To maximize the detection sensitivity, losartan and losartan carboxylic acid were monitored in a separate MRM time segment. The MS parameters for specific analyte ions are listed in Table 3.1. Data acquisition was performed using MassHunter B.07.01 and data analysis was conducted using the accompanied MassHunter qualitative and quantitative software (version B.07.00, Agilent Technologies).

Table 3.1: Mass spectrometer ion transitions for the 11 analytes. Transitions shown in bold were used for analyte quantification.

Analyte	Precursor (*m*/*z*)	Product (*m*/*z*)	Collision energy (eV)
Caffeine	195.2	123.0	20
	195.2	**138.0**	23
Paraxanthine	181.1	55.1	25
	181.1	**123.9**	27
Omeprazole	346.1	151.3	3
	346.1	**197.8**	15
5-hydroxyomeprazole	362.0	152.2	10
	362.0	**213.9**	25
Losartan	**423.2**	**207.2**	7
	423.2	405.1	15
Losartan carboxylic acid	**437.2**	**179.8**	37
	437.2	206.1	39
Dextromethorphan	**272.3**	**147.1**	30
	272.3	171.0	25
	272.3	213.2	25
Dextrorphan	258.3	133.1	25
	258.3	**157.1**	35
	258.3	199.2	30
Midazolam	326.2	222.8	30
	326.2	**291.1**	37
α-hydroxymidazolam	342.1	203.1	25
	342.1	**323.9**	29
Phenacetin (IS)	180.2	93.1	20
	180.2	**110.1**	27

3.2.5 Method validation

This assay was validated with reference to the US FDA guidelines for bioanalytical studies, which outline acceptable criteria for assay selectivity, sensitivity, accuracy and precision . All assay parameters were validated using drug-free human plasma which was donated by healthy volunteers who had abstained from all study medicines (including sources of dietary caffeine) for at least 72 hours. Calibrators and QCs were prepared by spiking drug-free plasma with known amounts of analytes; different stock and working solutions were used to make calibrators and QCs respectively. Peak area ratios of analytes to the IS across 8 concentrations in plasma were used to generate calibration curves for each analyte.

The linearity of the curves (Table 3.2) was assessed using least-squares regression, while accuracy of the calibrators was assessed by comparing their calculated concentrations with nominal concentrations (relative error; RE). QC samples were prepared at low, middle and high concentrations (Table 3.3) and were analysed in replicates of five across three different days. Inter- and intra-day accuracy were calculated using the RE of QC samples and inter- and intra-day precision was assessed using their relative standard deviation (RSD). Accuracy and precision were deemed acceptable if deviations at a given concentration were ≤ 15%, except at the lower limit of quantification (LLOQ), which could deviate up to 20%. The LLOQ was defined as the lowest point on the analyte calibration curves that met the above accuracy and precision criteria.

Recovery and matrix effects were also investigated. Recovery was assessed by calculating the RE of analyte peak areas in plasma spiked pre-extraction relative to analyte peak areas in blank plasma spiked post-extraction. Matrix effects were evaluated using the RE of analyte peak areas in blank plasma spiked post-extraction relative to analyte peak areas in

water:methanol:acetonitrile (2:1:1, v/v) that contained the same amount of analyte. Formal stability studies were not conducted as these have previously shown no significant changes in analyte response for this cocktail during various sample storage and handling situations (Ghassabian *et al.*, 2009; Grangeon *et al.*, 2017; Oh *et al.*, 2012; Ryu *et al.*, 2007; Yin *et al.*, 2004), however, analyte response was monitored in the stock solutions, working solutions and reconstituted samples left in the autosampler at various times. All showed < 15% RE compared to concentrations derived from fresh calibration curves. Of interest, when left in acetonitrile at room temperature for up to a week, phenacetin showed concentration deviations of < 5% RE, indicating high stability in this solvent.

Table 3.2: Retention times, LLOQs, calibration curve ranges and least-squares regression results. Linearity assessed over 3 different runs.

Analyte	Retention time (min)	LLOQ (ng/mL)	Calibration range (ng/mL)	R^2 (mean ± SD) (*n* = 3)
Caffeine	1.19	23.4	23.4-3000	0.983 ± 0.011
Paraxanthine	1.32	23.4	23.4-3000	0.991 ± 0.010
Omeprazole	2.78	1.95	1.95-250	0.999 ± 0.001
5-hydroxyomeprazole	1.70	7.81	7.81-1000	0.999 ± 0.001
Losartan	6.32	3.58	3.58-458.7	0.993 ± 0.003
EXP-3174	8.76	7.81	7.81-1000	0.993 ± 0.001
Dextromethorphan	2.46	1.09	1.09-139.8	0.985 ± 0.012
Dextrorphan	1.30	0.78	0.78-100	0.994 ± 0.004
Midazolam	2.75	0.78	0.78-100	0.999 ± 0.000
α-hydroxymidazolam	3.70	0.78	0.78-100	0.998 ± 0.001

3.2.6 Sample preparation and analyte extraction

All spiked and clinically-acquired plasma samples were stored at -80 °C until the time of analysis. Plasma aliquots (100 µL) were mixed with 400 µL of acetonitrile containing 2 ng IS and vortex-mixed for 1 min to precipitate plasma proteins, then centrifuged for 10 min at 20 817 *g*. The resulting supernatant was transferred to a 2 mL Eppendorf tube and evaporated under vacuum at 45 °C using a centrifugal concentrator, followed by

reconstitution in 1 mL water and 1 min of vortex-mixing. The reconstituted samples (1 mL) were then loaded onto SPE cartridges which had been conditioned with 1 mL methanol followed by 1 mL. The cartridges were washed with 2 mL water followed by 2 mL 10% methanol in water (v/v) before elution of the analytes with 2 mL methanol. The eluent was evaporated under vacuum at 45 °C, reconstituted in 100 µL water:methanol:acetonitrile (2:1:1, v/v), vortex-mixed for 1 min and centrifuged for 10 min at 20 817 *g* before transferring to an autosampler vial containing a 200 µL insert.

3.2.7 Clinical application

Following validation, this assay was successfully used to analyse samples and phenotype six healthy volunteers. This study was approved by Sydney Local Health District Human Research Ethics Committee and required the participant to provide informed written consent during a face-to-face interview. In brief, after an overnight fast, a venous cannula was inserted into a forearm vein and a baseline t = 0 h blood sample was collected into a lithium-heparinised 10 mL tube (BD, North Ryde, NSW, Australia). Immediately afterwards, the participant was orally administered the CYP-phenotyping cocktail with 250 mL of plain water: caffeine 100 mg (No-Doz® tablets; Key Pharmaceuticals, NSW, Australia), omeprazole 20 mg (Ozmep® enteric-coated tablets; Medis Pharma, NSW, Australia), losartan 25 mg (Cozavan® tablets; Alphapharm, NSW, Australia), dextromethorphan 30 mg (Bisolvon Dry® 10 mg/5 mL liquid; Sanofi-Aventis Healthcare, QLD, Australia) and midazolam 2 mg (Midazolam Sandoz® 5 mg/5 mL vials for injection; Sandoz, NSW, Australia). Caffeine, omeprazole and losartan were administered as tablets, while dextromethorphan (15 mL) and midazolam (2 mL) liquids were swallowed immediately after being mixed together in a small disposable cup. Blood samples were then serially collected at t = 1, 2, 4 and 6 h post-administration of the phenotyping medicines. Plasma was harvested from whole blood

samples by centrifuging the 10 mL collection tubes at 2000 *g* for 10 min followed by removal of the supernatant and storage at -80 °C until analysis. The remaining haematocrit was kept and stored at -80 °C for genotyping purposes. Participants were allowed to consume a small snack after the 2-h sample and lunch after the 4-h sample to minimise any potential food-effects on the pharmacokinetics of the probe drugs.

3.2.8 CYP-phenotyping and pharmacokinetic analyses

The following plasma concentration-time-derived metrics were used to estimate *in vivo* CYP activity: CYP1A2 = paraxanthine/caffeine concentration ratio at 4-h; CYP2C19 = 5-hydroxyomeprazole/ omeprazole concentration ratio at 4- or 6-h (due to variable lag in absorption); CYP2C9 = losartan carboxylic acid $AUC_{0\text{-}6\ h}$/losartan $AUC_{0\text{-}6\ h}$ ratio; CYP2D6 = dextrorphan $AUC_{0\text{-}6\ h}$/dextromethorphan $AUC_{0\text{-}6\ h}$ ratio; and CYP3A4 = α-hydroxymidazolam/midazolam concentration ratio at 4-h. The previous validation of these metrics was discussed in Chapter 1.

3.3 Results

3.3.1 Selectivity and sensitivity

Analyte-free plasma from six different healthy volunteers underwent sample extraction and was checked for interference at the mass transitions and retention times of the 10 analytes and IS. No overlapping peaks or signal abnormalities were detected. LLOQs were determined by choosing analyte concentrations that had peaks at least 5-times higher than the response of a blank sample, and that displayed accuracy of 80-120% and a precision of ≤ 20%. Amounts of analyte injected on-column ranged from 7.80-234.4 pg, representing up to an 80-fold improvement in sensitivity compared to similar assays (Ghassabian *et al.*, 2009; Ryu *et al.*, 2007; Yin *et al.*, 2004). Analyte retention times and LLOQs are displayed in

Table 3.2, and blank plasma and analyte LLOQ chromatogram overlays are depicted in Figure 3.1.

3.3.2 Calibration curves and linearity

Linear equations with 1/x weighting provided the best-fit regression models for all analytes. All calibration curves had coefficients of determination (R^2) of 0.983 or higher and spanned large concentration ranges (Table 3.2).

3.3.3 Accuracy and precision

Intra- and inter-day accuracy and precision for the 10 analytes and IS are shown in Table 3.3. All analytes had intra-day accuracy (RE) and precision (RSD) ranging between 90.7-110.2% and 0.46-11.4% respectively. Inter-day accuracy and precision ranged between 87.0-110.5% and 1.36-11.2% respectively.

3.3.4 Recovery and matrix effects

Recovery ranged from 34.1-104.9% across the analytes and IS at all tested concentrations with high reproducibility and consistency (RSD range 0.48-7.9%), indicating that quantification was not adversely affected for drugs with lower recoveries. Matrix effects varied widely across the analytes and IS at the tested concentrations (range 23.4-251.3%). The most marked ion enhancement was seen for α-hydroxymidazolam (233.0-251.3%), with caffeine (30.8-41.5%), paraxanthine (23.4-27.6%) and dextrorphan (37.4-40.3%) displaying significant ion suppression. In a similar fashion to recovery, matrix effects were consistent and reproducible across batches and plasma sources (RSD range 0.48-10.8%) and did not affect successful quantification of analytes across the tested concentration ranges.

3.3.5 Clinical application of assay

A representative concentration-time profile for each probe drug and their metabolites in a single healthy volunteer receiving the CYP-phenotyping cocktail is shown in Figure 3.3. Calibrators and QCs from these batches all met accuracy (RE 85-115%) and precision (RSD < 15%) requirements.

Table 3.3: Accuracy and precision data for each analyte and the IS. Intra-day accuracy and precision n = 5 for each concentration. Inter-day accuracy and precision n = 15 for each concentration (5 x replicates across 3 different runs).

Analyte	Nominal concentration (ng/mL)	Intra-day accuracy (RE %)	Inter-day accuracy (RE %)	Intra-day precision (RSD %)	Inter-day precision (RSD %)
Caffeine	93.8	97.2	92.9	1.44	6.05
	750	107.1	100.4	4.42	2.98
	3000	93.2	89.9	2.62	3.54
Paraxanthine	93.8	95.2	99.5	8.67	11.2
	750	90.7	95.1	4.54	8.85
	3000	94.4	97.3	3.40	9.00
Omeprazole	7.81	103.8	103.8	2.54	3.95
	62.5	107.6	107.1	1.24	1.36
	250	97.6	97.4	2.00	1.61
5-hydroxyomeprazole	31.3	100.8	95.8	8.90	4.28
	250	98.3	99.1	1.82	2.20
	1000	104.1	101.9	2.63	2.20
Losartan	14.3	97.8	101.4	0.58	2.96
	114.7	96.1	96.6	3.20	3.68
	458.7	101.2	110.5	1.29	3.07
EXP-3174	31.3	98.4	101.4	2.55	3.50
	250	97.4	96.6	11.4	6.59
	1000	110.2	110.5	1.30	3.69
Dextromethorphan	4.37	93.1	104.3	7.88	7.80
	35.0	99.4	102.0	0.46	5.11
	139.8	101.6	102.1	8.31	6.32
Dextrorphan	3.13	99.6	94.2	5.69	6.10
	25	104.3	98.2	7.00	5.84
	100	93.5	87.0	1.69	4.96
Midazolam	3.13	102.4	104.3	3.57	3.45
	25	104.3	101.5	1.13	2.14
	100	98.2	97.9	1.18	1.63
α-hydroxymidazolam	3.13	103.9	100.5	3.82	3.17
	25	99.2	97.5	1.53	2.17
	100	102.1	102.1	1.45	1.99
Phenacetin (IS)	20	N/A	N/A	2.87	5.71

Figure 3.1: Chromatogram overlays of the 10 analytes and internal standard phenacetin as a mixture in human plasma.

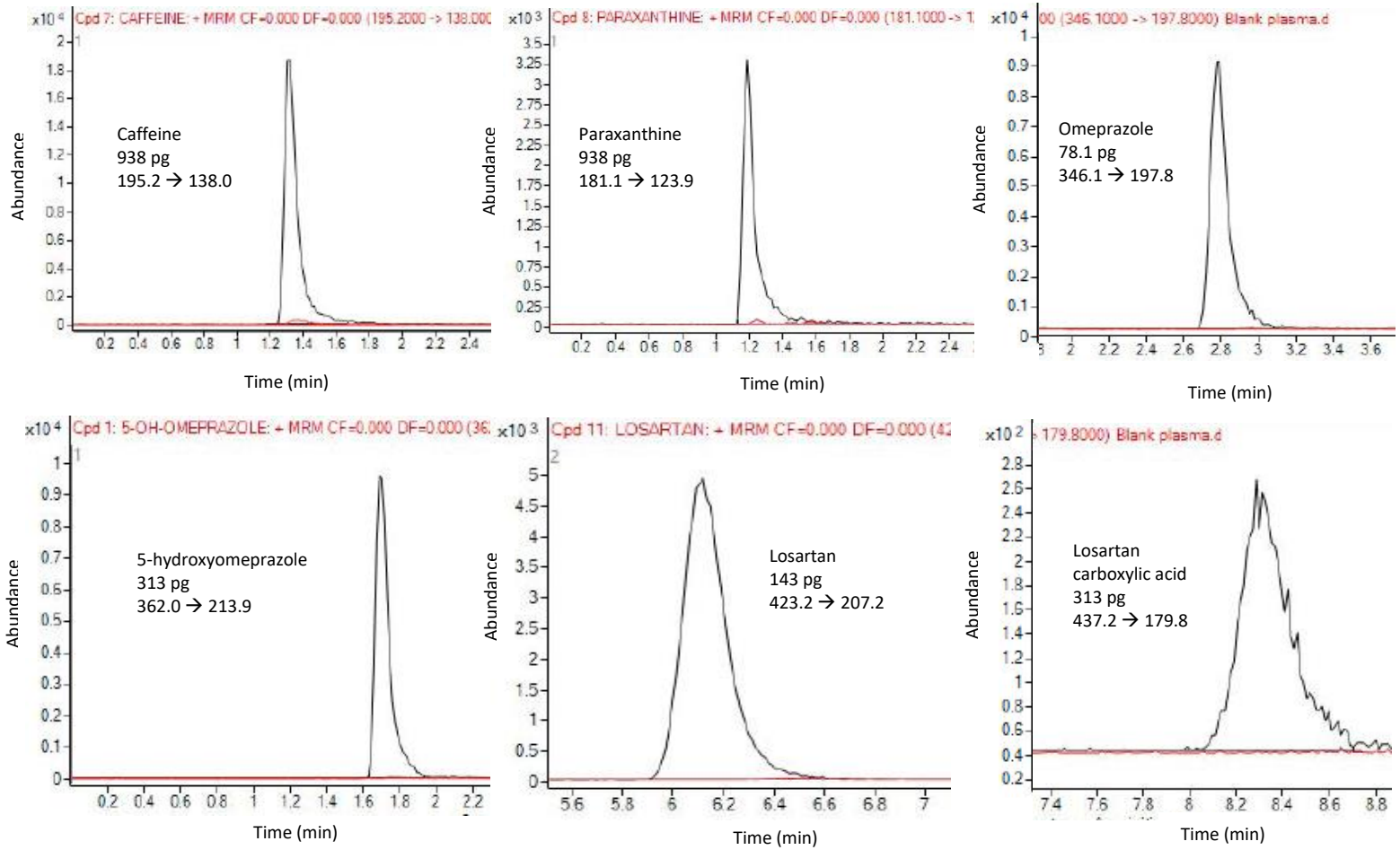
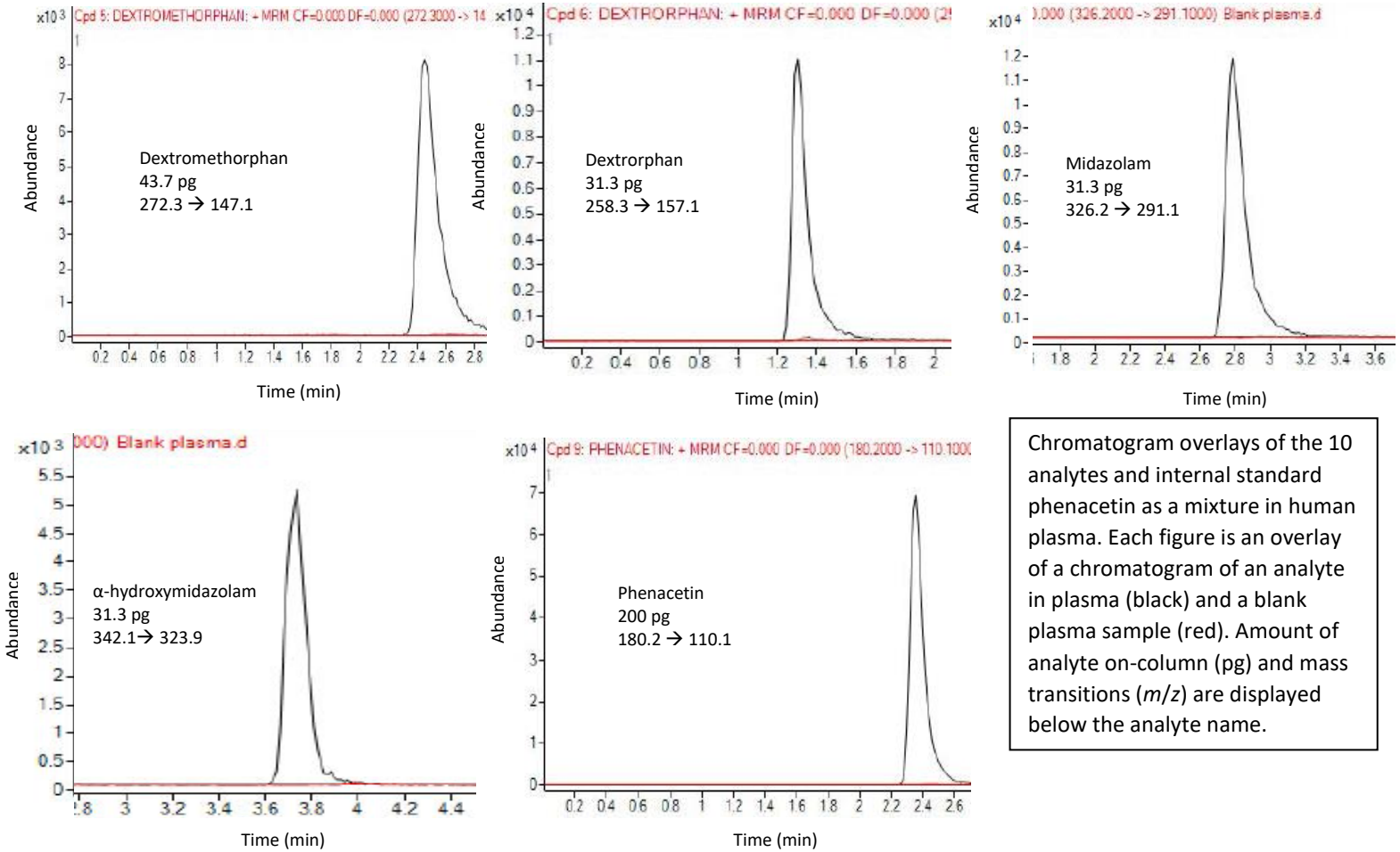
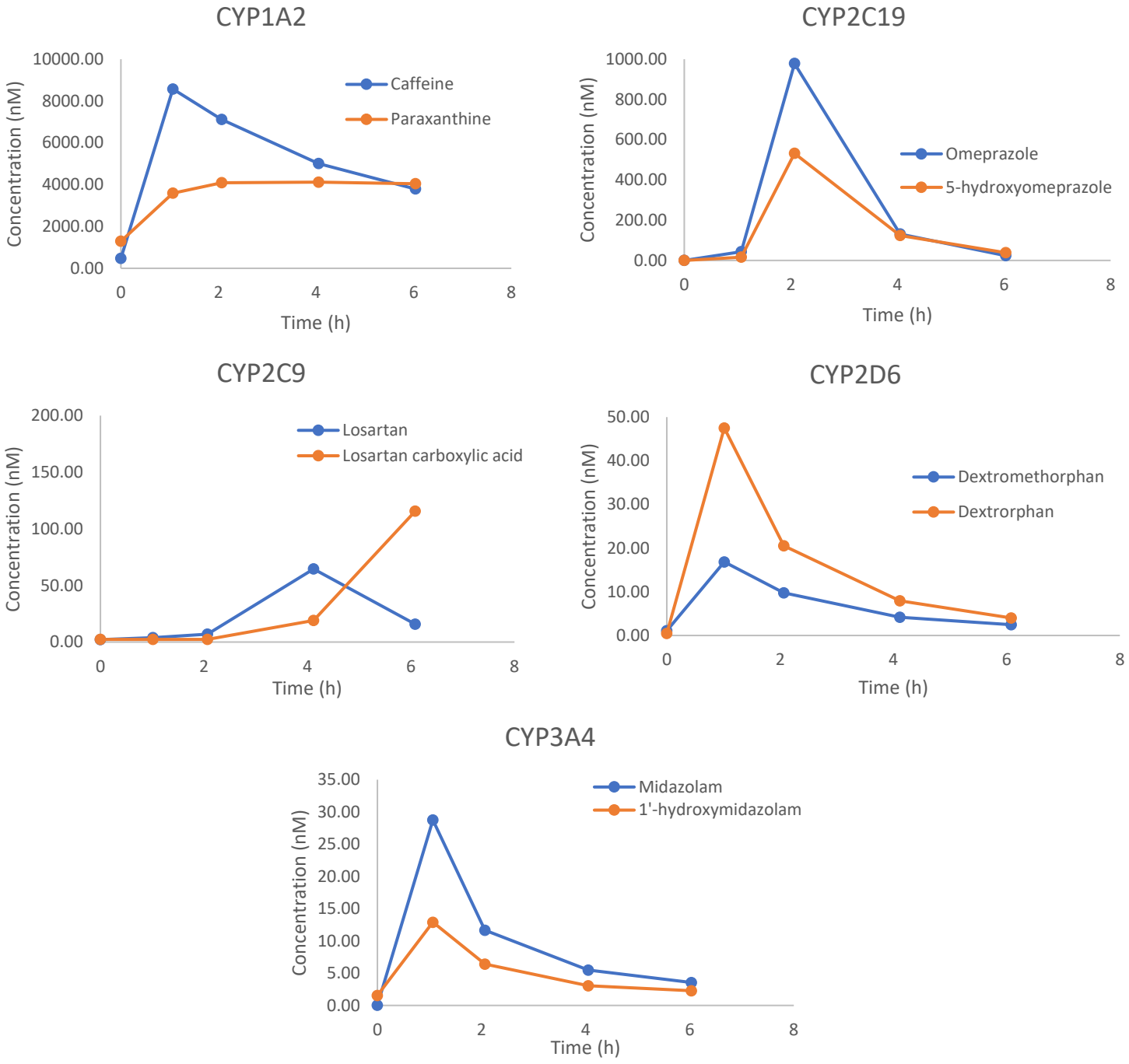


Figure 3.2 (cont.): Chromatogram overlays of the 10 analytes and internal standard phenacetin as a mixture in human plasma.



Chromatogram overlays of the 10 analytes and internal standard phenacetin as a mixture in human plasma. Each figure is an overlay of a chromatogram of an analyte in plasma (black) and a blank plasma sample (red). Amount of analyte on-column (pg) and mass transitions (*m*/*z*) are displayed below the analyte name.

Figure 3.3: Representative concentration-time profiles of the five probe drugs and their primary CYP-catalysed metabolites.



Representative concentration-time profiles of the five probe drugs and their primary CYP450-mediated metabolites. Data shown from one healthy participant who was administered caffeine 100 mg, omeprazole 20 mg, losartan 25 mg, dextromethorphan 30 mg and midazolam 2 mg.

3.4 Discussion

This improved and optimised analytical technique to measure a cocktail of CYP450 probe drugs (Ghassabian *et al.*, 2009; Ryu *et al.*, 2007) meets FDA-recommended specifications for specificity, sensitivity, accuracy and precision. Further, it has been used to analyse the samples from an investigator-initiated clinical trial of the probe drugs demonstrating its ability to be readily applied to clinical research. These methods allow for *in vivo* analysis of five CYP-phenotyping probe drugs, their metabolites and an internal standard in human plasma without evidence of low recovery or significant matrix effects impeding quantification.

Several recently published assays simultaneously analyse more than five CYP-phenotyping probe drugs and their metabolites (Bosilkovska *et al.*, 2014a; Donzelli *et al.*, 2014; Grangeon *et al.*, 2017), namely CYP2B6 and CYP2E1 substrates in addition to CYP1A2, CYP2C19, CYP2C9, CYP2D6 and CYP3A4, otherwise known as 'the main five CYP enzymes' (Zanger & Schwab, 2013). However, the CYP2B6 and CYP2E1 enzymes are estimated to contribute to only 10% of drug metabolism in humans (Zanger & Schwab, 2013), and including them in a cocktail of probe drugs can necessitate an analytical technique with multiple sample extraction steps and chromatographic conditions, along with duplicate sample injections (Grangeon *et al.*, 2017). In fact, the added preparatory and chromatographic complexity when determining these two enzymes in addition to the main five CYP enzymes is significant. Grangeon *et al.* (2017) report two separate, multi-step analyte extraction protocols (one for caffeine and another for the remaining analytes) and three separate chromatographic runs per sample in order to analyse the activity of all seven CYP enzymes. Further, one of the extraction protocols has multiple evaporation and reconstitution sub-

steps, each involving different reconstitution solutions depending on the analytes in question. This compares with the currently presented method, which uses a two-step extraction method (protein precipitation followed by SPE) and one set of chromatographic conditions for all analytes, considerably saving time and money by reducing labour and resource costs per sample.

This optimised assay uses a non-isotopically-labelled IS (phenacetin) for quantification of the five probe drugs and their primary metabolites. Grangeon *et al.* (2017) suggest that using isotopically-labelled analytes as internal standards aids in minimizing matrix effects, which can be true in certain circumstances (Li *et al.*, 2013). However, the extent to which this occurs would be negligible if the analytes do not affect the other analytes present, as is the case for the currently presented assay (Ryu *et al.*, 2007). Further, Ghassabian *et al.* (2009) demonstrated that phenacetin serves as a suitable 'all-rounder' surrogate for the physicochemical profiles of the 10 cocktail analytes, and its use as an internal standard did not adversely affect the accuracy or precision of their quantification; a finding replicated in this improved version of the assay.

Low recovery and significant matrix effects reduce the efficiency of the LC-MS/MS bioanalytical technique, which in turn can translate into a reduction in sensitivity, precision and accuracy (Taylor, 2005). Lower recoveries for paraxanthine, dextromethorphan and dextrorphan, and matrix effects for caffeine, paraxanthine, dextrorphan and α-hydroxymidazolam were noted. While further improving the analytical efficiency of these analytes would be of value and interest, the recoveries and matrix effects were highly consistent within and across batches such that quantification was unaffected, even at the lowest concentrations. Of interest, nearly all similarly-designed cocktail assays report high

recoveries and little or no matrix effects for these analytes in human plasma (Grangeon *et al.*, 2017; Lammers *et al.*, 2016; Oh *et al.*, 2012; Tanaka *et al.*, 2014), which is in contrast with the current study. Only Wohlfarth *et al.* (2012) report ion suppression for dextrorphan (37-58%) at similar values. Further, neither Oh *et al.* (2012) or Ghassabian *et al.* (2009) published matrix effect values. Most studies fail to report RSDs of the matrix effects, and Grangeon *et al.* (2017) report using calibration curves to calculate matrix effects, which should instead be calculated using peak area ratios of samples spiked post-extraction (or from a post-column infusion) to pure analytes in solvent (Van Eeckhaut *et al.*, 2009). Therefore, the true variability of the matrix effects for these analytes remains unknown, and future studies should report means, standard deviations and RSDs of any ion enhancement and suppression observations.

This assay uses both protein precipitation and SPE to prepare the sample for UHPLC-MS/MS analysis. While this adds time and cost for sample preparation compared to simple dilution techniques, the addition of these steps was necessary when routinely injecting large numbers of samples onto the UHPLC system. Preliminary tests injecting untreated plasma on-column caused high-pressure issues and needle seat blockages as injecting untreated, diluted plasma on-column is widely accepted as being problematic for UHPLC-MS/MS systems in a general sense, including negative implications for sensitivity, selectivity and matrix effects (Bonfiglio *et al.*, 1999; Müller *et al.*, 2002). Even when using protein precipitation and SPE, large numbers of samples still caused some high-pressure issues, at a lower frequency and slower rate of pressure rise, highlighting the importance of sample clean-up steps when using sensitive, high-throughput setups Some recent cocktail assays injecting untreated human plasma directly on-column report no adverse effects on

sensitivity, selectivity or matrix effects (Bosilkovska *et al.*, 2014a; Zadoyan *et al.*, 2012), however these techniques do not discuss the effects on assay robustness.

3.5 Conclusions

In conclusion, the currently-presented analytical technique successfully uses UHPLC-MS/MS to simultaneously analyse five CYP phenotyping probe drugs, their primary CYP-mediated metabolites and an internal standard in human plasma. This technique can support the simultaneous *in vivo* phenotyping of the activity of CYP1A2, CYP2C19, CYP2C9, CYP2D6 and CYP3A4. This method meets US FDA recommendations for bioanalytical methods including selectivity, sensitivity, accuracy and precision. This assay's relative simplicity and applicability compares favourably to other published assays for CYP probe drugs. This method represents an improved and optimised version of the internally- and externally-validated cocktails used in the Inje (Ryu *et al.*, 2007) and Ghassabian (Ghassabian *et al.*, 2009) assays, with increases in sensitivity of up to 80-fold, and a significant reduction in sample handling and preparatory complexity.

Following validation of this assay, it was used as described at the end of Chapter 2 to estimate CYP activity in 21 people of either European or South Asian geographic ancestry before, during and after consuming a broccoli-enriched diet (Chapter 5). In a similar fashion, the next chapter outlines a second bioanalytical method that was needed to measure sulforaphane exposure when testing the other hypotheses set out in Chapter 5.

4 An improved UHPLC-MS/MS assay for measuring sulforaphane in human plasma following a broccoli-enriched diet

4.1 Introduction

The importance of the isothiocyanates (ITCs) and their effects on drug-metabolising enzymes were discussed in section 1.7.1 of this thesis. Briefly recapping, these compounds are found in relatively high concentrations in cruciferous vegetables such as broccoli, cabbage and watercress, which feature almost ubiquitously in many diets across the globe (Steinkellner *et al.*, 2001). These compounds induce phase I and II drug-metabolising enzymes, which significantly contribute to the metabolism, and therefore the clearance, of carcinogenic compounds and other xenobiotics (Cheung & Kong, 2010; Thornalley, 2002; Zhang, 2004). Most studies investigating these effects are designed to test hypotheses in a cancer-focussed context, with the two most represented ITCs being sulforaphane (SUL) and phenethyl isothiocyanate (PEITC) (Lamy *et al.*, 2011).

Food-drug interaction studies require selective, sensitive, accurate and precise bioanalytical methods to quantify molecules of interest in biological matrices, which is usually achieved with high-performance liquid chromatography (HPLC) or liquid chromatography-tandem mass spectrometry (LC-MS/MS). Studies to validate and implement assays that quantify SUL that meet these criteria have been used with varying levels of success over the years (Agrawal *et al.*, 2006; Alumkal *et al.*, 2015; Ares *et al.*, 2015; Clarke *et al.*, 2011; Gasper *et al.*, 2005; Hauder *et al.*, 2011; Janobi *et al.*, 2006; Kumar & Sabbioni, 2010; Platz *et al.*, 2015). However, these assays are not without their issues, including 13-35 minute chromatographic run times (Agrawal *et al.*, 2006; Alumkal *et al.*, 2015; Janobi *et al.*, 2006) and relatively large plasma volumes (0.5 mL) (Hauder *et al.*, 2011; Janobi *et al.*, 2006).

Therefore, as outlined in the main objectives of this thesis (section 1.9), the aim of this study was to design, optimise and validate a simplified ultra-performance liquid chromatography (UHPLC)-MS/MS assay for sulforaphane in human plasma, which addresses the above issues and can readily be set up for use in food-drug and ITC-drug interaction studies.

4.2 Methods

4.2.1 Chemicals and reagents

Phenacetin (Internal standard) was purchased from Sigma-Aldrich (Castle Hill, NSW, Australia); (*R*,*S*)-sulforaphane was purchased from Abcam (Melbourne, VIC, Australia). Acetonitrile, methanol, water, formic acid and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). All solvents and reagents were HPLC-grade or above. Discovery C18 solid-phase extraction (SPE) cartridges (1 mL, 100 mg) were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia).

4.2.2 Stock and working solutions

Stock solutions of SUL and the internal standard (IS) phenacetin were prepared at 1 mg/mL by weighing and dissolving the compounds in acetonitrile. All stock solutions were stored at -20 °C in silanised vials (Shimadzu, Rydalmere, NSW, Australia). Working solutions for preparation of plasma calibrators and quality control (QC) samples were made by mixing microlitre aliquots of stock solutions and serially diluting with 0.1% formic acid in water (v/v), such that 10 µL of working solution when added to 100 µL plasma produced analyte concentrations used to construct standard curves. Working solutions were prepared fresh at the start of a given batch of samples and stored at -20 °C in 2 mL Eppendorf tubes (POCD, Artarmon, NSW, Australia).

4.2.3 Chromatographic conditions

Analyte separation was achieved on an Agilent Zorbax Eclipse XDB-C18 column (4.6 x 50 mm, 1.8 µm) fitted with a UHPLC Zorbax Eclipse XDB-C18 (4.6 x 5 mm, 1.8 µm) guard column. Mobile phase A contained 0.1% formic acid in water (v/v) and mobile phase B consisted of 0.1% formic acid in acetonitrile (v/v). A gradient was started at 50% B and

maintained isocratically for 5 min, then increased to 100% B over 1 min and maintained for 1 min, returning to 50% B over 0.5 min followed by 1 min of equilibration. The injection volume was 10 µL with a total run time of 8.5 min at a flow rate of 0.2 mL/min. Column temperature was set at 30 °C and the autosampler was kept at 4 °C.

4.2.4 Mass spectrometer settings

Analyses were performed on an Agilent 1290 series UHPLC system in tandem with 6460A triple quadrupole mass spectrometers (Agilent Technologies, Santa Clara, CA, USA). Tandem MS was conducted using electrospray ionisation equipped with jet stream technology in positive ion mode. The gas temperature was optimised at 350 °C with a flow rate of 12 L/min, while the sheath gas was 375 °C with a flow rate of 11 L/min. Capillary voltage was 3500 °C and the nebuliser pressure was 25 psi. SUL and the IS were detected in multiple reaction monitoring (MRM) mode with fragment voltages set at 60 V and 80 V, respectively, using nitrogen as the collision gas. For both molecules, one quantitative and one qualitative ion transition were monitored. A detailed summary of the MS parameters for specific analyte ions is presented in Table 4.1. Data was acquired using MassHunter B.07.01 and data analysis was conducted using the accompanied MassHunter qualitative and quantitative software (version B.07.00, Agilent Technologies).

Table 4.1: Mass spectrometer ion transitions for SUL and the phenacetin (IS). Transitions shown in bold were used for analyte quantification.

Analyte	Precursor (*m/z*)	Product (*m/z*)	Collision energy (eV)
Sulforaphane	**178.0**	**72.0**	40
	178.0	114.1	15
Phenacetin (IS)	180.1	65.1	35
	180.1	**110.0**	25

4.2.5 Method validation

This assay was validated with reference to the US FDA guidelines for bioanalytical studies, which outline acceptable criteria for assay selectivity, sensitivity, accuracy and precision. All assay parameters were validated using ITC/drug-free human plasma, which was donated by healthy volunteers who had abstained from all medicines and cruciferous vegetables for at least one week. Calibrators and QCs were made by spiking ITC/drug-free plasma with known amounts of SUL; different stock and working solutions were used to make calibrators and QCs respectively. The peak area ratios of SUL to the IS across eight concentrations in plasma were used to generate calibration curves for the purpose of quantification. The linearity of the curve was assessed using least-squares regression, while accuracy of the calibrators was assessed by comparing their calculated concentrations with nominal concentrations (relative error; RE).

QC samples were prepared at low, middle and high concentrations and were analysed in replicates of five across three different days. Inter- and intra-day accuracy were calculated using the RE of QC samples and inter- and intra-day precision was assessed using their relative standard deviation (RSD). Accuracy and precision were deemed acceptable if deviations at a given concentration were ≤ 15%, except at the lower limit of quantification (LLOQ), which could deviate up to 20%. Recovery and matrix effects were also investigated. Recovery was assessed by calculating the RE of SUL or IS peak areas in plasma spiked pre-extraction with analyte peak areas in blank plasma spiked post-extraction. Matrix effects were evaluated using the RE of analyte peak areas in blank plasma spiked post-extraction, to analyte peak areas in 0.1% formic acid in water (v/v) that contained the same amount of analyte.

Formal stability studies in plasma were not conducted as these have been reported extensively by others (Janobi *et al.*, 2006; Platz *et al.*, 2015). However, SUL and IS responses were monitored in stock solutions, working solutions and reconstituted samples left in the autosampler at various times, all showing < 15% RE compared to concentrations determined from fresh calibration curves.

4.2.6 Sample preparation and analyte extraction

All spiked and clinically-acquired plasma samples were stored at -80 °C. Plasma aliquots (100 µL) were mixed with 10 µL of acetonitrile containing 5.5 ng IS and briefly vortex-mixed. Then, 20 µL of TFA was added to the samples followed by vortex-mixing for 1 min to precipitate plasma proteins, which were then centrifuged for 10 min at 20 817 *g*, 4 °C. The samples then underwent SPE: cartridges were conditioned with 1 mL methanol followed by 1 mL 0.1% formic acid in water (v/v) prior to loading of the above supernatant (approx. 140 µL). The cartridges were washed with 1 mL 5% methanol in water (v/v) before elution of the analytes with 2 x 0.5 mL 90% acetonitrile + 0.1% formic acid in water (v/v). The eluent was evaporated under vacuum at 45 °C, reconstituted in 100 µL 0.1% formic acid in water (v/v), vortex-mixed for 1 min then centrifuged for 10 min at 20 817 *g*, 4 °C, before transferring to an autosampler vial containing a 200 µL insert.

4.2.7 Clinical application

Following validation, this assay was successfully used to analyse samples provided by healthy volunteers on a broccoli-enriched diet (n = 21), which formed part of a larger study detailed in Chapter 5. The study had ethics approval from the Sydney Local Health District Ethics Committee and required participants to provide written informed consent during a face-to-face interview. Sample collection was as follows: after providing a baseline sample

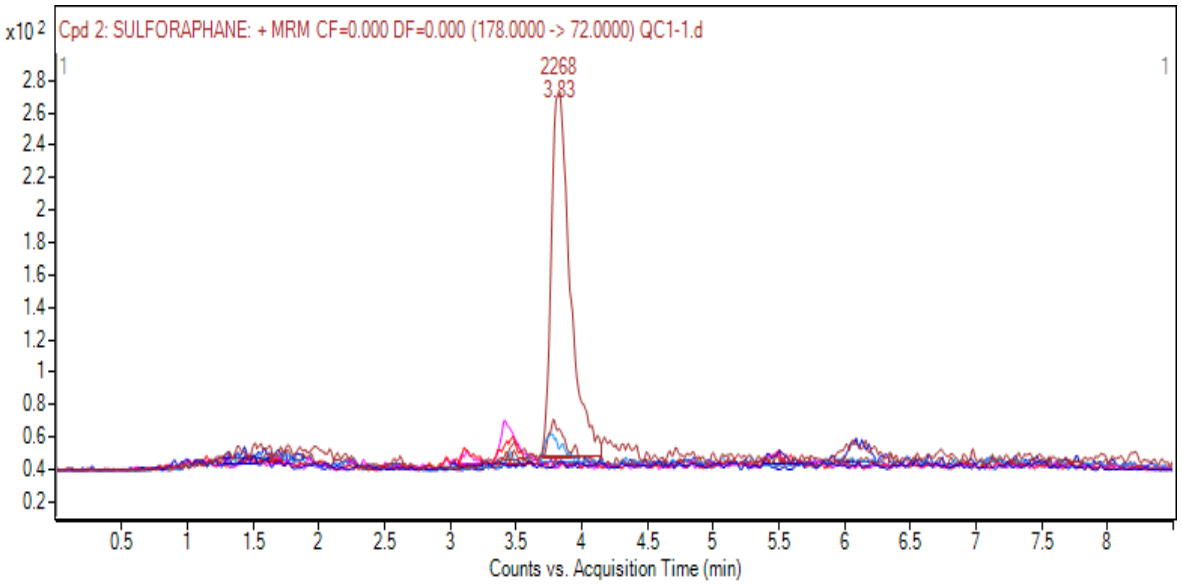
and an overnight fast, participants ate a 200 g microwave-steamed broccoli meal followed by insertion of a venous cannula into a forearm vein. Serial blood samples were then collected into lithium-heparinised 10 mL tubes (BD, North Ryde, NSW, Australia) at t = 2, 3, 4, 6 and 8 h post-broccoli consumption. Plasma was harvested from whole blood samples by centrifuging the 10 mL collection tubes at 2000 *g* for 10 min followed by removal of the supernatant and storage at -80 °C until analysis. Participants were allowed to have a small snack after the 4 h sample and lunch after the 6 h sample to minimise any potential food-effects on the study endpoints.

4.3 Results

4.3.1 Selectivity and sensitivity

Analyte-free plasma from six different volunteers underwent sample extraction and was checked for interference at the mass transitions and retention times of SUL and the IS. No overlapping peaks or signal abnormalities were detected. LLOQs were determined by choosing analyte concentrations that had peaks at least 5-times higher than the response of a blank sample and that displayed accuracy of 80-120% and a precision of ≤ 20%. Amounts of analyte injected on-column ranged from 7.8-100 pg, representing up to a 227% increase in sensitivity compared to similar assays (Gasper *et al.*, 2005). Retention times for SUL and the IS were 3.42 min and 4.42 min, respectively. The LLOQ for SUL was 0.78 ng/mL (7.8 pg on-column). Chromatogram overlays of blank plasma and SUL at low concentrations are shown in Figure 4.1.

Figure 4.1: Overlay of seven different sulforaphane chromatograms demonstrating assay selectivity and sensitivity.



Six chromatograms are of blank plasma from six different volunteers and one internal control plasma with sulforaphane added at the low-QC concentration (3.13 ng/mL, 31.3 pg on-column; retention time = 3.83 min, peak area 2268).

4.3.2 Calibration curves, linearity, accuracy and precision

A linear equation with 1/x weighting provided the best-fit regression model for SUL. The coefficients of determination (R^2) were of 0.989 or higher with concentrations ranging from 0.78-100 ng/mL.

Intra- and inter-day accuracy and precision for SUL and the IS are shown in Table 4.2. All SUL had intra-day accuracy (RE) and precision (RSD) ranging between 86.4-106.7% and 2.61-10.3% respectively. Inter-day accuracy and precision ranged between 91.3-97.0% and 3.99-7.11% respectively.

Table 4.2: Accuracy and precision data for SUL and the phenacetin (IS).

Analyte	Nominal concentration (ng/mL)	Measured mean concentration (ng/mL) (mean ± SD) (*n* = 15)	Intra-day accuracy (RE %) (*n* = 3)	Inter-day accuracy (RE %) (*n* = 3)	Intra-day precision (RSD %) (*n* = 15)	Inter-day precision (RSD %) (*n* = 15)
SUL	100 25 3.125	92.4 ± 6.48 23.9 ± 1.32 3.03 ± 0.32	106.7 101.2 96.3	97.0 95.6 91.3	7.18 2.61 3.73	7.11 3.99 6.40
IS	55	N/A	N/A	N/A	7.48	2.94

4.3.3 Recovery and matrix effects

Recovery was low for both SUL and the IS (Table 4.3). However, both molecules had reproducible and consistent recoveries across all tested concentrations (RSD range 6.82-11.0%), therefore quantification was not adversely affected. With regards to matrix effects, ion suppression was seen for SUL and the IS (Table 4.3), although as with recovery, matrix effects were consistent and reproducible across batches and plasma sources (RSD range 6.59-10.6%) and did not affect successful quantification of SUL across the tested concentration ranges.

Table 4.3: Recovery (RSD% ± SD) and matrix effect data (mean ± SD) for sulforaphane and the phenacetin (IS).

Analyte	Nominal concentration (ng/mL)	Recovery (mean ± SD)	Recovery RSD %	Absolute matrix effect (mean ± SD)	Absolute matrix effect RSD %
SUL	100 25 3.125	34.7 ± 2.36 25.2 ± 2.78 26.4 ± 2.30	6.82 11.0 8.69	62.3 ± 4.11 78.2 ± 8.29 82.1 ± 7.29	6.59 10.6 8.88
IS	55	46.3 ± 4.39	9.50	75.0 ± 7.52	10.0

4.3.4 Clinical application of assay

This assay has been used to phenotype 21 participants (> 150 plasma samples) in the above-mentioned clinical trial (these data are further explored and discussed in Chapter 5). Calibrators and QCs from these batches all met accuracy (RE = 85-115%) and precision (RSD < 15%) requirements.

4.4 Discussion

This validated UHPLC-MS/MS assay meets appropriate specifications for selectivity, sensitivity, accuracy and precision, and has successfully been used to analyse SUL in over 150 clinical trial samples, demonstrating its ability to be readily applied to dietary intervention studies. These methods allow for *in vivo* analysis of SUL in studies involving a cruciferous vegetable or SUL intervention, and provide evidence that low recovery and ion suppression do not impede analyte quantification.

Other assays that analyse SUL and its metabolites have relatively long chromatographic run times of 13-35 min (Agrawal *et al.*, 2006; Alumkal *et al.*, 2015; Janobi *et al.*, 2006), which is a barrier to high-throughput and efficiency when analysing large numbers of samples. The per-sample run time of the currently presented assay is 8.5 min, which represents up to a 4-fold improvement compared to similar assays.

In an effort to further reduce the resource demands and costs of this assay, a non-isotopically-labelled IS (ILIS) was used for quantification of SUL in plasma. ILISs can help to reduce matrix effects (Grangeon *et al.*, 2017; Li *et al.*, 2013). However, phenacetin has been used as a suitable IS for a range of analytes with varying physicochemical properties (Ghassabian *et al.*, 2009) (Chapter 3). Its use as an internal standard produced appropriate accuracy and precision for analyte quantification in these studies, which has also been observed for this SUL assay.

As discussed in Chapter 3, sensitivity, precision and accuracy can be reduced by low recovery and significant matrix effects (Taylor, 2005). The presented methods demonstrate low recoveries and ion suppression for SUL and the IS. However, the recoveries and matrix effects were highly consistent within and across batches such that quantification was

unaffected, even at the lowest concentrations. Unfortunately, matrix effect comparisons with other studies are not possible, as published SUL assays with similar analyte extraction techniques do not report matrix effect data (Alumkal *et al.*, 2015; Clarke *et al.*, 2011; Gasper *et al.*, 2005; Hauder *et al.*, 2011; Janobi *et al.*, 2006; Platz *et al.*, 2015). Future studies should report the means, standard deviations and RSDs of any observed matrix effects in order to better assess whether they affect the quantification of the analytes being investigated.

This assay uses both protein precipitation and SPE to prepare the sample for UHPLC-MS/MS analysis, which adds to sample preparation time in contrast to dilution and filtration methods. However, we found the addition of these steps necessary when injecting large numbers of samples onto the UHPLC system, as preliminary tests of injecting undiluted plasma on-column caused frequent high-pressure issues and needle seat blockages, consistent with previous reports that injection of untreated, diluted plasma on-column is problematic for UHPLC-MS/MS systems (Bonfiglio *et al.*, 1999; Müller *et al.*, 2002) (Chapter 3).

The sulforaphane concentration-time profiles generated using this assay are presented in Chapter 5, and highlight the utility of this assay in clinical studies that have cruciferous vegetable or isothiocyanate extract interventions. The sensitivity and resolution of this assay allow for quantification down to 7.8 pg on-column, representing plasma concentrations of 0.78 ng/mL—high enough to detect sulforaphane up to 8 hours post-consumption. Due to its ready applicability, the use of this validated, simplified assay is encouraged when conducting future cruciferous vegetable/ITC studies.

4.5 Conclusions

In conclusion, the currently-presented assay successfully uses UHPLC-MS/MS to analyse SUL and an internal standard in human plasma. The methods described meet recommendations for bioanalytical methods concerning selectivity, sensitivity, accuracy and precision (Booth & Kadavil, 2001). This assay's relative simplicity and short chromatographic run times compared to other published SUL assays has been demonstrated by its use in analysing over 150 samples. The presented method demonstrates an increase in sensitivity of up to 227% alongside reductions in sample handling and resource costs.

Following validation of this assay, it was used as described at the end of Chapter 2 to measure the plasma concentrations and exposure profiles of SUL in 21 people of either European or South Asian geographic ancestry before, during and after consuming a broccoli-enriched diet (Chapter 5). This assay and the CYP-phenotyping cocktail assay presented in Chapter 3 were both used successfully to test hypotheses generated from the thesis objectives (section 1.9), and these data are analysed and discussed in the next chapter.

5 The effects of broccoli consumption, sulforaphane exposure and genetics on the activity of drug-metabolising enzymes in people of European and South Asian ancestry

5.1 Introduction

Thus far, this thesis on variability in drug metabolism has explored the effects of cruciferous vegetables on phase I and II enzymes, identifying that in particular, CYP1A2 is significantly induced in persons consuming diets rich in these foods. The theme of geographic ancestry has also been introduced in Chapter 1, and understanding geographic ancestry as a composite of known and unknown intrinsic and extrinsic factors that affect the pharmacokinetics of drugs has been discussed. Suitable bioanalytical methods are required in order to conduct prospective human trials that explore the interactions between cruciferous vegetable-enriched diets, ancestry and drug metabolism; the design, optimisation and validation of two such assays have been described in Chapters 3 and 4.

This chapter unites all of the themes and research presented thus far, by demonstrating the application of the presented assays to answering hypotheses involving geographic ancestry, genetics, cruciferous vegetable-enriched diets and drug metabolism.

CYP1A2 activity has been shown to vary in populations of European or South Asian ancestry and the presence or absence of *Cruciferae* in the diet (Chapters 1 and 2). In one particular observational study, broccoli consumption was associated with increased CYP1A2 activity in the European cohort, but lower activity in the South Asian cohort (Perera *et al.*, 2012a). It was hypothesised that differences in food preparation, including the currying of foods, could be producing this difference in enzyme activity, alongside other unknown ancestry-specific

differences. Of note, these findings have not been confirmed in a prospective, controlled trial.

Further, CYP2C19, CYP2C9, CYP2D6 and CYP3A4 have not been investigated in this context (Chapter 2) and no studies were identified that have investigated the effects of both geographic ancestry and *Cruciferae* on these enzymes. There are common overlaps between the CYPs with respect to induction mechanisms and hence a molecule that induces CYP1A2 could also, via the same pathways, induce other CYP enzymes (Tompkins & Wallace, 2007). Therefore, the known effects of broccoli on increased CYP1A2 activity may also be reflected in the effects on other CYP enzymes represented in the phenotyping cocktail.

Therefore, the aims of this clinical trial were to:

1) Investigate the acute and medium-term effects of a broccoli-enriched diet on CYP1A2, CYP2C19, CYP2C9, CYP2D6 and CYP3A4 activities;
2) Establish if the above effects vary between healthy subjects of European and South Asian ancestry;
3) Explore the effects of diet and genetics on SUL exposure, and see if these vary between Europeans and South Asians;
4) Confirm that CYP1A2 is induced in Europeans on a broccoli-enriched diet, with no or a reduced change in South Asians; and
5) Explore the contribution of diet, genetics and geographic ancestry on CYP1A2, CYP2C19, CYP2C9, CYP2D6 and CYP3A4 activities in the two ancestry groups.

5.2 Methods

This trial received general ethics approval and site-specific approval (SSA) from the Sydney Local Health District (SLHD) ethics committee at Concord Repatriation General Hospital (CRGH) (Approval ID HREC/12/CRGH/206) (Appendices 8.3 and 8.4). The study was conducted in an outpatient clinic at CRGH. The trial was prospectively registered with the Australian New Zealand Clinical Trials Registry (ANZCTR) (Trial ID ACTRN12613001112752) (Appendix 8.5), which is a World Health Organisation International Clinical Trials Registry Platform (WHO ICTRP). The Therapeutic Goods Administration (TGA) was notified of the use of the study medicines as CYP enzyme probe drugs (Appendix 8.6).

5.2.1 Participants

The study population consisted of healthy, community-dwelling males aged between 18-55 years (*n* = 21; see section 5.2.7.1 for sample size calculations). Participants were of European (*n* = 11) and South Asian (*n* = 10) geographic ancestry who were willing to participate in a dietary intervention trial. All participants were screened for study eligibility at a face-to-face interview during which a questionnaire was completed (Appendix 8.7), ensuring that the inclusion criteria were met and no exclusion criteria were met (Figure 5.1). Patients were labelled as 'self-reported healthy' if they reported no diseases or concurrent therapeutic substance use as per the questionnaire. After eligibility was confirmed, written, informed consent to participate was obtained (Appendix 8.8) and a copy of the Participant Information Sheet (PIS) was given (Appendix 8.9).

Previous reports of differences in CYP activity by sex are varied, with findings being divided between no difference and activity being higher in men than women (Nehlig, 2018). Further, CYP1A2 activity can be influenced by sex hormones, including the contraceptive pill (Perera

et al., 2012a). Therefore, to reduce the potential for confounding by the influence of sex differences, it was decided to recruit only male participants in this study in order to achieve higher confidence in and better explain the observed interactions between diet, ancestry and genetics.

Geographic ancestry was self-determined by each participant based on the geographic origins of the participant's four grandparents. Participants were eligible to participate if all four of their grandparents (explicitly, their biological parents' biological parents) were of the same geographic ancestry.

Participants were recruited via university noticeboards and web distribution services by means of a poster (Appendix 8.10) and electronic advertisements approved by the HREC. On successful and full completion of the study, participants received A$500 to compensate them for their time and effort.

Participants were assigned a random number and thus de-identified in paper and electronic records. Hard copy consent forms and survey questionnaires were stored in a locked cabinet in a secured room. All information obtained from participants was coded in spreadsheets and password protected. Data will be kept for at least six years and then disposed of through deletion (in the case of computer files), shredded (in the case of hardcopy documents) or disposed in clinical waste (for clinical samples). All procedures were in accordance with the University of Sydney Research Data Management Policy, 2014 (http://sydney.edu.au/policies/showdoc.aspx?recnum=PDOC2013/337&RendNum=0, retrieved 17/01/18) and Research Data Management Procedures, 2015 (http://sydney.edu.au/policies/showdoc.aspx?recnum=PDOC2014/366, retrieved 17/01/18).

Figure 5.1: Participant inclusion and exclusion criteria.

Inclusion criteria

i. Male, aged 18-55 years
ii. European or South Asian geographic ancestry
iii. Non-smoker or ex-smoker that has abstained from smoking for at least 6 months
iv. Ability to comply with the planned procedures and provide written informed consent which requires a level of English language competency to effectively understand the protocol and adequately meet the definition of informed consent
v. Self-reported healthy with no reported or diagnosed acute or chronic health conditions
vi. Access to a microwave with an appropriate cooking setting

Exclusion criteria

i. Known hypersensitivity or intolerance to any of the five drugs used in the study
ii. History of clinical signs of hypotension, specifically fainting, light-headedness and/or a blood pressure lower than 80/60 mmHg
iii. Female
iv. Known food allergies or intolerances that limit participation in this study
v. Oral antimicrobial use within the past 3 months
vi. Body mass index (BMI) outside of 18.5-32.5 $kg.m^{-2}$
vii. Currently taking or using any prescription, over-the-counter, herbal, complementary or illicit medicines
viii. Given blood or participating in another clinical study in the last 3 months

5.2.2 Study design

This study adopted an open-label, 3-period, sequential, crossover dietary intervention trial design. Participants completed two 3-day food diaries (Appendices 8.11 and 8.12) over the three days leading up to Day 1 and during Days 6-8, inclusive. Figure 5.2 outlines the study design. On Day 1, the CYP probe cocktail (see section 5.2.3 below) was administered following an overnight fast. All food and beverages were provided on-site during the study days; a plain muffin, choice of a chicken and salad or plain salad sandwich and water were offered to the participants. Each study day, this food was ordered from the same on-site canteen at the same time of day to minimise the introduction of any potential variability. The muffin was offered after collecting the 2-hour blood sample and the sandwich was offered after the 4-hour blood sample was collected. Water was freely available throughout the study days.

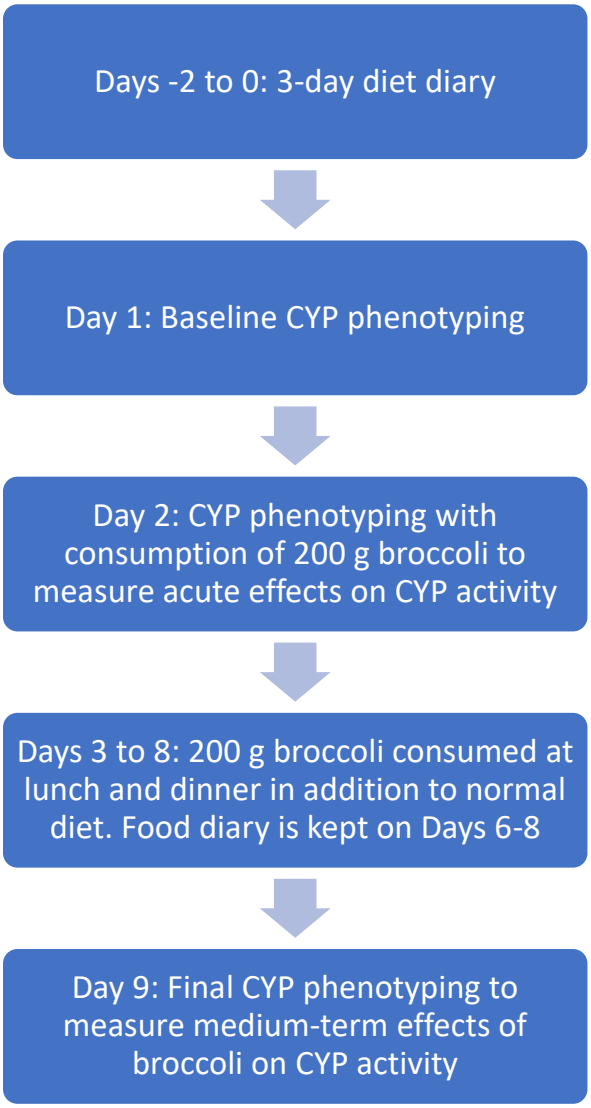
On Day 2, 200 g of microwave-steamed broccoli (see 5.2.3 below) was consumed 2 hours before the CYP probe cocktail was administered to assess the acute effects of broccoli consumption on CYP activity. After the last venous blood sample was collected at six hours post-dose, participants were provided with pre-packaged and labelled 'snap-lock' bags containing weighed broccoli servings to be microwaved and consumed for the next six days (Days 3-8).

From Days 3-8, participants consumed 200 g of broccoli with lunch and dinner whilst following their usual dietary habits. Participants were instructed to maintain their usual diet throughout the study and make no major changes to the types and quantities of food that they usually consume. They were also asked to avoid consumption of grapefruit juice throughout the study, have no more than 4 standard drinks of alcohol on any one occasion

and avoid caffeine on the mornings that the CYP phenotyping cocktail was being administered. As mentioned above, a second food diary was completed during this period to monitor foodstuffs consumed and encourage adherence to the study protocol. On Day 9, participants returned following an overnight fast for the final administration of the CYP probe cocktail.

To ensure participant safety, pressure and pulse (via automated blood pressure monitor), respiratory rate and level of sedation were monitored hourly throughout the study period. This monitoring process is described in Appendix 8.13.

Figure 5.2: Summary diagram of sequential timeline of the clinical study.



5.2.3 Broccoli procurement and preparation

Broccoli was purchased fresh from one local supplier (Stewart Dickson Produce Pty Ltd) to reduce inter-batch variability. For the duration of the study, an arrangement was made with the supplier that broccoli would come from the same two growers, who had neighbouring farms. The broccoli was washed for 5 seconds under running tap water and then cut at the short part of the stem with the florets still attached into easily eaten pieces. The pieces were then weighed and divided into 200 g quantities, placed in snap-lock sealable plastic bags and stored at 4 °C until use. Participants were instructed to ensure that the broccoli

was kept refrigerated until consumed. Participants were provided with a BPA-free steaming container and instructed to microwave the broccoli at a wattage and time specified below in Table 5.1. These recommendations were based on Verkerk *et al.* (2009) and Song and Thornalley (2007) which suggested that microwave steaming for 3 min at 1000 W produced the lowest decrease in ITC content. During informal palatability testing it was determined that this could be further decreased to 2 min at 1000 W, which was then linearly scaled to produce the times in Table 5.1.

Table 5.1: Recommended broccoli cooking times based on available microwave power and published evidence of broccoli constituent stability (Chapter 1).

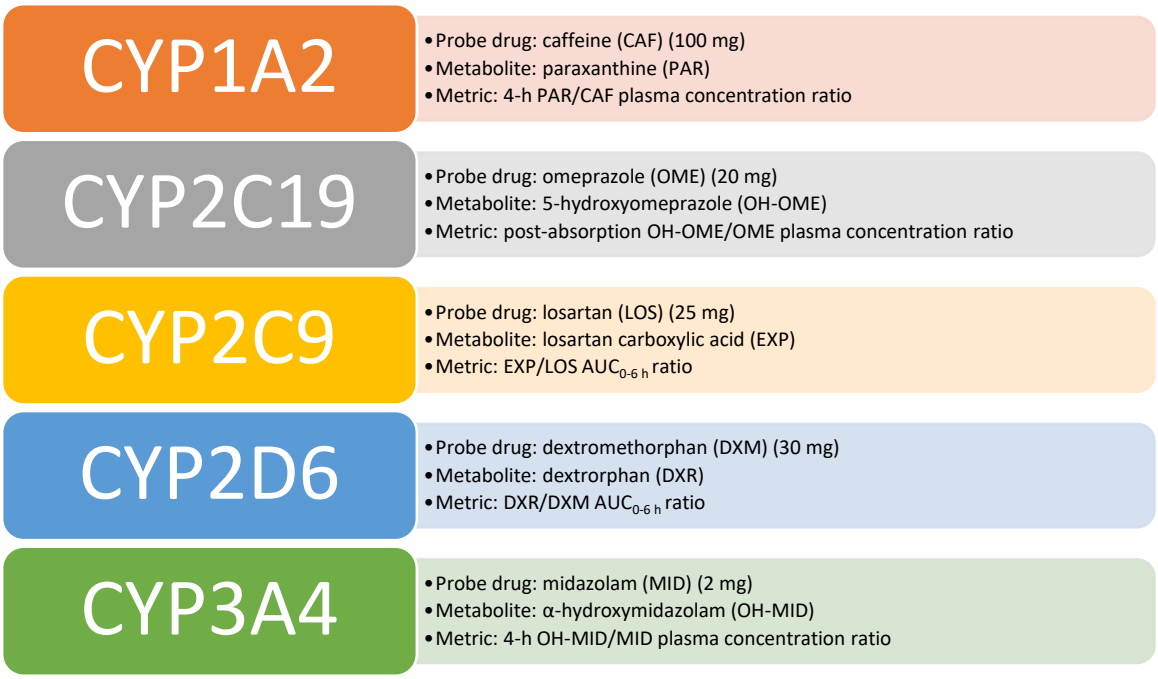
Microwave power (watts)	Recommended cooking time (seconds)
700	150 (2 min 30 s)
800	135 (2 min 15 s)
900	130 (2 min 10 s)
1000	120 (2 min)
1200	110 (1 min 50 s)

Participants consumed the entire contents of the 200 g broccoli sample on each occasion. If this method of eating the broccoli was unappealing to the participant to the point of non-compliance or drop-out, a small amount (< 10 mL) of salad dressing was permitted to be added before eating. Details on how to handle, prepare and consume the broccoli were given to participants in a handout (Appendix 8.14).

5.2.4 CYP-phenotyping procedure

The CYP probe drugs, metabolites, phenotyping metrics and sampling procedure used are described in section 3.2.7 of this thesis and summarised in Figure 5.3 below.

Figure 5.3: CYP probe drugs, metabolites and phenotyping metrics.



5.2.5 Analytical methods

UHPLC-MS/MS analytical methods for the 5 probe drugs, their 5 enzyme-specific metabolites (Chapter 3) (Figure 5.3) and sulforaphane (SUL) (Chapter 4) have been described in this thesis. Samples for CYP phenotyping were collected on all 3 study days, with SUL being analysed in the baseline D1 and D9 samples, as well as in all six samples on D2 to explore exposure after consuming a broccoli meal (200 g).

5.2.6 Genotyping

Each participant was genotyped for variants known to affect CYP activity or SUL metabolism (Chapter 1). The full list of SNPs and copy number assays for the relevant drug-metabolising enzymes in this study are listed in Appendix 8.16. However, only variants where genotype varied between participants were included in the analyses. DNA extraction/purification and subsequent genotyping was conducted by the Australian Genome Research Facility (Brisbane, QLD). DNA was purified and extracted from participant blood samples using the

methods outlined in Appendix 8.15. Genotyping was performed using the Agena Bioscience MassARRAY platform and the iPLEX ADME PGx panel according to the manufacturer's protocols (Agena Bioscience, San Diego, CA) (Lee *et al.*, 2016).

5.2.7 Data and statistical analyses

5.2.7.1 Sample size calculations

When designing this study, the sample size was based on the assumption that a paired-samples *t* test or non-parametric equivalent would be used. CYP1A2 activity metrics across the study days and between ancestry groups were used in the variability calculation due to familiarity with its variability and access to previous raw data. Using this information and a standard deviation of 0.30 for CYP1A2 activity (Ghassabian *et al.*, 2009), a sample size of n = 14 participants in each ancestry group (total n = 28) was calculated to detect up to a 25% difference in CYP1A2 activity with 80% power and a type I error (α) of 0.05. Because CYP1A2 activity displays the largest intra- and inter-subject variability (Perera *et al.*, 2012a), it was deemed a suitable surrogate for sample size approximation for the other CYP enzymes being simultaneously studied, n = 28 subjects allowed for detection of up to a 25% difference in CYP2C19, CYP2C9, CYP2D6 and CYP3A4 activity with 80% power and a type I α of 0.05.

Mixed-effects models have been widely recommended due to their utility when analysing repeated-measured data arising from crossover trials (Goh *et al.*, 2010; Kakuda *et al.*, 2014; Nordmark *et al.*, 2014; Turpault *et al.*, 2009). Some of the reasons for this are their superior power compared to other types of analyses, their ability to include statistically-controlling covariates, which can greatly reduce the sample sizes needed to detect significant differences in endpoints, and control of familywise error across multiple comparisons (Gueorguieva & Krystal, 2004; Putt & Chinchilli, 1999). Because the method of sample size

calculation depends on the choice of planned statistical tests in a study's analyses, sample size was also calculated based on the use of multiple mixed-effects models.

As preliminary results had been collected by the time of this realisation, *post hoc* sample size re-calculations were possible. These were performed using the GLIMMPSE sample size calculator for crossover studies with repeat-measures found at http://glimmpse.samplesizeshop.org (Kreidler *et al.*, 2013). The parameters constant across the 5 enzyme activities were power = 0.8 and $\alpha = 0.1$ (for 90% confidence intervals, in a similar fashion to a bioequivalence design) (Kakuda *et al.*, 2014; Nordmark *et al.*, 2014) to detect a difference in enzyme activity of up to 25%. For each CYP enzyme activity, estimates of the standard deviations and within-subject correlation coefficients were derived from the preliminary data of this study, as well as from Turpault *et al.* (2009) (CYP2C19), Vogl *et al.* (2015) (CYP2C9), Dorado *et al.* (2012) (CYP2D6) and Dorne *et al.* (2003) (CYP3A4). The results are presented in Table 5.2, and indicate that *n* = 10-12 participants in each ancestry group is appropriate to test the proposed hypotheses (section 5.1) for most of the CYP enzymes.

Table 5.2: GLIMMPSE sample size calculations from a linear mixed-effects model. Calculated *n* is for Study Day, Ancestry and Study Day*Ancestry in the linear model for each geographic ancestry group, unless specified otherwise.

CYP enzyme	Power	Type I error	Difference in metric (%)	Calculated *n*
CYP1A2	0.8	0.1	25	6
CYP2C19	0.8	0.1	25	8
CYP2C9	0.8	0.1	25	12
CYP2D6	0.8	0.1	25	6
CYP3A4	0.8	0.1	25	16

5.2.7.2 Pharmacokinetic analyses

Non-compartmental methods were used to calculate pharmacokinetic parameters. Area under the concentration-time curves (AUCs) for each probe drug, metabolite and SUL were calculated from plasma concentrations using the linear trapezoidal rule.

5.2.7.3 Food diary analyses

The food diaries were analysed using the methods presented by Perera *et al.* (2012a). Participants were categorised based on their consumption of foods known to inhibit or induce CYP1A2 activity (Table 5.3) as well as their curry consumption (Table 5.4). The two-tailed Pearson's correlation coefficient was used to assess correlations between diet sub-categories and CYP enzyme activity.

5.2.7.4 Statistical analyses

All data were analysed in SPSS Statistics for Windows, version 23 (IBM Corp., Armonk, N.Y., USA). Normality of endpoint and residual distributions was assessed using the Shapiro-Wilk test and by visually observing the distributions. Differences in food consumption category proportions were analysed using a 2-way chi-square test with a Bonferroni-corrected type I error to account for familywise error across multiple comparisons.

Table 5.3: Foodstuffs known to inhibit or induce CYP1A2. Methods by Perera *et al*. (2012a).

CYP1A2 Inducers	CYP1A2 Inhibitors	Other Dietary Factors
Bok Choy	Carrots	Chocolate consumption
Broccoli	Celeriac	Tea consumption
Brussels sprouts	Celery	Soft drink/energy drink consumption
Cabbage	Coriander	Coffee consumption
Cauliflower	Cumin	Alcohol consumption
Char-grilled meats	Grapefruit juice	Any dietary restrictions
Radish	Parsnip	Predominantly curry diet
Rocket	Parsley	Vegetarian
Wasabi		

Table 5.4: Categories used to define subject diets based on CYP1A2 inducer, CYP1A2 inhibitor and curry consumption.

Category	Definition
High CYP inducer consumption	Greater than 8 servings per week (across both food diaries)
High CYP inhibitor consumption	Greater than 6 servings per week (across both food diaries)
Predominantly curry diet	At least 1 serving of curry per day for a whole food diary

The various CYP phenotyping metrics were natural log-transformed prior to statistical analysis to reduce variability and approximate normal distributions (Shen *et al.*, 2006). A linear mixed-effects model was created for each CYP enzyme (with the exception of CYP2D6, whose analyses required three separate models—see section 5.3.4.4.3) to produce estimated marginal means (EMMs) (least-squares means) of log-transformed metrics. In the models, Study Day, Ancestry, Genotype and their various interactions were fixed effects and

the intercept of each participant was a random effect. Post-hoc tests for significant factor interactions were performed using the Least Significant Difference (LSD) method. The type I error was set at 0.1 to produce 90% confidence intervals (CIs) for differences in log-transformed EMMs between study days and between ancestry groups. These CIs were log-back-transformed to produce geometric EMM (GEMM) ratios with corresponding 90% confidence intervals (see section 5.2.7.1 for a rationale supporting the use of these methods). The model structures, repeat-measures covariance structures, random-effects covariance structures and SPSS syntaxes are presented in Appendix 8.17.

For SUL analyses, the two-tailed Pearson's correlation coefficient was used to assess correlations between the various SUL time points and D2 $AUC_{0\text{-}8\ h}$ after natural log-transformation. A linear mixed-effects model was used to assess the differences in D2 SUL $AUC_{0\text{-}8\ h}$ between geographic ancestry groups and explore the effects of genetics on SUL exposure. As with CYP activity metrics, the liberal type I error of 0.1 was used to assess statistical significance of differences in SUL concentrations and AUCs between ancestry groups.

5.3 Results

All enrolled participants completed the study in full with no serious adverse events. Transient drowsiness and light-headedness were reported by some participants, which were attributed to fasting and taking the midazolam, dextromethorphan and losartan combination.

5.3.1 Participant demographics

Participant age, weight, height, BMI and ancestry are presented in Table 5.5. Differences in demographics between ancestry groups were not statistically analysed, as this study was not powered or designed to detect differences in these parameters. However, there were no major differences in the means and standard deviations of these demographics between the groups.

5.3.2 Dietary data

The proportions of those who reportedly consumed diets high in CYP1A2 inducers, CYP1A2 inhibitors and curry are shown in Table 5.6, Table 5.7 and Table 5.8, respectively. Of those consuming low amounts of CYP1A2 inducer foods, 14% were European and 86% were South Asian ($P < 0.05$). With regards to high consumption of inducer foods, 71% were European and 29% were South Asian ($P < 0.05$). For CYP1A2 inhibitor foods, there were no significant differences between ancestry groups. However, with regards to curry consumption, 69% of low curry consumers were European and 31% were South Asian. Of those consuming a predominantly curry diet, 100% were South Asian ($P < 0.05$).

Table 5.5: Participant demographics.

ID	Geographic ancestry	Age (years)	Weight (kg)	Height (m)	BMI ($kg.m^{-2}$)
1	European	33	77.6	1.78	24.5
2	South Asian	51	70.0	1.62	26.7
3	European	18	62.0	1.60	24.2
4	South Asian	18	79.8	1.78	25.3
5	South Asian	43	71.2	1.77	22.9
6	South Asian	19	92.9	1.76	30.0
7	South Asian	24	63.4	1.78	20.0
8	European	20	72.2	1.81	22.0
9	European	22	56.0	1.77	17.9
10	European	23	64.0	1.80	19.8
11	European	19	72.1	1.86	20.9
12	European	20	85.3	1.83	25.5
13	European	23	89.8	1.84	26.4
14	European	19	87.3	1.81	26.6
15	European	23	62.7	1.81	19.1
16	European	28	84.7	1.83	25.4
17	South Asian	23	82.7	1.77	26.3
18	South Asian	20	66.0	1.70	22.9
19	South Asian	22	82.5	1.80	25.4
20	South Asian	33	73.0	1.72	24.7
21	South Asian	20	90.0	1.70	31.1
	Mean ± SD (total) *n* = 21	24.8 ± 8.59	75.5 ± 10.8	1.77 ± 0.07	24.2 ± 3.40
	Mean ± SD (European) *n* = 11	22.5 ± 4.46	74.0 ± 11.77	1.80 ± 0.07	22.9 ± 3.13
	Mean ± SD (South Asian) *n* = 10	27.3 ± 11.35	77.2 ± 9.98	1.70 ± 0.05	25.5 ± 3.31

Table 5.6: Consumption of CYP1A2 inducers by ancestry.

			Geographic ancestry		
			European	South Asian	Total
CYP inducer diet	Low consumption of CYP inducers	Count (*n*)	1	6	7
		% within CYP inducer diet	14.3%	85.7%	100.0%
		% within Geographic ancestry	9.1%	60.0%	33.3%
		% of Total	4.8%	28.6%	33.3%
	High consumption of CYP inducers	Count (*n*)	10	4	14
		% within CYP inducer diet	71.4%	28.6%	100.0%
		% within Geographic ancestry	90.9%	40.0%	66.7%
		% of Total	47.6%	19.0%	66.7%
Total		Count (*n*)	11	10	21
		% within CYP inducer diet	52.4%	47.6%	100.0%
		% within Geographic ancestry	100.0%	100.0%	100.0%
		% of Total	52.4%	47.6%	100.0%

Table 5.7: Consumption of CYP1A2 inhibitors by ancestry.

			Geographic ancestry		
			European	South Asian	Total
CYP inhibitor diet	Low consumption of CYP inhibitors	Count	9	8	17
		% within CYP inhibitor diet	52.9%	47.1%	100.0%
		% within Geographic ancestry	81.8%	80.0%	81.0%
		% of Total	42.9%	38.1%	81.0%
	High consumption of CYP inhibitors	Count	2	2	4
		% within CYP inhibitor diet	50.0%	50.0%	100.0%
		% within Geographic ancestry	18.2%	20.0%	19.0%
		% of Total	9.5%	9.5%	19.0%
Total		Count	11	10	21
		% within CYP inhibitor diet	52.4%	47.6%	100.0%
		% within Geographic ancestry	100.0%	100.0%	100.0%
		% of Total	52.4%	47.6%	100.0%

Table 5.8: Counts and frequencies of a predominantly curry diet by ancestry.

			Geographic ancestry		
			European	South Asian	Total
Predominantly curry diet	Low curry consumption	Count	11	5	16
		% within Predominantly curry diet	68.8%	31.3%	100.0%
		% within Geographic ancestry	100.0%	50.0%	76.2%
		% of Total	52.4%	23.8%	76.2%
	Predominantly curry diet	Count	0	5	5
		% within Predominantly curry diet	0.0%	100.0%	100.0%
		% within Geographic ancestry	0.0%	50.0%	23.8%
		% of Total	0.0%	23.8%	23.8%
Total		Count	11	10	21
		% within Predominantly curry diet	52.4%	47.6%	100.0%
		% within Geographic ancestry	100.0%	100.0%	100.0%
		% of Total	52.4%	47.6%	100.0%

5.3.3 Sulforaphane exposure

Sulforaphane (SUL) concentrations and AUCs after broccoli ingestion showed remarkable variability both overall and in the two ancestry groups, and required natural log-transformation to achieve a normal distribution (Table 5.10). Across the seven time points where SUL was measured and D2 $AUC_{0\text{-}8\ h}$, CV% of log-transformed data ranged from 12-76% in the European ancestry group, 18-55% in the South Asians and 15-71% overall. Mean concentration-time plots of SUL on D2 are shown in Figure 5.4.

In the mixed-effects model, *GSTM1* and *GSTP1* genotypes had a significant effect on SUL $AUC_{0\text{-}8\ h}$ ($P < 0.05$ for both), whereas *GSTT1*, *GSTT2*, *UGT1A1* and geographic ancestry had no effect. The differences in SUL exposure by *GSTM1* and *GSTP1* genotypes are reported in Table 5.9. There was a genotype-exposure relationship, with SUL exposure being the highest in *GSTM1* and *GSTP1* null homozygotes, intermediate in *GSTM1* and *GSTP1* null heterozygotes, and lowest in those with two functional alleles.

All natural log-transformed SUL concentration-time points and the D2 $AUC_{0\text{-}8\ h}$ were correlated. In particular, the 3- and 4-h post-broccoli time points were strongly correlated with the $AUC_{0\text{-}8\ h}$ (correlation coefficients 0.981 and 0.982, respectively [both $P < 0.01$]). Of note, SUL $AUC_{0\text{-}8}$ was significantly correlated with a predominantly curry diet (correlation coefficient 0.441 [$P = 0.045$]). SUL $AUC_{0\text{-}8}$ did not significantly correlate with the various activity metrics for all 5 CYP enzymes across the three study days.

Table 5.9: Back-transformed geometric EMM ratios of SUL $AUC_{0\text{-}8\ h}$ activity with 90% CIs between *GSTM1* and *GSTP1* genotypes.

***GSTM1* rs1065411**	**CC~/CG#**	**CC/GG$**	**GC/GG**
Overall	4.93 (1.97, 12.4)[1]	2.00 (1.07, 3.74)[2]	2.46 (1.09, 5.57)[3]
***GSTP1* rs1695a**	**AA^/AG&**	**AA/GG***	**AG/GG**
Overall	4.79 (2.39, 9.57)[4]	1.30 (0.40, 4.21)	3.70 (1.05, 13.0)[5]

GSTM1 CC = homozygous null allele, CG = heterozygous null allele, GG = homozygous functional allele

GSTP1 AA = homozygous null allele, AG = heterozygous null allele, GG = homozygous functional allele

~: *n* = 8

#: *n* = 2

$: *n* = 11

^: *n* = 14

&: *n* = 6

*: *n* = 1

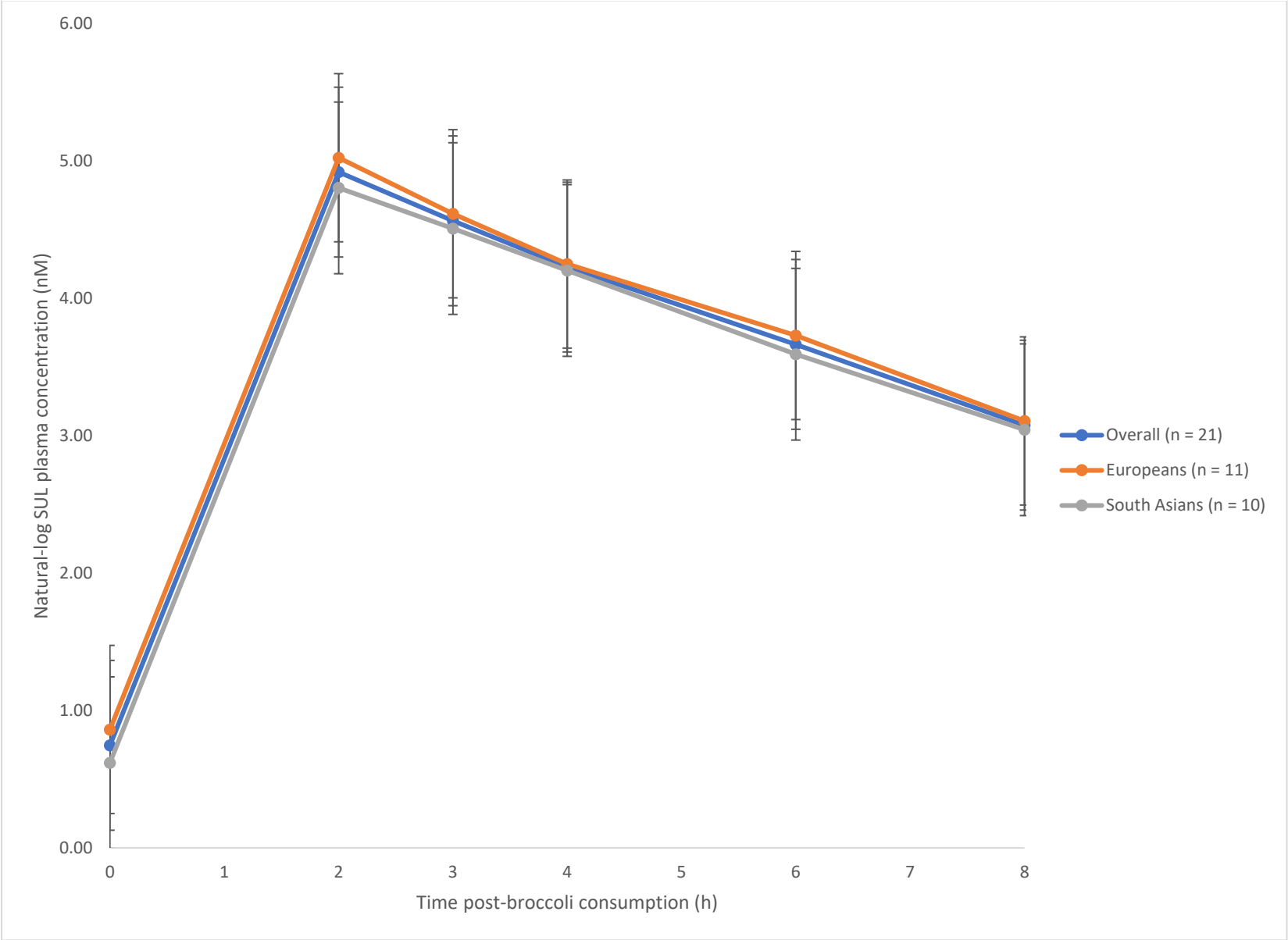
1. *P* = 0.007
2. *P* = 0.069
3. *P* = 0.072
4. *P* = 0.001
5. *P* = 0.088

Table 5.10: Natural log-transformed sulforaphane plasma concentrations and D2 $AUC_{0\text{-}8\ h}$ by participant and ancestry.

Participant ID	Geographic ancestry	D1 baseline (nM)	D2 2-h (nM)	D2 3-h (nM)	D2 4-h (nM)	D2 6-h (nM)	D2 8-h (nM)	$AUC_{0\text{-}8\ h}$ (nM.h)	D9 (nM)
1	European	1.94	5.13	4.81	4.40	4.18	3.58	6.52	2.58
3	European	1.08	4.60	4.74	4.20	3.67	2.61	6.20	1.19
8	European	1.91	5.57	5.32	5.03	4.67	3.74	7.03	3.52
9	European	0.35	3.45	3.00	2.85	2.85	2.71	5.03	3.30
10	European	1.59	5.90	5.49	5.38	5.08	4.59	7.41	3.29
11	European	0.35	5.69	5.20	4.67	4.18	3.49	6.92	2.85
12	European	0.39	4.95	4.49	4.19	3.54	2.71	6.19	2.64
13	European	0.35	5.79	5.36	5.04	4.14	3.43	7.02	1.85
14	European	0.72	5.31	4.38	3.76	3.36	2.54	6.26	1.50
15	European	0.35	4.22	3.16	2.99	1.55	1.49	5.24	2.66
16	European	0.42	4.63	4.79	4.23	3.80	3.29	6.26	2.84
	Mean (Europeans)	**0.86**	**5.02**	**4.61**	**4.25**	**3.73**	**3.11**	**6.37**	**2.57**
	SD (Europeans)	**0.66**	**0.75**	**0.84**	**0.81**	**0.95**	**0.81**	**0.74**	**0.75**
	CV%	**76.39**	**14.97**	**18.19**	**19.00**	**25.44**	**26.21**	**11.58**	**29.35**
2	South Asian	0.44	5.84	5.36	5.01	4.16	3.39	7.05	2.40
4	South Asian	1.12	4.66	3.53	3.45	3.37	3.29	5.86	3.21
5	South Asian	1.21	6.83	6.69	5.99	5.64	4.26	8.16	2.86
6	South Asian	0.98	6.25	6.05	5.75	5.09	4.76	7.67	4.18
7	South Asian	0.43	5.47	5.26	4.87	4.14	3.45	6.84	3.46
17	South Asian	0.42	3.69	3.40	3.23	2.44	2.19	5.11	2.22
18	South Asian	0.35	3.08	2.64	2.62	2.42	2.13	4.65	2.54
19	South Asian	0.45	4.07	4.58	4.37	3.53	2.74	6.00	2.50
20	South Asian	0.45	4.06	3.92	3.49	2.79	2.46	5.56	1.84
21	South Asian	0.34	4.07	3.64	3.23	2.35	1.75	5.39	2.20
	Mean (South Asians)	**0.62**	**4.80**	**4.51**	**4.20**	**3.59**	**3.04**	**6.23**	**2.74**

Participant ID	Geographic ancestry	D1 baseline (nM)	D2 2-h (nM)	D2 3-h (nM)	D2 4-h (nM)	D2 6-h (nM)	D2 8-h (nM)	$AUC_{0\text{-}8\ h}$ (nM.h)	D9 (nM)
	SD (South Asians)	0.34	1.23	1.30	1.16	1.15	0.97	1.15	0.70
	CV%	54.90	25.59	28.84	27.68	32.15	31.86	18.48	25.58
	Mean (Total)	0.75	4.92	4.56	4.23	3.66	3.08	6.30	2.65
	SD (Total)	0.53	0.99	1.06	0.97	1.03	0.87	0.93	0.72
	CV%	71.40	20.08	23.15	22.88	28.03	28.25	14.83	27.03

Figure 5.4: Plasma concentration-time profile for SUL on D2 (natural log-transformed data). *T* = 0 data taken from baseline D1 SUL plasma sample, as participants consumed no *Cruciferae* between this sample and the D2 sampling window.



5.3.4 CYP activity

5.3.4.1 CYP1A2

5.3.4.1.1 Caffeine

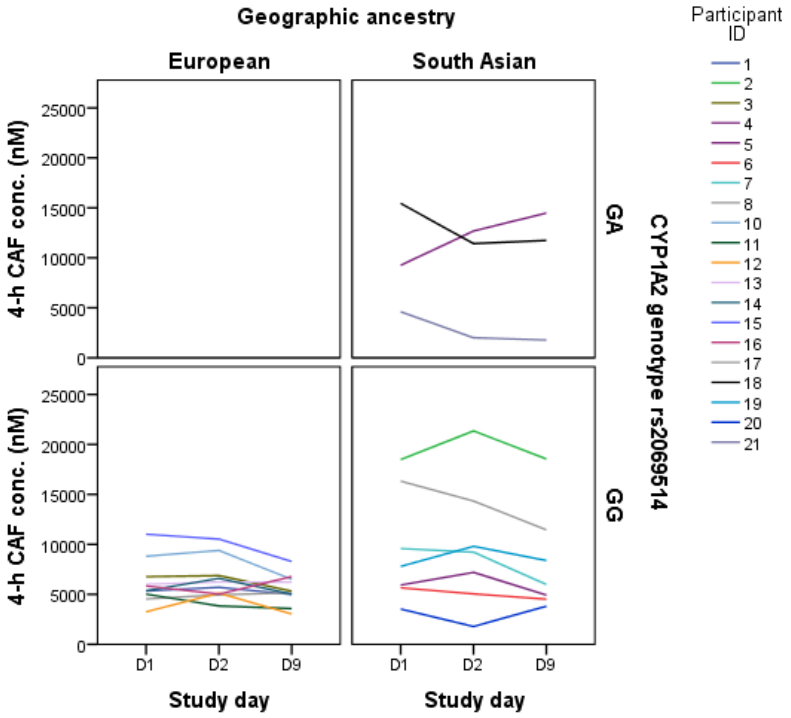
Changes in caffeine 4-h plasma concentrations and the CYP1A2 activity metric across study days showed marked variability, both overall and when stratified by ancestry (Table 5.11). The coefficient of variation (CV%) of 4-h caffeine plasma concentrations ranged from 27-38% in Europeans and 55-63% in South Asians. Interestingly, these CV% varied little within ancestry groups across the study days, validating the choice of a repeated-measures prospective design for this trial. Individual participant caffeine plasma concentrations by ancestry and genotype across study days are shown in Figure 5.5. Caffeine plasma concentrations were higher throughout the study in the South Asian cohort compared to the Europeans (9,235 ± 5,535 nM vs 6,345 ± 2,131 nM, respectively). In the Europeans, mean caffeine concentrations increased immediately after a broccoli meal (D1 to D2), and decreased after 6 days of broccoli consumption (D1 to D9). In the South Asian ancestry group, mean caffeine concentrations decreased between D1 and D2, and decreased between D1 and D9.

Table 5.11: CYP1A2 data across study day by ancestry.

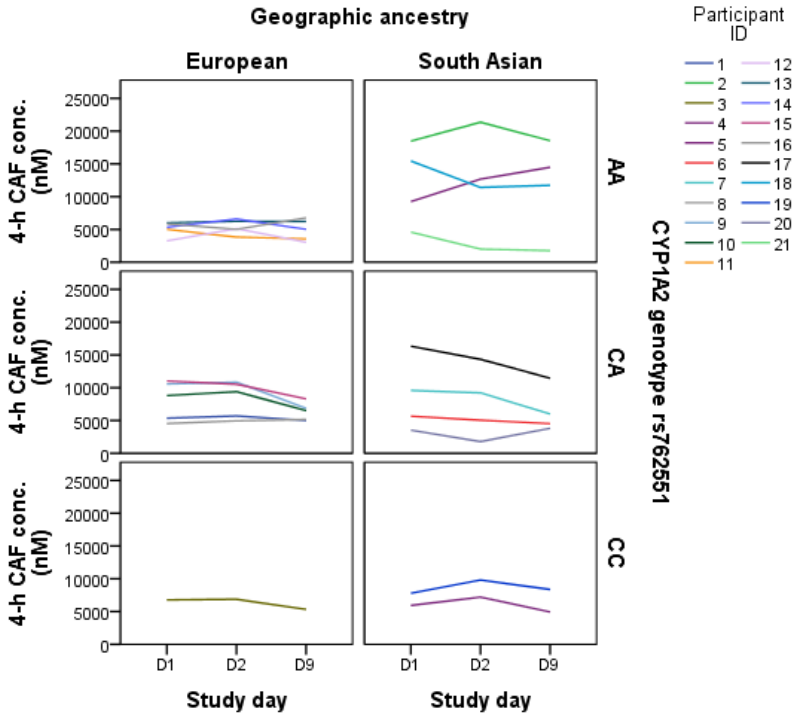
	Genotypes			CYP1A2								
				D1			D2			D9		
ID	*CYP1A2*1C* rs2069514 -3860G>A	*CYP1A2*1F* rs762551 -163C>A	Geographic ancestry	4-h CAF conc. (nM)	4-h PAR conc. (nM)	PAR/CAF 4-h conc. ratio	4-h CAF conc. (nM)	4-h PAR conc. (nM)	PAR/CAF 4-h conc. ratio	4-h CAF conc. (nM)	4-h PAR conc. (nM)	PAR/CAF 4-h conc. ratio
1	GG	AC	European	5350	3923	0.733	5696	3880	0.681	5003	4121	0.824
3	GG	CC	European	6773	4190	0.619	6874	5632	0.819	5318	4660	0.876
8	GG	AC	European	4532	541	0.119	4945	597	0.121	5160	598	0.116
9	Fail	AC	European	10568	910	0.086	10845	1031	0.095	6859	1036	0.151
10	GG	AC	European	8810	712	0.081	9392	549	0.058	6509	946	0.145
11	GG	AA	European	5016	1344	0.268	3837	1275	0.332	3572	1091	0.305
12	GG	AA	European	3255	3406	1.046	5120	4246	0.829	3042	3547	1.166
13	GG	AA	European	6008	3257	0.542	6236	4550	0.730	6217	4487	0.722
14	GG	AA	European	5352	5305	0.991	6599	4873	0.738	5023	5119	1.019
15	GG	CA	European	11017	5541	0.503	10527	6351	0.603	8291	4289	0.517
16	GG	AA	European	5840	5027	0.861	5024	3283	0.654	6778	7050	1.040
			Mean (Europeans)	**6593**	**3105**	**0.532**	**6827**	**3297**	**0.515**	**5616**	**3359**	**0.626**
			SD (Europeans)	**2494**	**1914**	**0.357**	**2382**	**2101**	**0.303**	**1518**	**2125**	**0.395**
			CV%	**37.8**	**61.7**	**67.1**	**34.9**	**63.7**	**58.8**	**27.0**	**63.3**	**63.1**
2	GG	AA	South Asian	18478	8207	0.444	21355	7751	0.363	18541	10152	0.548
4	GG	CC	South Asian	5929	5489	0.926	7201	4320	0.600	4929	3615	0.733
5	AG	AA	South Asian	9261	6916	0.747	12680	9302	0.734	14486	7738	0.534
6	GG	AC	South Asian	5649	718	0.127	5045	380	0.075	4516	400	0.089
7	GG	AC	South Asian	9584	763	0.080	9223	781	0.085	5985	596	0.100
17	GG	CA	South Asian	16329	17165	1.051	14330	18093	1.263	11454	14636	1.278
18	GA	AA	South Asian	15448	11003	0.712	11419	8848	0.775	11739	7180	0.612

	Genotypes			CYP1A2								
				D1			D2			D9		
ID	*CYP1A2*1C* rs2069514 -3860G>A	*CYP1A2*1F* rs762551 -163C>A	Geographic ancestry	4-h CAF conc. (nM)	4-h PAR conc. (nM)	PAR/CAF 4-h conc. ratio	4-h CAF conc. (nM)	4-h PAR conc. (nM)	PAR/CAF 4-h conc. ratio	4-h CAF conc. (nM)	4-h PAR conc. (nM)	PAR/CAF 4-h conc. ratio
19	GG	CC	South Asian	7800	4179	0.536	9806	7896	0.805	8381	5556	0.663
20	GG	CA	South Asian	3524	2100	0.596	1781	1328	0.745	3808	1551	0.407
21	GA	AA	South Asian	4595	3652	0.795	2001	1355	0.677	1776	1651	0.930
			Mean (South Asians)	**9660**	**6019**	**0.601**	**9484**	**6005**	**0.612**	**8562**	**5308**	**0.589**
			SD (South Asians)	**5298**	**5110**	**0.317**	**5952**	**5547**	**0.358**	**5354**	**4674**	**0.357**
			CV%	**54.9**	**84.9**	**52.7**	**62.8**	**92.4**	**58.4**	**62.5**	**88.1**	**60.5**
			Mean (Total)	**8053**	**4493**	**0.565**	**8092**	**4587**	**0.561**	**7018**	**4287**	**0.608**
			SD (Total)	**4267**	**3976**	**0.332**	**4542**	**4240**	**0.325**	**4041**	**3617**	**0.368**
			CV%	**53.0**	**88.5**	**58.8**	**56.1**	**92.4**	**58.0**	**57.6**	**84.4**	**60.5**

Figure 5.5: Caffeine 4-h plasma concentrations across study days by ancestry and *CYP1A2*1C* (rs2069514) (A) and *CYP1A2*1F* (rs762551) (B) genotypes.



A



B

5.3.4.1.2 Paraxanthine

Paraxanthine concentrations were more variable than caffeine concentrations: CV% of paraxanthine concentrations ranged from 62-64% in Europeans and 85-92% in South Asians, which were similar within the ancestry groups across study days. Individual participant paraxanthine plasma concentrations by ancestry, and genotype across study days are depicted below in Figure 5.6. Paraxanthine plasma concentrations were higher throughout the study in the South Asian cohort compared to the Europeans (5,777 ± 5,110 nM vs 3,254 ± 2,047 nM, respectively). In the Europeans, mean paraxanthine concentrations increased immediately after a broccoli meal (D1 to D2), and increased overall after 6 days of broccoli consumption (D1 to D9). In the South Asian ancestry group, mean paraxanthine concentrations decreased slightly between D1 and D2 and decreased overall between D1 and D9.

5.3.4.1.3 CYP1A2 activity

The 4-h paraxanthine/caffeine ratio displayed large variability: CV% of CYP1A2 activity ranged from 59-68% in Europeans and 53-61% in South Asians, but this variability remained consistent within the ancestry groups across study days. There was a significant effect between Study Day and Ancestry in the mixed-effects model for the European cohort (F = 3.499, P = 0.040). When examined further, there was a significant 20% increase in CYP1A2 activity after 6 days of broccoli consumption (D1 to D9) in the Europeans (Table 5.12), but no changes in the South Asian cohort. With respect to genetics, overall, the *CYP1A2*1C* genotype did not significantly affect enzyme activity (F = 0.310, P = 0.584), however there was a significant 2.67-fold difference in activity between those who had the CC and CA *CYP1A2*1F* genotypes, respectively (GEMM ratio 2.69 [1.19, 6.10], P = 0.049) (Table 5.13). SUL exposure had no significant effect on CYP1A2 activity across the study days, in that

there was no significant correlation between SUL exposure and CYP1A2 activity. Individual changes in CYP1A2 activity by ancestry and genotype across study days are shown in Figure 5.7.

Table 5.12: Back-transformed geometric EMM ratios of CYP1A2 activity between study days with 90% CIs by ancestry group.

	D2/D1	D9/D1	D9/D2
Europeans (*n* = 11)	0.98 (0.85, 1.13)	1.20 (1.04, 1.38)*	1.22 (1.06, 1.41)
South Asians (*n* = 10)	0.96 (0.84, 1.11)	0.96 (0.83, 1.10)	0.99 (0.86, 1.14)
Overall (*n* = 21)	0.97 (0.87, 1.07)	1.03 (0.93, 1.14)	1.06 (0.96, 1.18)

*P = 0.038

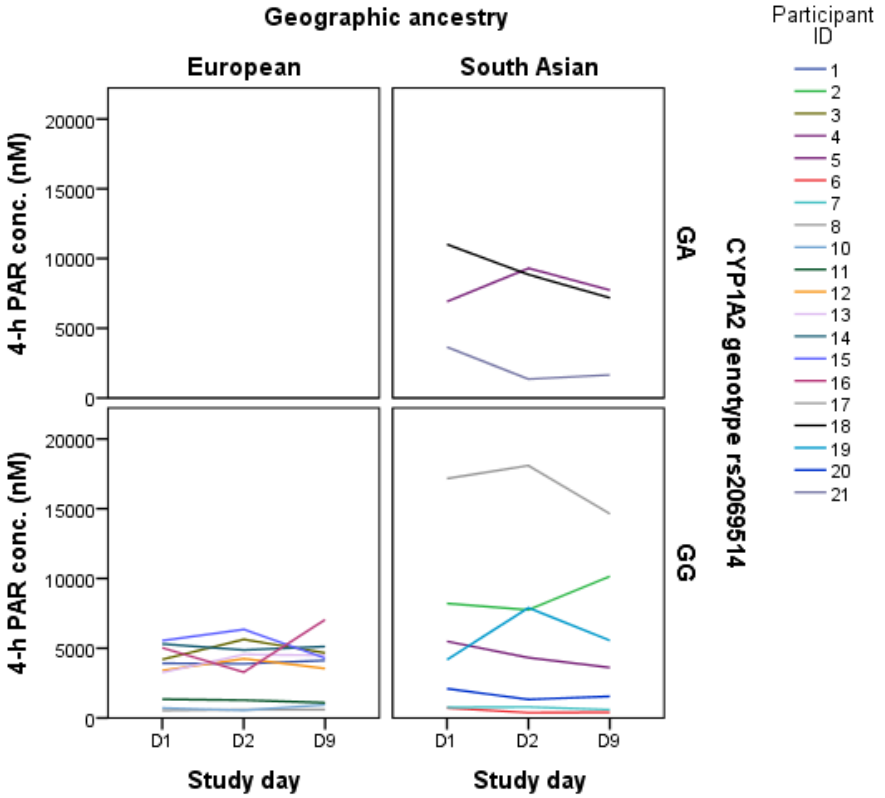
Table 5.13: Back-transformed geometric EMM ratios of CYP1A2 activity with 90% CIs between *CYP1A2*1F* (rs762551) genotypes.

	CC[1]/AC[2]	CC/AA[3]	AA/AC
Overall	2.69 (1.19, 6.10)*	1.40 (0.48, 4.06)	1.93 (0.76, 4.85)

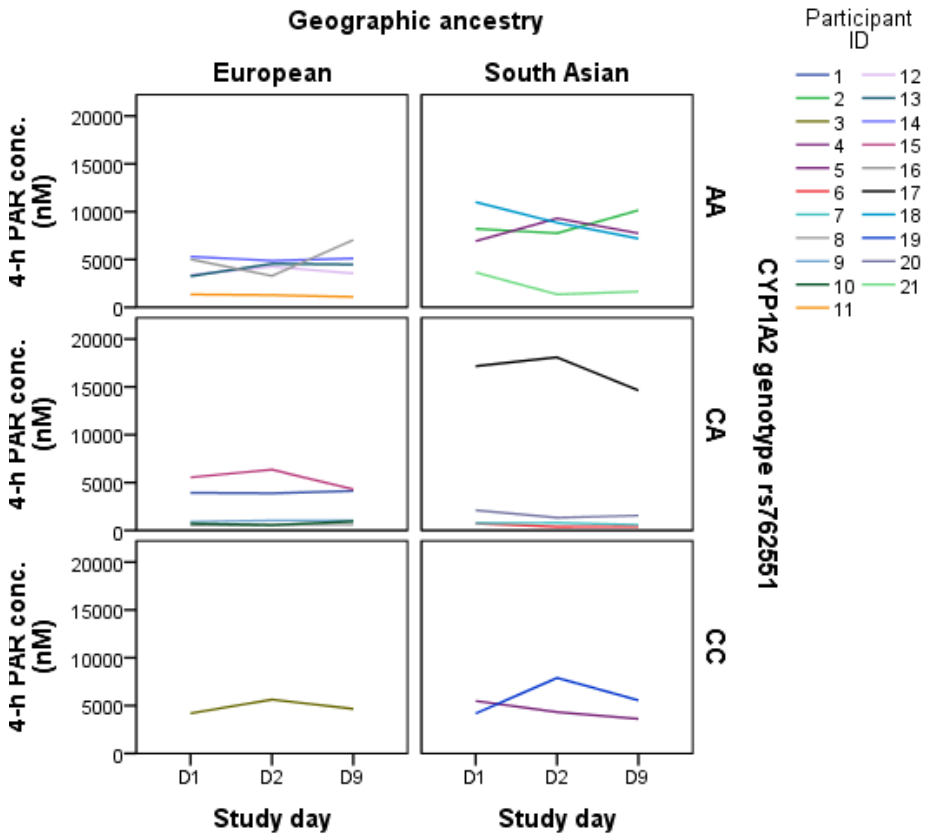
1. N = 3
2. N = 9
3. N = 9

*P = 0.049

Figure 5.6: Paraxanthine 4-h plasma concentrations across study days by ancestry and *CYP1A2*1C* (rs2069514) (A) and *CYP1A2*1F* (rs762551) (B) genotypes.

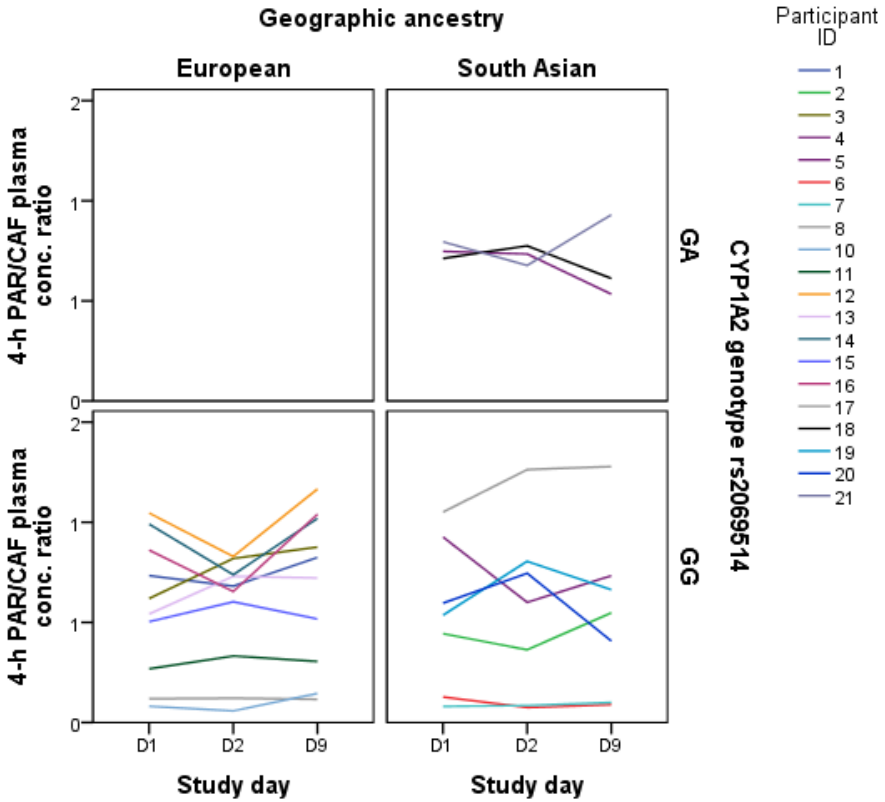


A



B

Figure 5.7: CYP1A2 activity across study days by ancestry and *CYP1A2*1C* (rs2069514) (A) and *CYP1A2*1F* (rs762551) (B) genotypes.



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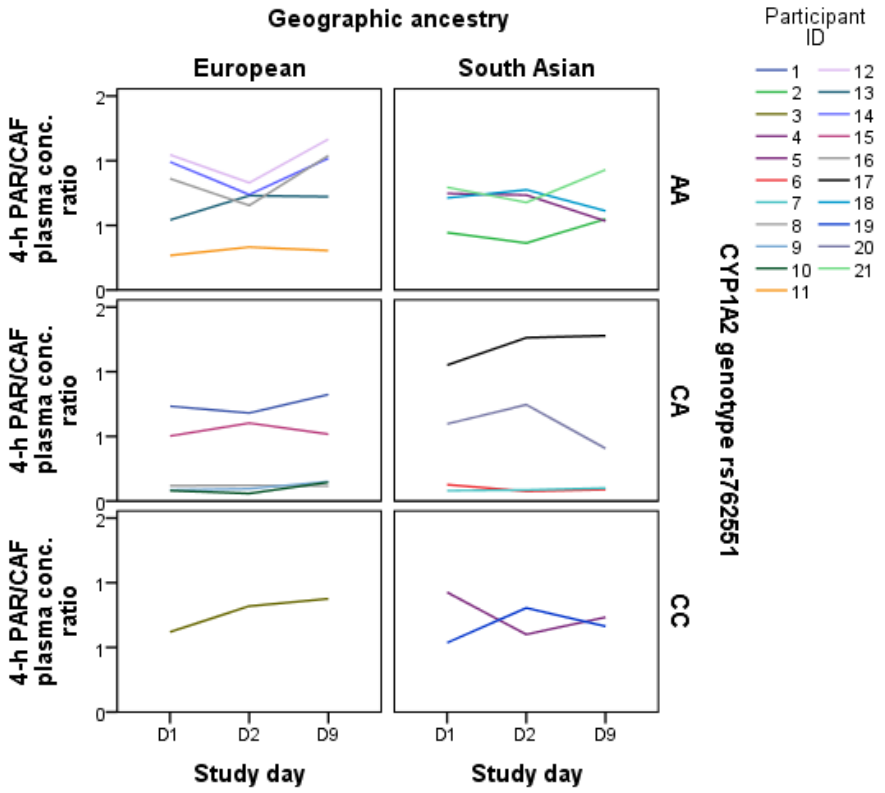


B

5.3.4.2 CYP2C19

5.3.4.2.1 Omeprazole

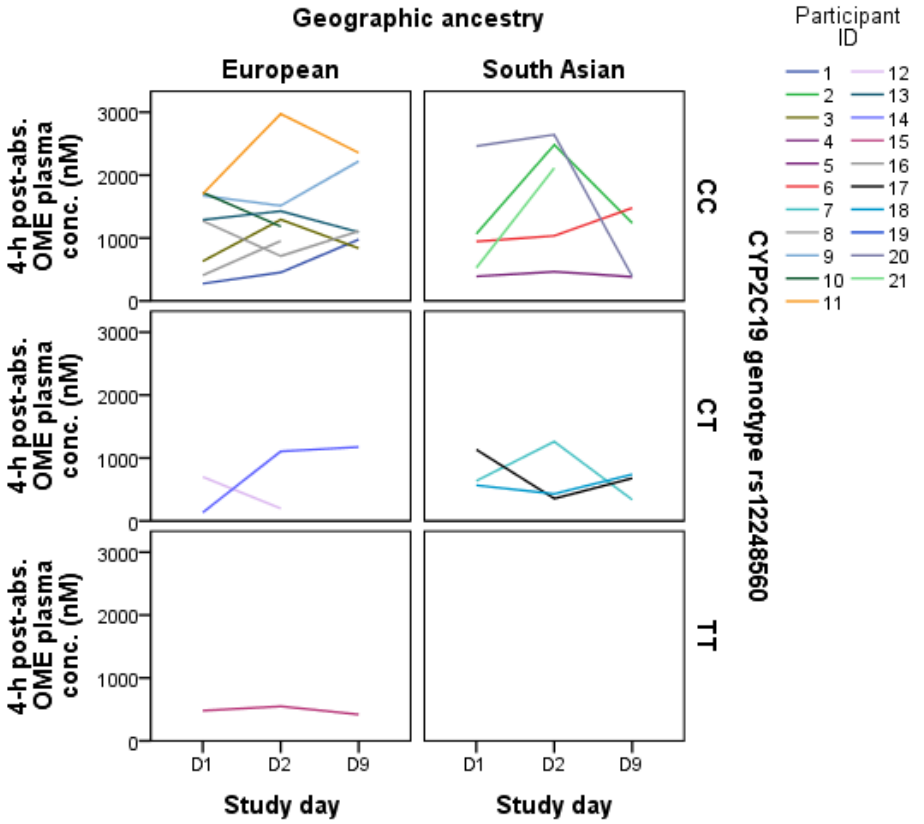
As discussed in Chapter 3, omeprazole showed erratic absorption lag-times, such that concentrations are missing for some participants, who presumably did not absorb an appreciable amount of omeprazole in the 6-hour sampling window. Further, the post-absorption plasma concentrations of omeprazole and 5-hydroxyomeprazole and their corresponding ratio were chosen to investigate CYP2C19, as these parameters displayed less variability than the calculated $AUC_{0\text{-}6\ h}$. The omeprazole post-absorption plasma concentrations showed marked variability, both overall and when stratified by ancestry (Table 5.14). Omeprazole CV% ranged from 53-66% in Europeans and 60-69% in South Asians; CV% varied little within ancestry groups across the study days. Individual participant omeprazole data by ancestry and genotype across study days are depicted in Figure 5.8. Omeprazole plasma concentrations were higher throughout the study in the European cohort compared to the South Asians (1,111 ± 674 nM vs 1,020 ± 662 nM, respectively). In the Europeans, mean omeprazole concentrations increased immediately after a broccoli meal (D1 to D2) and increased after 6 days of broccoli consumption (D1 to D9). In the South Asian ancestry group, they increased between D1 and D2 and decreased between D1 and D9. Omeprazole concentration data was available for *n* = 19, *n* = 21 and *n* = 15 participants on D1, D2 and D9, respectively.

Table 5.14: CYP2C19 data across study days by ancestry. Missing data marked with '-'. Continues onto next page.

	Genotype				CYP2C19								
					D1			D2			D9		
ID	*CYP2C19*1C* rs3758581 80161A>G	*CYP2C19*2* rs4244285 19154G>A	*CYP2C19*17* rs12248560 -806C>T	Geographic ancestry	Post-abs. OME conc. (nM)	Post-abs. OH-OME conc. (nM)	OH-OME/OME post-abs. conc. ratio	Post-abs. OME conc. (nM)	Post-abs. OH-OME conc. (nM)	OH-OME/OME post-abs. conc. ratio	Post-abs. OME conc. (nM)	Post-abs. OH-OME conc. (nM)	OH-OME/OME post-abs. conc. ratio
1	GG	GG	CC	European	275	204	0.740	453	253	0.557	978	532	0.544
3	AG	GG	CC	European	632	465	0.736	1293	788	0.610	836	524	0.627
8	GG	GG	CC	European	408	114	0.278	954	557	0.584	-	-	-
9	GG	AG	CC	European	1673	341	0.204	1514	422	0.279	2221	754	0.340
10	GG	AG	CC	European	1724	662	0.384	1183	323	0.273	-	-	-
11	GG	GG	CC	European	1702	920	0.541	2973	899	0.302	2355	1221	0.519
12	GG	GG	CT	European	698	644	0.922	198	92	0.465	-	-	-
13	GG	AG	CC	European	1288	639	0.496	1428	637	0.446	1092	629	0.576
14	GG	GG	CT	European	133	152	1.144	1106	787	0.711	1174	946	0.806
15	AG	GG	TT	European	480	327	0.681	549	329	0.599	419	328	0.781
16	GG	GG	CC	European	1272	802	0.631	713	441	0.619	1109	690	0.622
				Mean (Europeans)	**935**	**479**	**0.614**	**1124**	**503**	**0.495**	**1273**	**703**	**0.602**
				SD (Europeans)	**609**	**273**	**0.277**	**744**	**254**	**0.153**	**670**	**277**	**0.149**
				CV%	**65.1**	**56.9**	**45.0**	**66.2**	**50.5**	**30.9**	**52.7**	**39.4**	**24.7**
2	GG	AG	CC	South Asian	1066	341	0.320	2482	547	0.220	1238	568	0.459
4	AG	GG	CC	South Asian	391	395	1.010	465	486	1.046	383	370	0.968
5	GG	AA	CC	South Asian	-	-	-	1918	105	0.055	-	-	-
6	GG	AG	CC	South Asian	945	218	0.231	1035	187	0.180	1477	192	0.130
7	GG	AG	CT	South Asian	636	330	0.520	1261	359	0.285	338	222	0.656
17	GG	GG	CT	South Asian	1134	676	0.596	355	135	0.381	678	310	0.458
18	GG	GG	CT	South Asian	566	400	0.707	430	629	1.462	739	647	0.876
19	GG	GG	CC	South Asian	-	-	-	762	459	0.601	-	-	-

	Genotype				CYP2C19								
					D1			D2			D9		
ID	*CYP2C19*1C* rs3758581 80161A>G	*CYP2C19*2* rs4244285 19154G>A	*CYP2C19*17* rs12248560 -806C>T	Geographic ancestry	Post-abs. OME conc. (nM)	Post-abs. OH-OME conc. (nM)	OH-OME/OME post-abs. conc. ratio	Post-abs. OME conc. (nM)	Post-abs. OH-OME conc. (nM)	OH-OME/OME post-abs. conc. ratio	Post-abs. OME conc. (nM)	Post-abs. OH-OME conc. (nM)	OH-OME/OME post-abs. conc. ratio
20	GG	AG	CC	South Asian	2461	188	0.076	2643	163	0.062	393	30	0.075
21	GG	AG	CC	South Asian	525	113	0.215	2111	214	0.101	-	-	-
				Mean (South Asians)	**965**	**333**	**0.459**	**1346**	**328**	**0.439**	**749**	**334**	**0.517**
				SD (South Asians)	**662**	**173**	**0.308**	**877**	**191**	**0.470**	**448**	**216**	**0.343**
				CV%	**68.6**	**51.9**	**67.1**	**65.2**	**58.2**	**106.9**	**59.8**	**64.7**	**66.2**
				Mean (Total)	**948**	**417**	**0.549**	**1230**	**420**	**0.468**	**1029**	**531**	**0.562**
				SD (Total)	**614**	**242**	**0.293**	**797**	**238**	**0.334**	**620**	**308**	**0.251**
				CV%	**64.7**	**57.9**	**53.3**	**64.8**	**56.7**	**71.4**	**60.2**	**58.0**	**44.7**

Figure 5.8: Omeprazole post-absorption plasma concentrations by study day, ancestry and *CYP2C19*17* (rs12248560) (A), *CYP2C19*1C* (rs3758581) (B) and *CYP2C19*2* (rs4244285) (C) genotypes.



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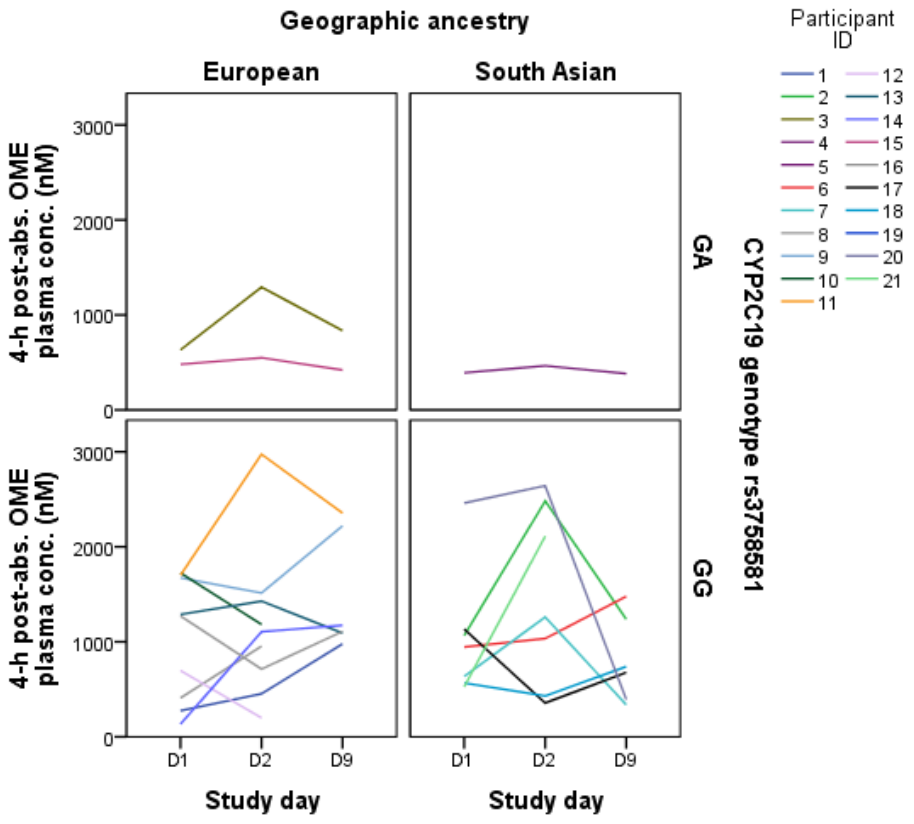
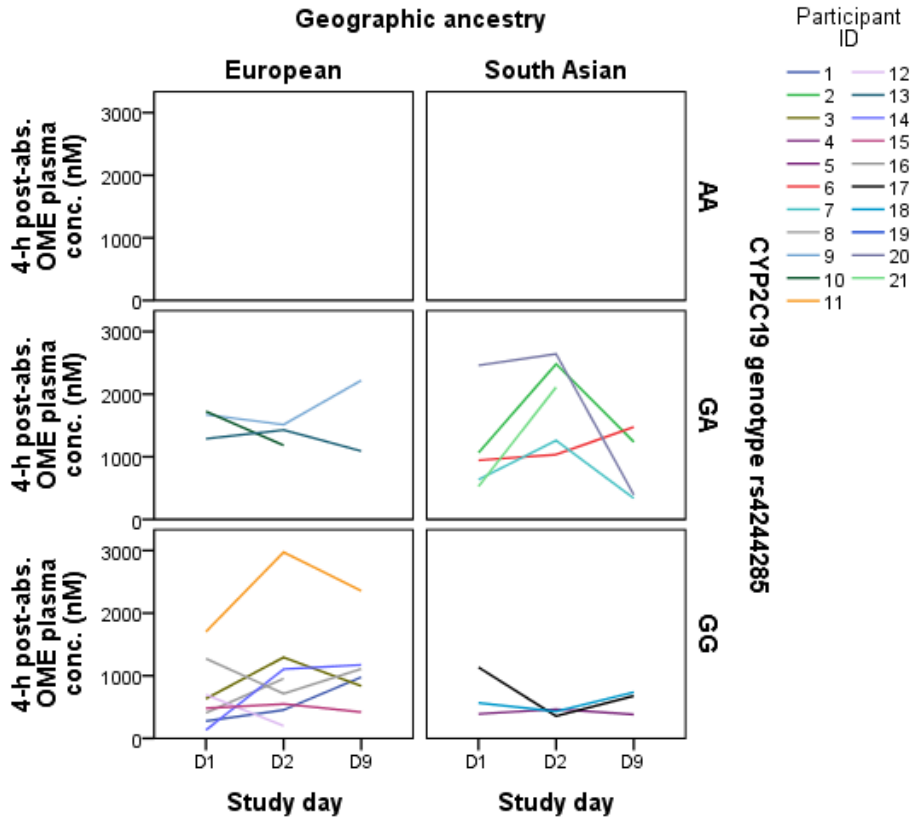


B

Figure 5.8: Omeprazole post-absorption plasma concentrations by study day, ancestry and *CYP2C19*17* (rs12248560) (A), *CYP2C19*1C* (rs3758581) (B) and *CYP2C19*2* (rs4244285) (C) genotypes. Continued from previous page.



C

5.3.4.2.2 5-hydroxyomeprazole

5-hydroxyomeprazole (OH-OME) concentrations had comparable variability to omeprazole: CV% ranged from 40-57% in Europeans and 52-65% in South Asians (Table 5.14). Individual participant OH-OME plasma concentrations by ancestry, and genotype across study days are depicted below in Figure 5.9. Plasma concentrations were higher throughout the study in the European cohort compared to the South Asians (562 ± 268 nM vs 332 ± 193 nM, respectively). Mean OH-OME concentrations increased over the duration of the study (D1 to D2 to D9) in the European ancestry group and remained near-constant in the South Asians. As with OME, OH-OME concentration data was available for *n* = 19, *n* = 21 and *n* = 15 participants on D1, D2 and D9, respectively.

5.3.4.2.3 CYP2C19 activity

In the European ancestry group, the 5-OH-OME/OME post-absorption ratio displayed relatively less variability than for other CYP enzymes, with CV% ranging from 25-45%. The opposite was observed for the South Asians, with CV% spanning 66-107%, attributed to two participants with higher values (4 and 18). However, the mixed effect model was able to account for missing data and adjust CYP2C19 activity according to ancestry and genotype, leading to detection of a significant effect across study days ($F = 2.835$, $P = 0.072$). When further explored, overall, CYP2C19 activity decreased 17% immediately after a broccoli meal (D1 to D2), then rebounded 18% by the end of the study after 6-days of broccoli consumption (D2 to D9) (Table 5.15). This pattern was reflected in the two ancestry groups, but did not achieve statistical significance (Study Day*Ancestry interaction $F = 1.450$, $P = 0.248$).

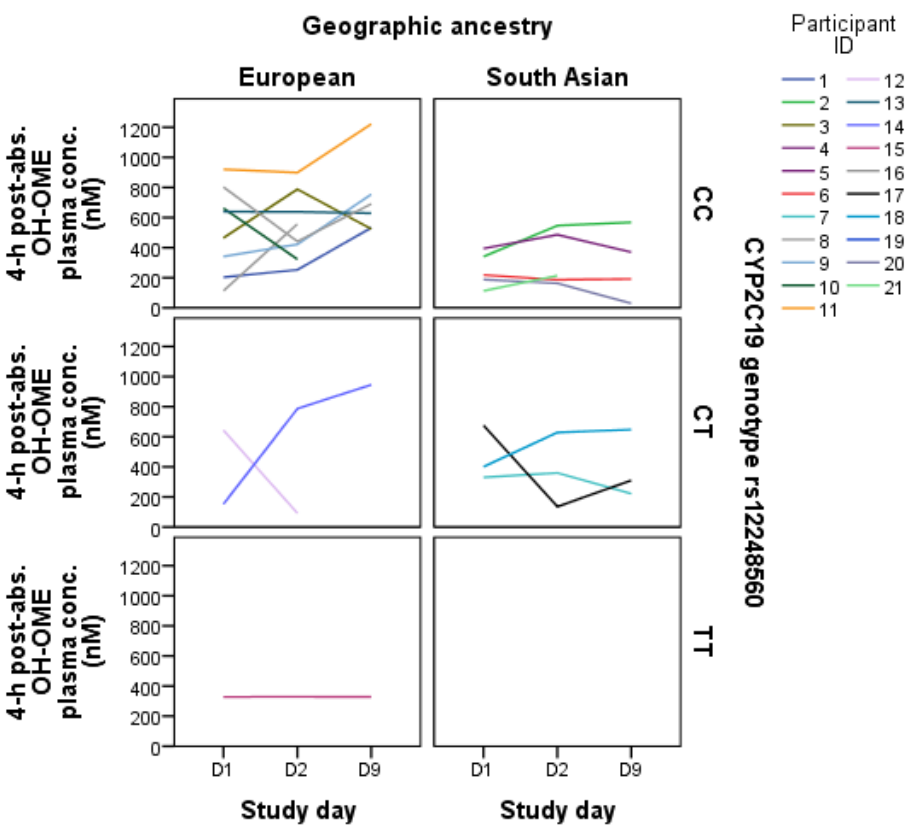
CYP2C19 activity significantly varied by genotype in the mixed-effects model for *CYP2C19*17* (F = 4.175, P = 0.029), *CYP2C19*1C* (F = 3.928, P = 0.060) and *CYP2C19*2* (F = 8.610, P = 0.001). The overall effects for each gene are listed in Table 5.16. Particular genotypes had large, significant effects on CYP2C19 activity as anticipated. For *CYP2C19*17*, CT individuals had 73% higher activity than those with the CC genotype; for *CYP2C19*1C*, GA individuals had 75% higher activity than GG individuals. For *CYP2C19*2*, there was roughly a dose-response relationship: CYP2C19 activity was 5.7-fold higher in GG individuals relative to AA individuals and 3.2-fold higher in GA individuals relative to those with the AA genotype.

Genotype also interacted with ancestry in the model, with *CYP2C19*17* CC Europeans having 90% higher CYP2C19 activity than CC South Asians (GEMM ratio 1.90 [1.16, 3.10], P = 0.035). Further, there was a difference in enzyme activity between Europeans and South Asians with the *CYP2C19*1C* GG genotype (GEMM ratio 2.19 [1.41, 3.39], P = 0.005). This pattern was not repeated across those with *CYP2C19*2* null alleles.

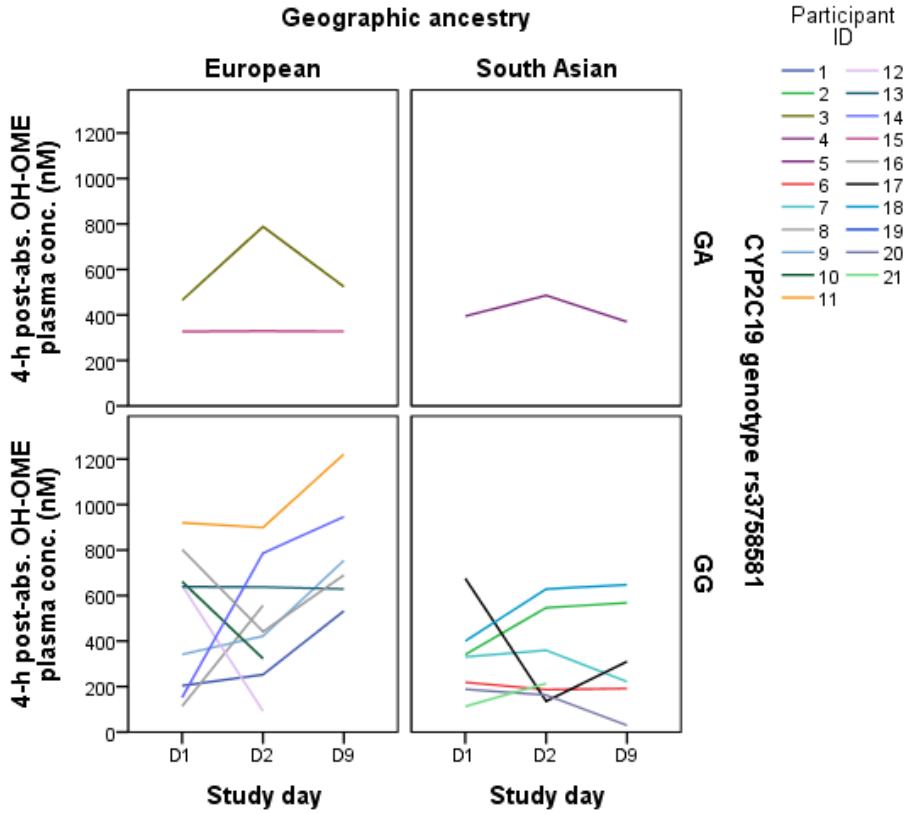
High consumption of CYP1A2 inducers was positively correlated with CYP2C19 activity (correlation coefficient 0.661, P = 0.001) and high consumption of CYP1A2 inhibitors was negatively correlated with activity (correlation coefficient -0.742, P = 0.002). SUL exposure did not significantly correlate with CYP2C19 activity across the three study days.

For better visualisation, individual changes in CYP2C19 activity by ancestry and genotype across study days are depicted below in Figure 5.10.

Figure 5.9: 5-hydroxyomeprazole post-absorption plasma concentrations by study day, ancestry and *CYP2C19*17* (rs12248560) (A), *CYP2C19*1C* (rs3758581) (B) and *CYP2C19*2* (rs4244285) (C) genotypes.

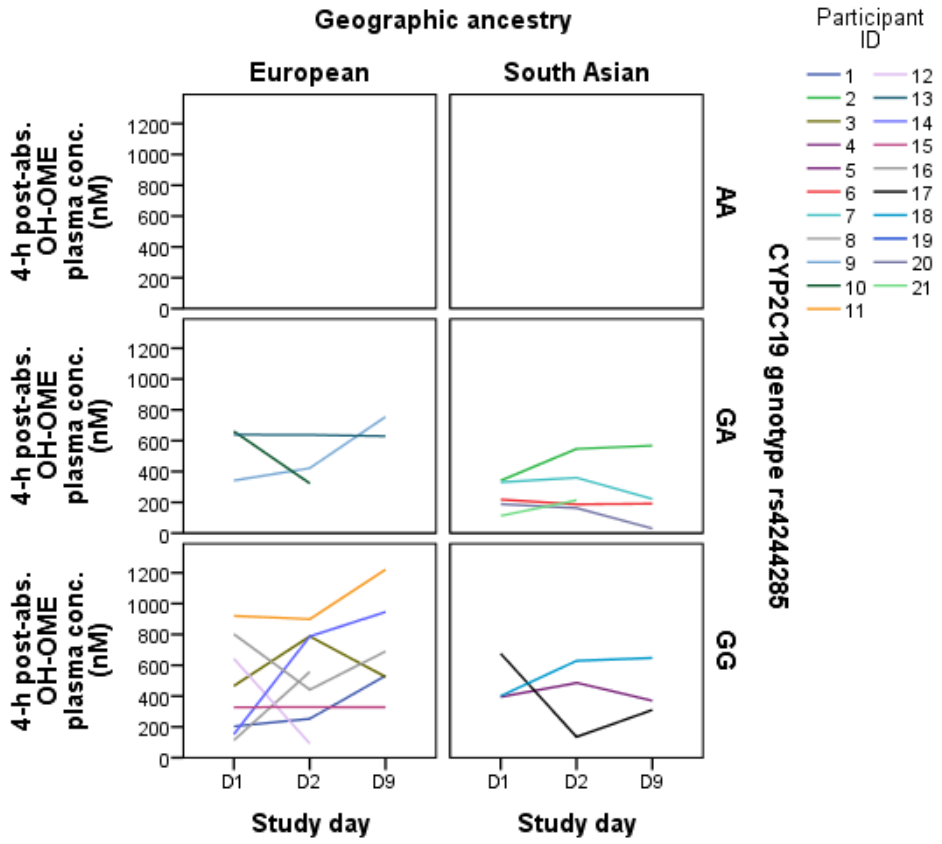


A



B

Figure 5.9: 5-hydroxyomeprazole post-absorption plasma concentrations by study day, ancestry and *CYP2C19*17* (rs12248560) (A), *CYP2C19*1C* (rs3758581) (B) and *CYP2C19*2* (rs4244285) (C) genotypes. Continued from previous page.



C

Table 5.15: Back-transformed geometric EMM ratios of CYP2C19 activity with 90% CIs across study days by ancestry group.

	D2/D1	D9/D1	D9/D2
Europeans (*n* = 11)	0.85 (0.71, 1.03)	1.02 (0.83, 1.25)	1.19 (0.97, 1.47)
South Asians (*n* = 10)	0.81 (0.66, 1.01)	0.96 (0.76, 1.20)	1.17 (0.94, 1.47)
Overall (*n* = 21)	0.83 (0.72, 0.96)[1]	0.99 (0.85, 1.15)	1.18 (1.02, 1.38)[2]

1. *P* = 0.036

2. *P* = 0.072

Table 5.16: Back-transformed geometric EMM ratios with 90% CIs of CYP2C19 activity between *CYP2C19*17*, *CYP2C19*1C* and *CYP2C19*2* genotypes.

***CYP2C19*17* (rs12248560)**		
CT˜/CC#	**CT/TT$**	**TT/CC**
1.73 (1.22, 2.46)[1]	1.13 (0.51, 2.54)	1.53 (0.74, 3.15)
***CYP2C19*1C* (rs3758581)**		
AG^/GG*		
1.75 (1.08, 2.84)[2]		
***CYP2C19*2* (rs4244285)**		
GGx/AAy	**GG/AGz**	**AG/AA**
5.66 (2.47, 12.9)[3]	1.78 (1.29, 2.47)[4]	3.17 (1.42, 7.05)[5]

˜: *n* = 5

#: *n* = 15

$: *n* = 1

^: *n* = 3

*: *n* = 18

x: *n* = 12

y: *n* = 1

z: *n* = 8

1. *P* = 0.013

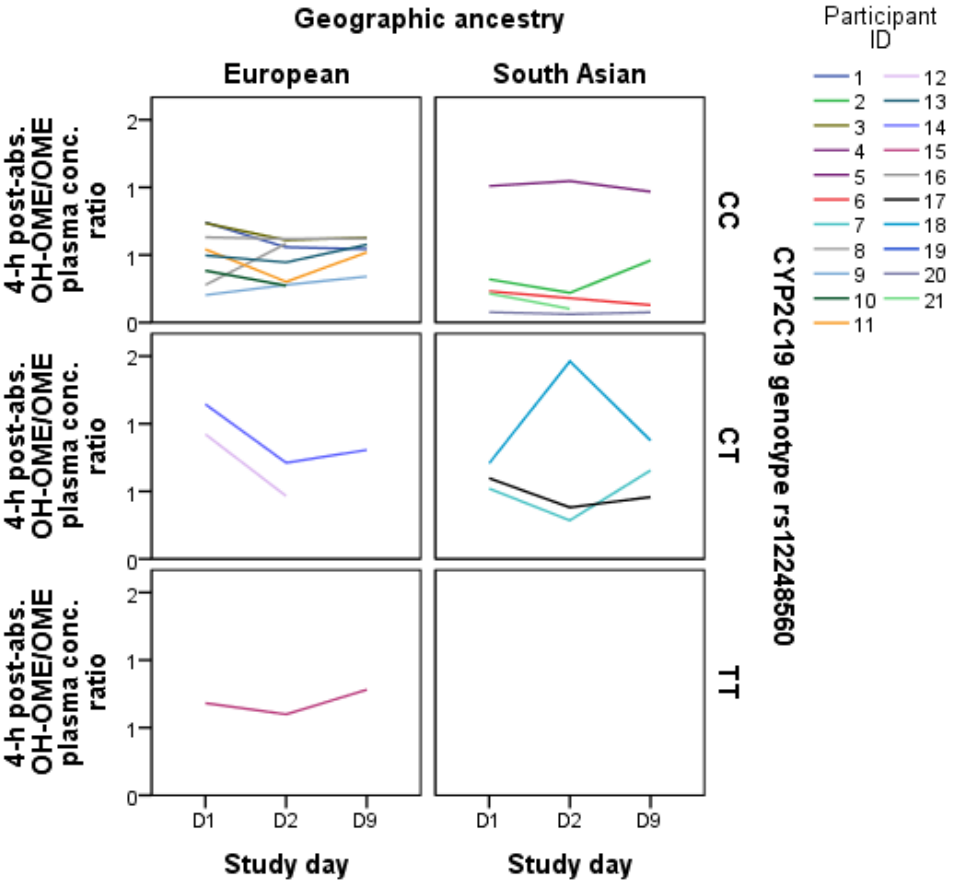
2. *P* = 0.060

3. *P* = 0.001

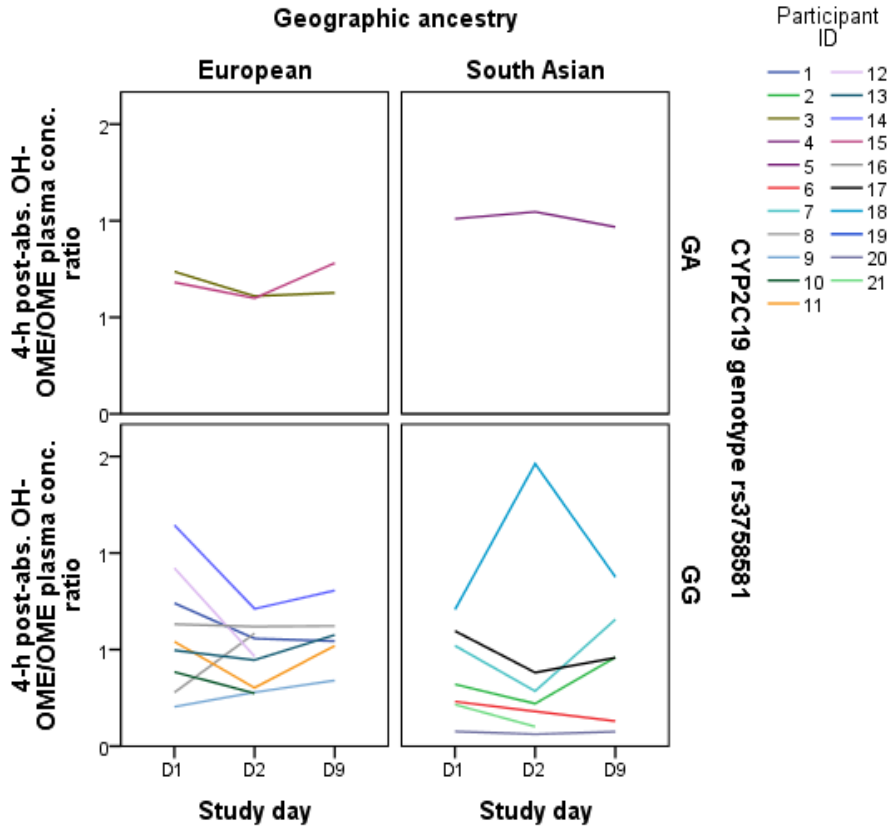
4. *P* = 0.006

5. *P* = 0.020

Figure 5.10: CYP2C19 activity across study days by ancestry and *CYP2C19*17* (rs12248560) (A), *CYP2C19*1C* (rs3758581) (B) and *CYP2C19*2* (rs4244285) (C) genotypes.

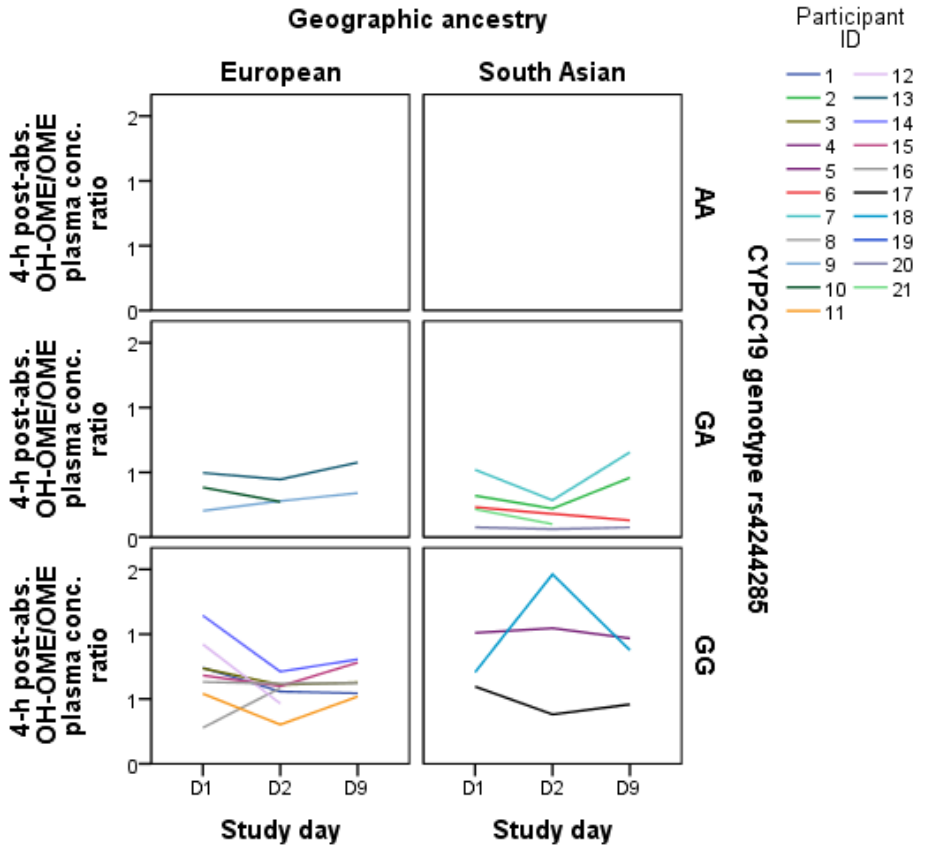


A



B

Figure 5.10: CYP2C19 activity across study days by ancestry and *CYP2C19*17* (rs12248560) (A), *CYP2C19*1C* (rs3758581) (B) and *CYP2C19*2* (rs4244285) (C) genotypes.



C

5.3.4.3 CYP2C9

5.3.4.3.1 Losartan

Losartan $AUC_{0\text{-}6\ h}$ CV% ranged from 41-49% in Europeans and 49-52% in South Asians; CV% varied little within ancestry groups across the study days (Table 5.17). Individual participant losartan data by ancestry and CYP2C9 genotype across study days are shown in Figure 5.11. Losartan plasma concentrations were similar in both ancestry groups (Europeans: 372 ± 162 nM.h, South Asians: 375 ± 191 nM.h). In the Europeans, mean losartan concentrations increased throughout the study after 6 days of broccoli consumption (D1 to D2 to D9); in the South Asian ancestry group, the concentrations were stable immediately after a broccoli meal (D1 to D2) and decreased slightly after 6 days of broccoli consumption (D2 to D9).

5.3.4.3.2 Losartan carboxylic acid

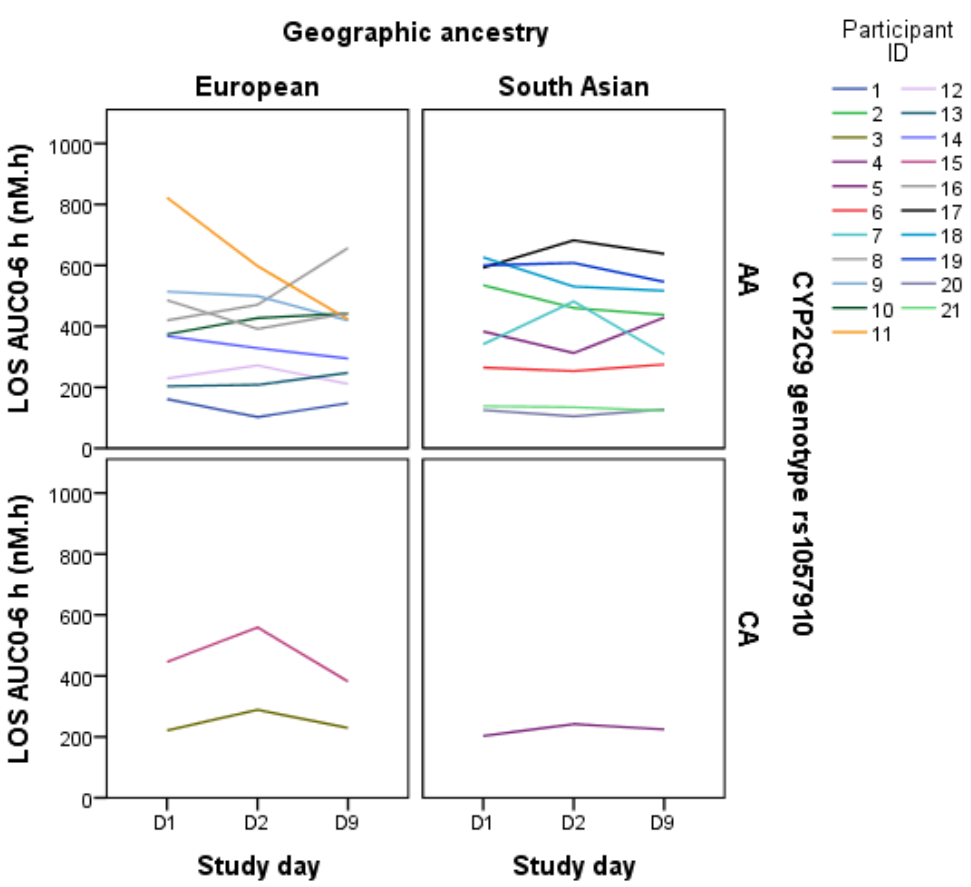
Losartan carboxylic acid (EXP) concentrations had comparable variability to losartan: CV% ranged from 41-53% in Europeans and 56-58% in South Asians (Table 5.17). Individual participant EXP plasma concentrations by ancestry, and genotype across study days are shown in Figure 5.9. Plasma concentrations were higher throughout the study in the South Asian cohort compared to the Europeans (912 ± 520 nM.h vs 563 ± 251 nM.h, respectively). Mean EXP concentrations increased immediately after a broccoli meal (D1 to D2) then decreased after 6 days of broccoli consumption (D2 to D9), and this pattern was also observed in the South Asian ancestry group.

Table 5.17: CYP2C9 data across study days by ancestry. Continues onto next page.

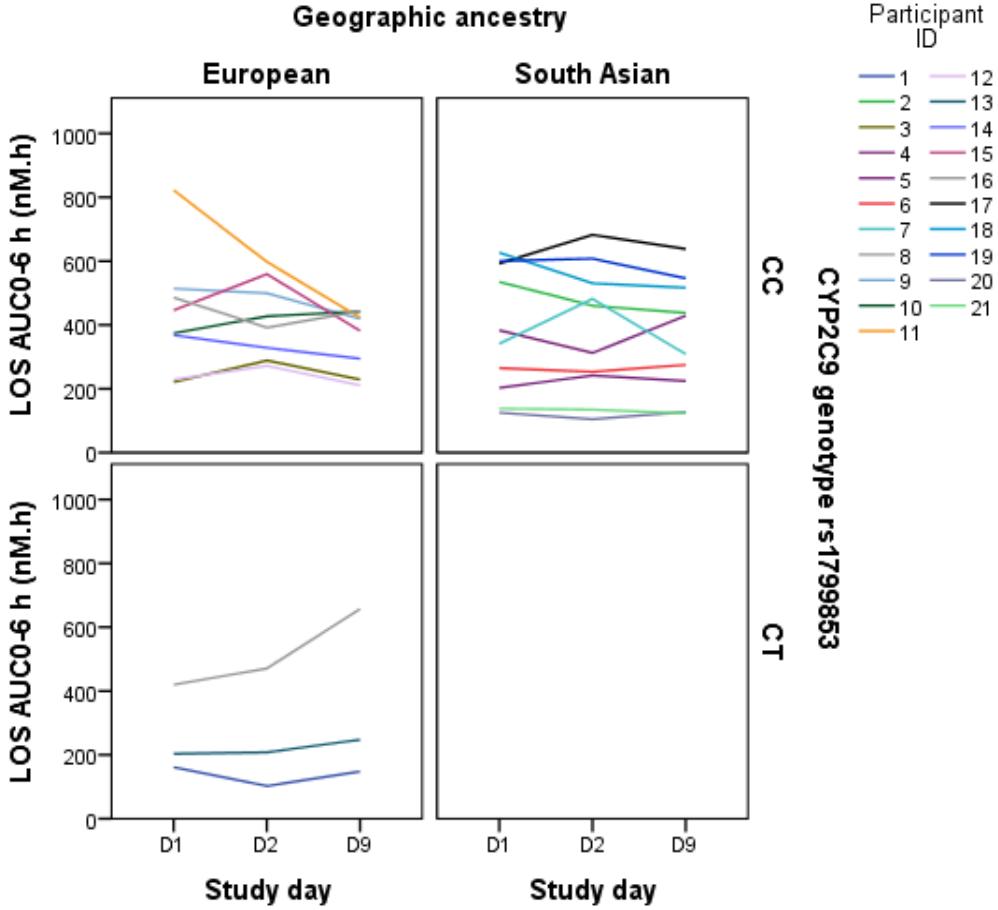
	Genotype			CYP2C9 activity								
				D1			D2			D9		
ID	*CYP2C9*2* rs1799853 3608C>T	*CYP2C9*3* rs1057910 42614A>C	Geographic ancestry	LOS $AUC_{0\text{-}6\ h}$ (nM.h)	EXP $AUC_{0\text{-}6\ h}$ (nM.h)	EXP/LOS $AUC_{0\text{-}6\ h}$ ratio	LOS $AUC_{0\text{-}6\ h}$ (nM.h)	EXP $AUC_{0\text{-}6}$ (nM.h)	EXP/LOS $AUC_{0\text{-}6\ h}$ ratio	LOS $AUC_{0\text{-}6\ h}$ (nM.h)	EXP $AUC_{0\text{-}6\ h}$ (nM.h)	EXP/LOS $AUC_{0\text{-}6\ h}$ ratio
1	CT	AA	European	161	159	0.988	102	336	3.280	148	597	4.035
3	CC	AC	European	221	230	1.039	289	471	1.632	229	300	1.310
8	CT	AA	European	420	397	0.946	471	655	1.391	657	631	0.961
9	CC	AA	European	514	595	1.157	499	789	1.580	420	402	0.958
10	CC	AA	European	374	544	1.455	427	459	1.075	442	447	1.011
11	CC	AA	European	822	987	1.201	598	958	1.602	423	648	1.530
12	CC	AA	European	229	489	2.136	272	565	2.080	211	484	2.292
13	CT	AA	European	203	208	1.021	208	321	1.543	248	247	0.997
14	CC	AA	European	368	892	2.424	328	1179	3.588	295	1041	3.534
15	CC	AC	European	446	492	1.103	559	598	1.070	382	423	1.108
16	CC	AA	European	486	785	1.616	392	593	1.512	443	653	1.475
			Mean (Europeans)	**386**	**525**	**1.371**	**377**	**629**	**1.850**	**354**	**534**	**1.747**
			SD (Europeans)	**189**	**276**	**0.497**	**153**	**260**	**0.833**	**145**	**218**	**1.086**
			CV%	**49.0**	**52.5**	**36.2**	**40.6**	**41.3**	**45.0**	**41.0**	**40.8**	**62.2**
2	CC	AA	South Asian	535	1642	3.068	460	1930	4.195	438	1395	3.188
4	CC	AC	South Asian	203	551	2.715	242	745	3.080	225	532	2.371
5	CC	AA	South Asian	383	1533	3.999	312	939	3.006	429	1813	4.229
6	CC	AA	South Asian	265	410	1.550	253	344	1.359	275	481	1.749
7	CC	AA	South Asian	341	404	1.185	482	571	1.186	309	457	1.482
17	CC	AA	South Asian	593	1242	2.096	682	1253	1.837	638	1145	1.795
18	CC	AA	South Asian	626	1393	2.224	530	1836	3.462	517	1172	2.267

	Genotype			CYP2C9 activity								
				D1			D2			D9		
ID	*CYP2C9*2* rs17998533608C>T	*CYP2C9*3* rs1057910 42614A>C	Geographic ancestry	LOS $AUC_{0\text{-}6\ h}$ (nM.h)	EXP $AUC_{0\text{-}6\ h}$ (nM.h)	EXP/LOS $AUC_{0\text{-}6\ h}$ ratio	LOS $AUC_{0\text{-}6\ h}$ (nM.h)	EXP $AUC_{0\text{-}6}$ (nM.h)	EXP/LOS $AUC_{0\text{-}6\ h}$ ratio	LOS $AUC_{0\text{-}6\ h}$ (nM.h)	EXP $AUC_{0\text{-}6\ h}$ (nM.h)	EXP/LOS $AUC_{0\text{-}6\ h}$ ratio
19	CC	AA	South Asian	600	978	1.630	608	884	1.454	546	842	1.542
20	CC	AA	South Asian	125	480	3.834	105	756	7.221	127	520	4.077
21	CC	AA	South Asian	138	372	2.700	135	471	3.491	123	251	2.036
			Mean (South Asians)	**381**	**901**	**2.500**	**381**	**973**	**3.029**	**363**	**861**	**2.474**
			SD (South Asians)	**197**	**514**	**0.946**	**200**	**543**	**1.808**	**178**	**503**	**1.013**
			CV%	**51.6**	**57.1**	**37.9**	**52.4**	**55.8**	**59.7**	**49.1**	**58.4**	**41.0**
			Mean (Total)	**384**	**704**	**1.909**	**379**	**793**	**2.412**	**358**	**690**	**2.093**
			SD (Total)	**188**	**440**	**0.927**	**172**	**445**	**1.477**	**158**	**407**	**1.091**
			CV%	**49.0**	**62.6**	**48.6**	**45.4**	**56.1**	**61.2**	**44.0**	**59.0**	**52.1**

Figure 5.11: Losartan $AUC_{0\text{-}6\ h}$ across study days by ancestry and *CYP2C9*3* (rs1057910) (A), *CYP2C9*2* (rs1799853) (B) genotypes.



A



B

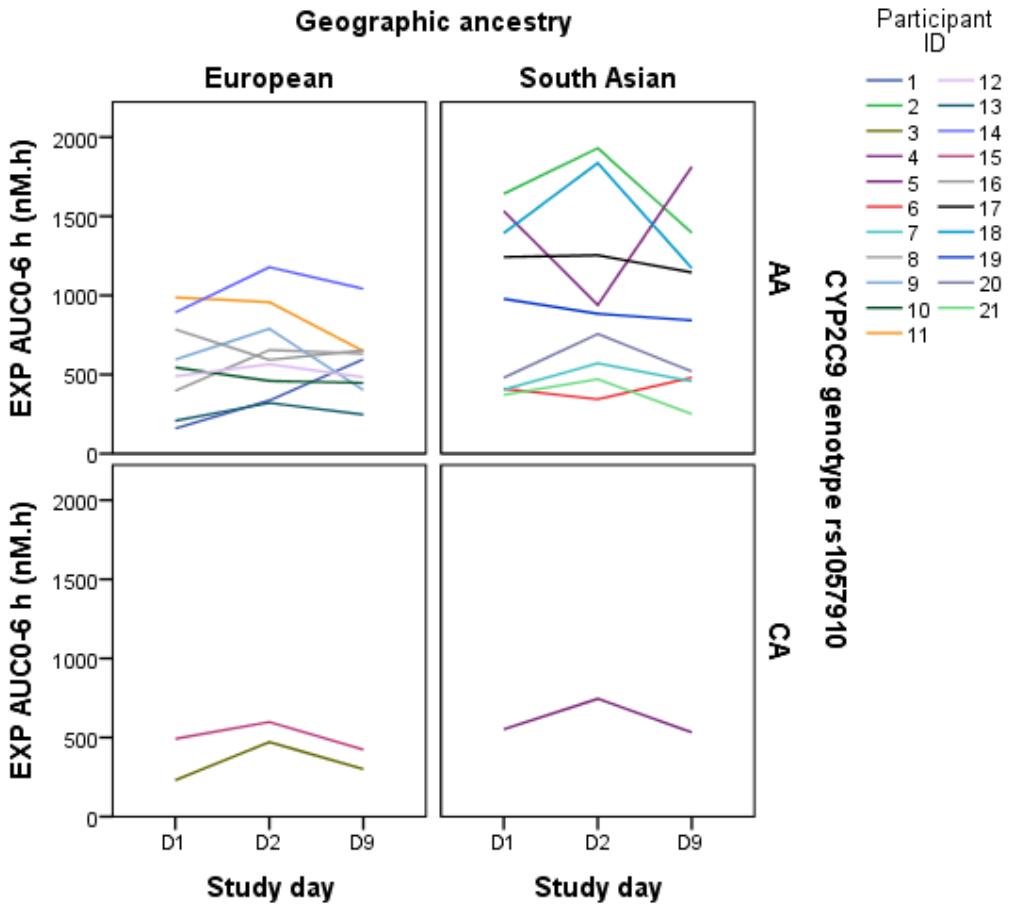
5.3.4.3.3 CYP2C9 activity

In the European ancestry group, the EXP/losartan $AUC_{0\text{-}6\ h}$ ratio CV% ranged from 36-62%. For the South Asian participants, CV% spanned 38-60%. There was a significant change in CYP2C9 activity across study days (F = 4.736, P = 0.014). When explored further, overall, CYP2C9 activity increased 25% from D1 to D2, but decreased 11% by the end of the study (D2 to D9) (Table 5.18). Ancestry had a significant overall effect in the model (F = 6.802, P = 0.016), with South Asians having 1.94-fold higher CYP2C9 activity than Europeans ([1.25, 3.00], P = 0.016). Further, there was a Study Day*Ancestry interaction in the European cohort (F = 4.180, P = 0.022): CYP2C9 activity increased 32% between D1 to D2 (Table 5.18).

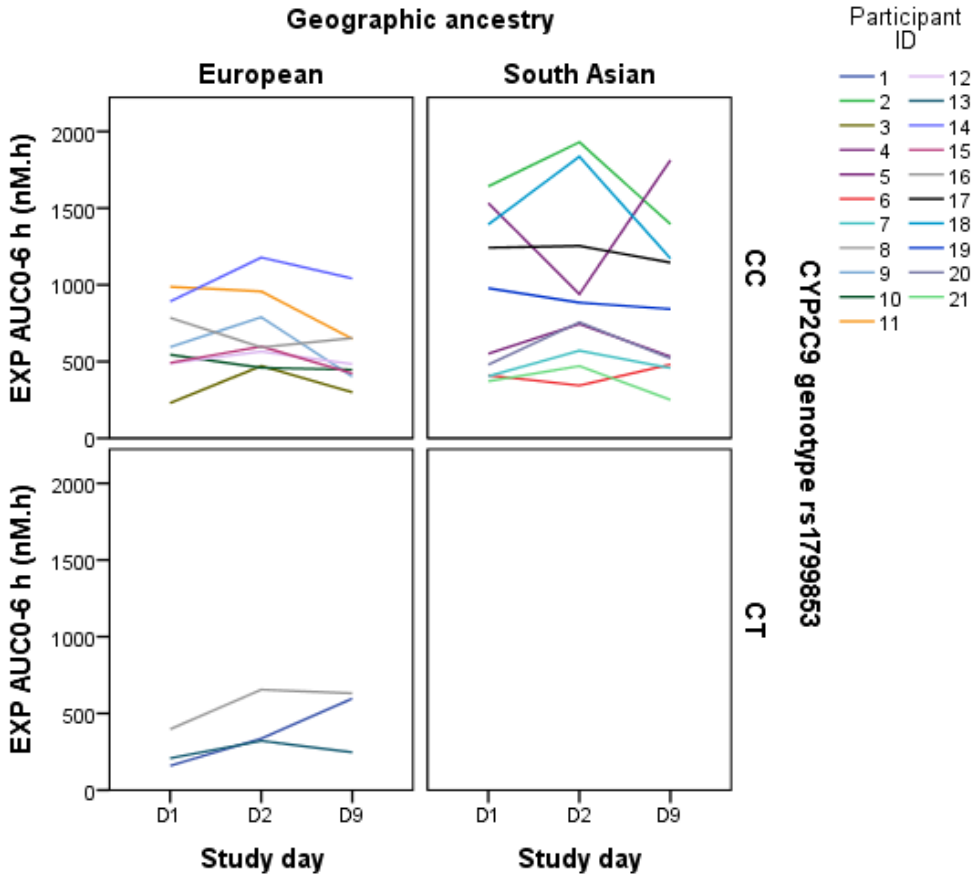
There were no significant overall effects in the model for differences in CYP2C9 activity between genotypes. However, the Ancestry*Genotype interaction in the model was significant, with CYP2C9 activity significantly varying by ancestry within genotypes for *CYP2C9*3* (F = 6.016, P = 0.023) and *CYP2C9*2* (F = 5.899, P = 0.024). The overall differences between the ancestry groups by genotype are listed in Table 5.19. For both variants, South Asians had higher activity than Europeans regardless of genotype. There was no significant correlation between SUL exposure and CYP2C9 activity across the study days.

Individual changes in CYP2C9 activity by ancestry and genotype across study days are shown in Figure 5.13.

Figure 5.12: Losartan carboxylic acid $AUC_{0\text{-}6\ h}$ across study days by ancestry and *CYP2C9*3* (rs1057910) (A), *CYP2C9*2* (rs1799853) (B) genotypes.



A



B

Table 5.18: Back-transformed geometric EMM ratios of CYP2C9 activity with 90% CIs across study days by ancestry group.

	D2/D1	D9/D1	D9/D2
Europeans (*n* = 11)	1.32 (1.12, 1.54)[1]	1.17 (0.99, 1.37)	0.89 (0.76, 1.04)
South Asians (*n* = 10)	1.12 (0.94, 1.32)	0.99 (0.84, 1.17)	0.89 (0.75, 1.05)
Overall (*n* = 21)	1.25 (1.10, 1.41)[2]	1.10 (0.98, 1.24)	0.89 (0.79, 1.00)[3]

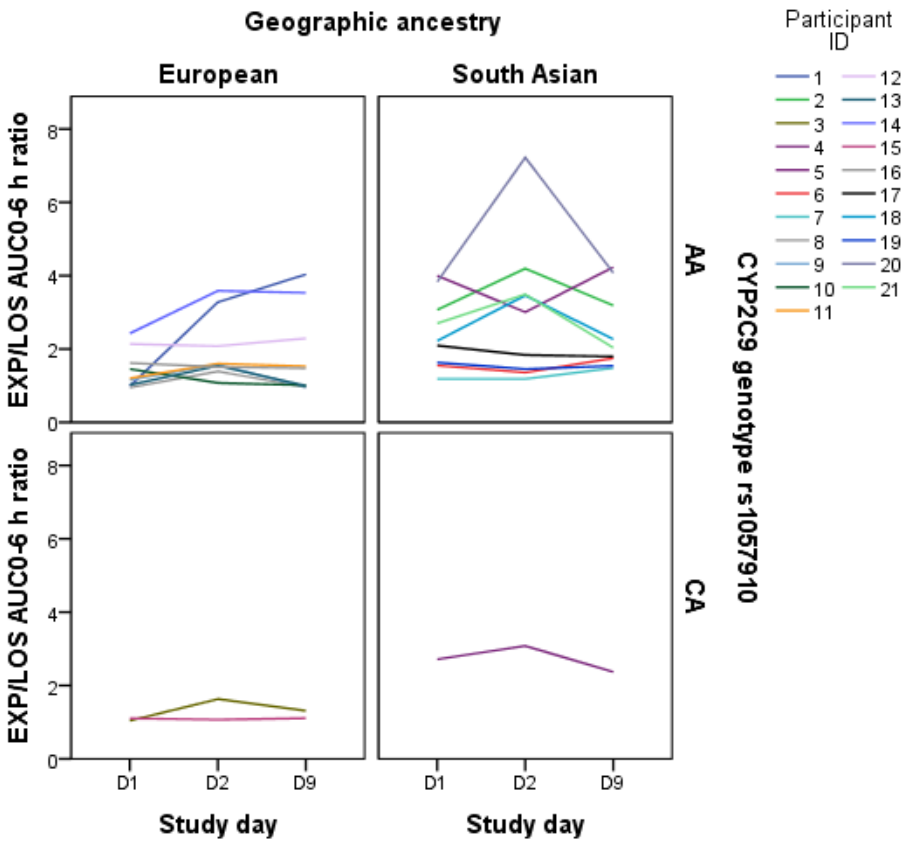
1. *P* = 0.006
2. *P* = 0.004
3. *P* = 0.097

Table 5.19: Back-transformed geometric EMM ratios of CYP2C9 activity with 90% CIs: differences between ancestry groups by *CYP2C9*3* and *CYP2C9*2* genotypes.

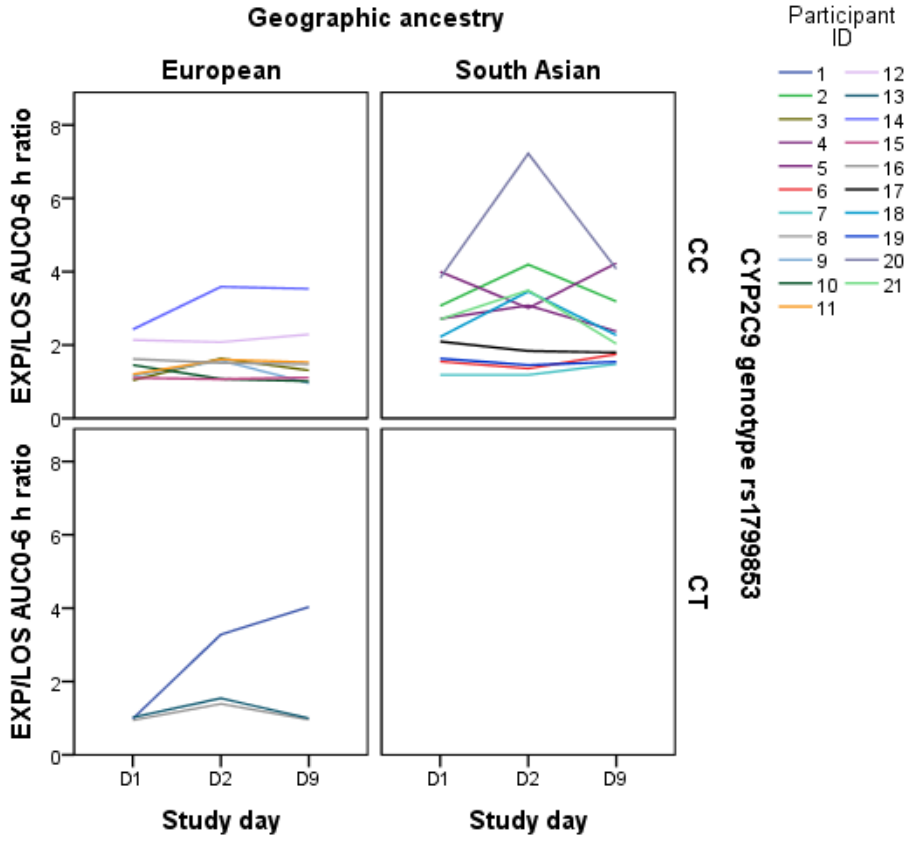
	CYP2C9*3		***CYP2C9*2***
Genotype	**South Asian/European**	**Genotype**	**South Asian/European**
AA~	1.54 (1.14, 2.09)[1]	**CC$**	1.80 (1.19, 2.74)[3]
AC#	2.44 (1.10, 5.41)[2]	**CT^**	n/a

~: South Asian *n* = 9, European *n* = 9
#: South Asian *n* = 1, European *n* = 2
$: South Asian *n* = 10, European *n* = 8
^: South Asian *n* = 3, European *n* = 0
1. *P* = 0.023
2. *P* = 0.069
3. *P* = 0.024

Figure 5.13: CYP2C9 activity across study days by ancestry and *CYP2C9*3* (rs1057910) (A), *CYP2C9*2* (rs1799853) (B) genotypes.



A



B

5.3.4.4 CYP2D6

5.3.4.4.1 Dextromethorphan

Dextromethorphan (DXM) $AUC_{0\text{-}6\ h}$ CV% ranged from 53-60% in Europeans and 60-66% in South Asians; CV% varied little within ancestry groups across the study days (Table 5.20). Because of the large number of genotypes present in the CYP2D6 analyses, individual participant DXM spaghetti plots by ancestry and genotype across study days are displayed in Appendix 8.18, instead of within this chapter. DXM AUC was slightly higher in the South Asian ancestry group compared to the Europeans (45.6 ± 29.2 nM.h vs 36.8 ± 20.6 nM.h, respectively). In the Europeans, mean DXM concentrations increased immediately after a broccoli meal and then increased further after 6 days of broccoli consumption (D1 to D2 to D9); this pattern was also found in the South Asian ancestry group. Frequencies of DXM variant genotypes are displayed in Appendix 8.19.

5.3.4.4.2 Dextrorphan

Dextrorphan (DXR) concentrations had lower variability than DXM: CV% ranged from 35-36% in Europeans and 43-46% in South Asians (Table 5.20). Individual participant DXR plasma concentrations by ancestry, and genotype across study days are depicted with the corresponding DXM data in Appendix 8.18. DXM AUC was higher throughout the study in the European cohort compared to the South Asians (96.8 ± 34.7 nM.h vs 84.0 ± 37.3 nM.h, respectively). In the Europeans, mean DXR concentrations decreased immediately after a broccoli meal and then decreased further after 6 days of broccoli consumption (D1 to D2 to D9); this pattern was also seen in the South Asian ancestry group. Frequencies of DXR variant genotypes are displayed in Appendix 8.19.

Table 5.20: CYP2D6 data across study days by ancestry. Continues onto next page.

		CYP2D6								
		D1			D2			D9		
Participant	Geographic ancestry	DXM $AUC_{0\text{-}6\ h}$ (nM.h)	DXR $AUC_{0\text{-}6\ h}$ (nM.h)	DXR/DXM $AUC_{0\text{-}6\ h}$ ratio	DXM $AUC_{0\text{-}6\ h}$ (nM.h)	DXR $AUC_{0\text{-}6\ h}$ (nM.h)	DXR/DXM $AUC_{0\text{-}6\ h}$ ratio	DXM $AUC_{0\text{-}6\ h}$ (nM.h)	DXR $AUC_{0\text{-}6\ h}$ (nM.h)	DXR/DXM $AUC_{0\text{-}6\ h}$ ratio
1	European	43.9	101.1	2.303	26.1	62.5	2.392	32.0	56.5	1.766
3	European	20.0	100.4	5.032	11.6	86.5	7.445	11.8	75.9	6.444
8	European	40.4	76.7	1.900	55.9	72.7	1.301	67.6	95.5	1.412
9	European	66.8	170.0	2.544	71.8	169.6	2.361	30.7	155.1	5.052
10	European	47.4	161.5	3.405	38.7	123.3	3.182	51.2	158.0	3.086
11	European	75.7	121.5	1.605	57.0	130.8	2.297	35.4	85.0	2.398
12	European	44.4	59.8	1.347	52.7	62.2	1.180	54.9	53.7	0.979
13	European	46.5	94.5	2.035	44.7	72.5	1.623	50.8	98.5	1.938
14	European	12.5	92.3	7.398	14.1	83.9	5.952	7.12	75.1	10.551
15	European	10.3	64.0	6.210	16.8	100.6	5.998	10.2	82.8	8.099
16	European	17.4	72.9	4.182	27.3	91.1	3.342	22.1	88.9	4.024
	Mean (Europeans)	**38.7**	**101.3**	**3.451**	**37.9**	**96.0**	**3.370**	**34.0**	**93.2**	**4.159**
	SD (Europeans)	**21.6**	**36.6**	**2.015**	**20.1**	**33.2**	**2.132**	**20.2**	**34.3**	**3.082**
	CV%	**55.8**	**36.1**	**58.4**	**53.2**	**34.6**	**63.3**	**59.5**	**36.9**	**74.1**
2	South Asian	27.8	76.2	2.738	28.8	109.9	3.813	32.8	99.5	3.030
4	South Asian	27.2	59.3	2.180	27.3	60.1	2.202	30.8	58.7	1.908
5	South Asian	29.6	145.1	4.905	34.1	126.7	3.710	26.8	83.0	3.092
6	South Asian	9.88	68.8	6.964	8.14	41.9	5.147	7.95	50.2	6.323
7	South Asian	10.0	60.8	6.071	22.0	72.1	3.279	19.3	105.9	5.479
17	South Asian	69.5	52.3	0.752	38.3	32.6	0.850	11.9	41.4	3.472
18	South Asian	80.4	128.2	1.595	59.0	104.4	1.768	50.0	116.7	2.331
19	South Asian	94.2	108.1	1.148	71.0	74.5	1.049	69.9	67.6	0.966

		CYP2D6								
		D1			D2			D9		
Participant	Geographic ancestry	DXM $AUC_{0\text{-}6\ h}$ (nM.h)	DXR $AUC_{0\text{-}6\ h}$ (nM.h)	DXR/DXM $AUC_{0\text{-}6\ h}$ ratio	DXM $AUC_{0\text{-}6\ h}$ (nM.h)	DXR $AUC_{0\text{-}6\ h}$ (nM.h)	DXR/DXM $AUC_{0\text{-}6\ h}$ ratio	DXM $AUC_{0\text{-}6\ h}$ (nM.h)	DXR $AUC_{0\text{-}6\ h}$ (nM.h)	DXR/DXM $AUC_{0\text{-}6\ h}$ ratio
20	South Asian	95.9	157.6	1.643	95.8	139.5	1.456	86.5	139.8	1.615
21	South Asian	72.3	53.6	0.741	73.1	49.0	0.670	57.3	36.7	0.640
	Mean (South Asians)	**51.7**	**91.0**	**2.874**	**45.8**	**81.1**	**2.394**	**39.3**	**79.9**	**2.886**
	SD (South Asians)	**34.1**	**40.2**	**2.279**	**27.6**	**37.0**	**1.512**	**25.8**	**34.7**	**1.842**
	CV%	**66.0**	**44.2**	**79.3**	**60.4**	**45.7**	**63.2**	**65.7**	**43.4**	**63.8**
	Mean (Total)	**44.9**	**96.4**	**3.176**	**41.6**	**88.9**	**2.906**	**36.5**	**86.9**	**3.553**
	SD (Total)	**28.3**	**37.8**	**2.111**	**23.7**	**35.0**	**1.885**	**22.6**	**34.3**	**2.589**
	CV%	**63.1**	**39.2**	**66.5**	**57.0**	**39.4**	**64.9**	**62.0**	**39.5**	**72.9**

5.3.4.4.3 CYP2D6 activity

Due to the large number of CYP2D6 variants and copy number variants tested, three linear mixed models were necessary to avoid over-parameterisation and allow for successful EMM generation. In the European ancestry group, the DXR/DXM $AUC_{0\text{-}6\ h}$ ratio CV% ranged from 58-74%. For the South Asian participants, CV% spanned 64-79%. There was a significant change in CYP2D6 activity across study days: activity increased 17% from D2 to D9 (F = 1.806, P = 0.052), although this was not supported by statistically significant changes between the other study days (Table 5.21). Ancestry did not have a significant overall effect in the model, nor did Study Day*Ancestry.

Genetic variants and gene copy number had no significant overall effects in the models due to the low numbers in each variant genotype sub-group. However, the Ancestry*Genotype interactions in the models were significant, with CYP2D6 activity significantly varying between ancestries by genotype for *CYP2D6*10* (F = 4.202, P = 0.061), *CYP2D6*4* (F = 6.725, P = 0.019) and copy number (F = 4.164, P = 0.059). The overall differences between the ancestry groups by genotype are listed in Table 5.22. For *CYP2D6*10* GG and *CYP2D6*4* CC individuals, Europeans had higher CYP2D6 activity than South Asians. For those individuals with one copy of the CYP2D6 gene, Europeans had 3-fold higher activity than South Asians.

Table 5.21: Back-transformed geometric EMM ratios of CYP2D6 activity with 90% CIs across study days by ancestry group.

	D2/D1	D9/D1	D9/D2
Europeans (*n* = 11)	0.95 (0.78, 1.15)	1.08 (0.89, 1.32)	1.14 (0.94, 1.38)
South Asians (*n* = 10)	0.91 (0.74, 1.11)	1.09 (0.89, 1.34)	1.20 (0.98, 1.47)
Overall (*n* = 21)	0.93 (0.81, 1.06)	1.09 (0.95, 1.24)	1.17 (1.02, 1.35)[1]

1. P = 0.052

Table 5.22: Back-transformed geometric EMM ratios of CYP2D6 activity with 90% CIs between ancestry by *CYP2D6*4* and *CYP2D6*10* genotypes and *CYP2D6* gene copy number.

	CYP2D6*4		***CYP2D6*10***		***CYP2D6* gene copy number**
Genotype	**European/South Asian**	**Genotype**	**European/South Asian**	**Category**	**European/South Asian**
GG~	2.02 (1.10, 3.70)[1]	**CC$**	2.13 (1.28, 3.52)[2]	**1 copy&**	3.02 (1.17, 7.81)[3]
AG#	0.43 (0.10, 1.73)	**CT^**	0.42 (0.14, 1.30)	**2 copies***	0.39 (0.12, 1.29)
				> 2 copiesx	1.70 (0.93, 3.10)

~: European *n* = 7, South Asian *n* = 9

#: European *n* = 4, South Asian *n* = 1

$: European *n* = 7, South Asian *n* = 9

^: European *n* = 4, South Asian *n* = 1

&: European *n* = 2, South Asian *n* = 3

*: European *n* = 3, South Asian *n* = 1

x: European *n* = 6, South Asian *n* = 6

1. *P* = 0.061

2. *P* = 0.019

3. *P* = 0.059

5.3.4.5 CYP3A4

5.3.4.5.1 Midazolam

The 4-h midazolam plasma concentration CV% ranged from 39-50% in Europeans and 36-50% in South Asians; CV% varied little within ancestry groups across the study days (Table 5.23). Individual participant midazolam data across study days by ancestry and genotype are shown in Figure 5.14. Mean 4-h midazolam plasma concentrations were similar in both ancestry groups (Europeans: 5.99 ± 2.68 nM, South Asians: 6.13 ± 2.73 nM). In the Europeans, mean 4-h midazolam concentrations increased immediately after a broccoli meal (D1 to D2), then decreased after 6 days of broccoli consumption (D2 to D9). Midazolam concentrations at 4-h increased from D1 to D2 to D9 in the South Asian ancestry group.

5.3.4.5.2 α-hydroxymidazolam

α-hydroxymidazolam (OH-MID) concentrations had larger variability than midazolam: CV% ranged from 37-64% in Europeans and 54-76% in South Asians (Table 5.23). Individual participant 4-h OH-MID plasma concentrations across study days by ancestry and genotype are shown in Figure 5.15. Mean 4-h plasma concentrations were very similar in both ancestry groups (Europeans: 2.93 ± 1.52 nM, South Asians: 2.42 ± 1.52 nM). Mean OH-MID concentrations increased between D1 to D2 then decreased between D2 to D9, and this pattern was also seen in the South Asian ancestry group.

Table 5.23: CYP3A4 data across study days by ancestry. Continues onto next page.

	Genotype		CYP3A4								
			D1			D2			D9		
Participant	*CYP3A4*22* rs35599367 15389C>T	Geographic ancestry	4-h MID conc. (nM)	4-h OH-MID conc. (nM)	OH-MID/MID 4-h conc. ratio	4-h MID conc. (nM)	4-h OH-MID conc. (nM)	OH-MID/MID 4-h conc. ratio	4-h MID conc. (nM)	4-h OH-MID conc. (nM)	OH-MID/MID 4-h conc. ratio
1	CC	European	4.08	2.43	0.595	5.87	4.98	0.848	5.48	3.06	0.558
3	CT	European	3.91	1.62	0.414	2.55	1.25	0.490	4.63	1.87	0.403
8	CC	European	2.84	1.70	0.598	3.71	2.44	0.657	5.13	3.79	0.739
9	CC	European	5.57	3.64	0.654	10.5	4.57	0.434	4.50	4.07	0.904
10	CC	European	9.09	2.99	0.329	10.7	3.68	0.345	8.89	2.77	0.312
11	CC	European	11.7	5.97	0.510	8.40	7.91	0.942	7.37	1.81	0.245
12	CC	European	2.71	1.12	0.413	4.20	0.99	0.235	3.92	1.25	0.318
13	CC	European	5.82	2.06	0.354	6.59	1.99	0.303	3.92	1.77	0.450
14	CC	European	3.89	3.13	0.804	3.01	2.28	0.759	2.53	2.17	0.858
15	CC	European	10.0	5.64	0.563	6.21	2.94	0.474	7.39	3.90	0.528
16	CC	European	6.72	2.32	0.345	6.31	1.92	0.304	9.45	2.51	0.266
		Mean (Europeans)	**6.03**	**2.97**	**0.507**	**6.19**	**3.18**	**0.526**	**5.75**	**2.63**	**0.508**
		SD (Europeans)	**3.04**	**1.58**	**0.151**	**2.79**	**2.02**	**0.240**	**2.22**	**0.97**	**0.235**
		CV%	**50.4**	**53.4**	**29.7**	**45.0**	**63.6**	**45.6**	**38.6**	**36.8**	**46.3**
2	CC	South Asian	3.38	2.60	0.768	5.08	3.34	0.657	4.57	2.34	0.511
4	CC	South Asian	1.76	1.41	0.799	3.31	2.28	0.691	2.21	1.56	0.707
5	CC	South Asian	4.01	1.86	0.464	5.36	2.54	0.474	3.35	1.77	0.528
6	CC	South Asian	6.09	2.06	0.338	7.05	2.34	0.332	8.71	2.37	0.272
7	CC	South Asian	7.02	3.09	0.440	4.76	1.97	0.415	10.7	7.09	0.660
17	CC	South Asian	7.95	2.95	0.371	7.07	2.66	0.377	6.73	2.39	0.356
18	CC	South Asian	8.36	4.36	0.521	5.95	3.76	0.632	7.51	3.65	0.486

	Genotype		CYP3A4								
			D1			D2			D9		
Participant	*CYP3A4*22* rs35599367 15389C>T	Geographic ancestry	4-h MID conc. (nM)	4-h OH-MID conc. (nM)	OH-MID/MID 4-h conc. ratio	4-h MID conc. (nM)	4-h OH-MID conc. (nM)	OH-MID/MID 4-h conc. ratio	4-h MID conc. (nM)	4-h OH-MID conc. (nM)	OH-MID/MID 4-h conc. ratio
19	CC	South Asian	10.8	2.80	0.260	11.2	5.45	0.486	12.5	3.05	0.244
20	CC	South Asian	3.94	0.27	0.069	4.94	0.84	0.169	4.53	0.59	0.129
21	CC	South Asian	4.31	0.32	0.074	5.23	0.69	0.133	5.45	0.28	0.052
		Mean (South Asians)	**5.76**	**2.17**	**0.410**	**5.99**	**2.59**	**0.436**	**6.63**	**2.51**	**0.395**
		SD (South Asians)	**2.76**	**1.27**	**0.248**	**2.14**	**1.39**	**0.193**	**3.28**	**1.91**	**0.220**
		CV%	**48.0**	**58.5**	**60.5**	**35.6**	**53.6**	**44.2**	**49.5**	**76.0**	**55.7**
		Mean (Total)	**5.90**	**2.59**	**0.461**	**6.10**	**2.90**	**0.484**	**6.17**	**2.57**	**0.454**
		SD (Total)	**2.84**	**1.46**	**0.204**	**2.44**	**1.73**	**0.218**	**2.74**	**1.45**	**0.229**
		CV%	**48.1**	**56.6**	**44.2**	**40.0**	**59.8**	**45.2**	**44.4**	**56.4**	**50.6**

Figure 5.14: 4-h midazolam plasma concentrations across study days by ancestry and *CYP3A4*22* (rs35599367) genotype.

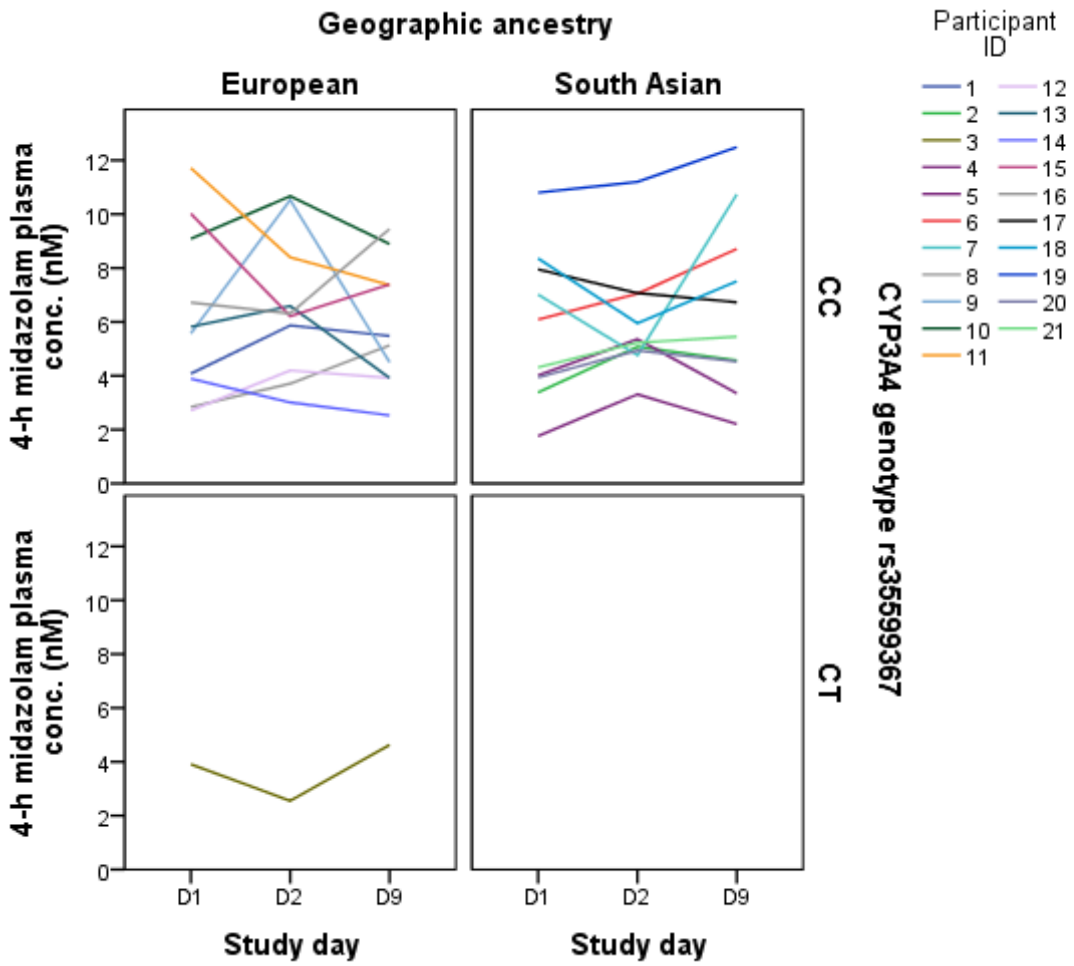
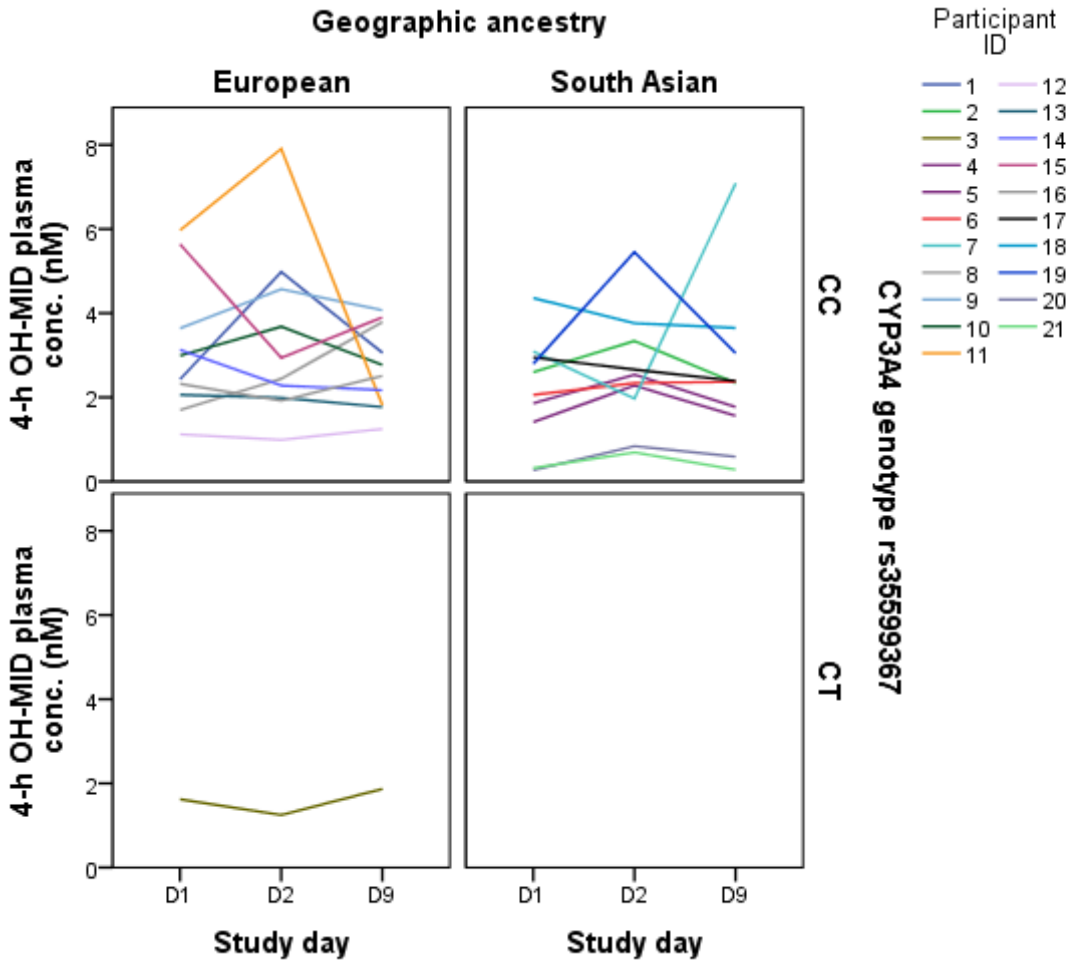


Figure 5.15: 4-h α-hydroxymidazolam plasma concentrations across study days by ancestry and *CYP3A4*22* (rs35599367) genotype.



5.3.4.5.3 CYP3A4 activity

In the European ancestry group, the 4-h OH-MID/MID plasma concentration ratio CV% ranged from 30-46% (Table 5.23). For the South Asian participants, CV% spanned 44-61%. There was a no significant effect of Study Day on the mixed-effects model, and therefore no statistically significant changes in CYP3A4 activity across study days (F = 0.588, P = 0.560). Similarly, Ancestry had no significant overall effect on the model (F = 0.713, P = 0.410). Further, there was no significant effect of Study Day*Ancestry (F = 1.845, P = 0.172), but the *post hoc* test panel indicated that CYP3A4 activity decreased 19% between D2 to D9 (Table 5.24). This should be interpreted cautiously due to a failed Study Day*Ancestry F test. Genotype had no significant overall effects on the model (F = 0.016, P = 0.902), nor was there an Ancestry*Genotype interaction (F = 1.825, P = 0.194)

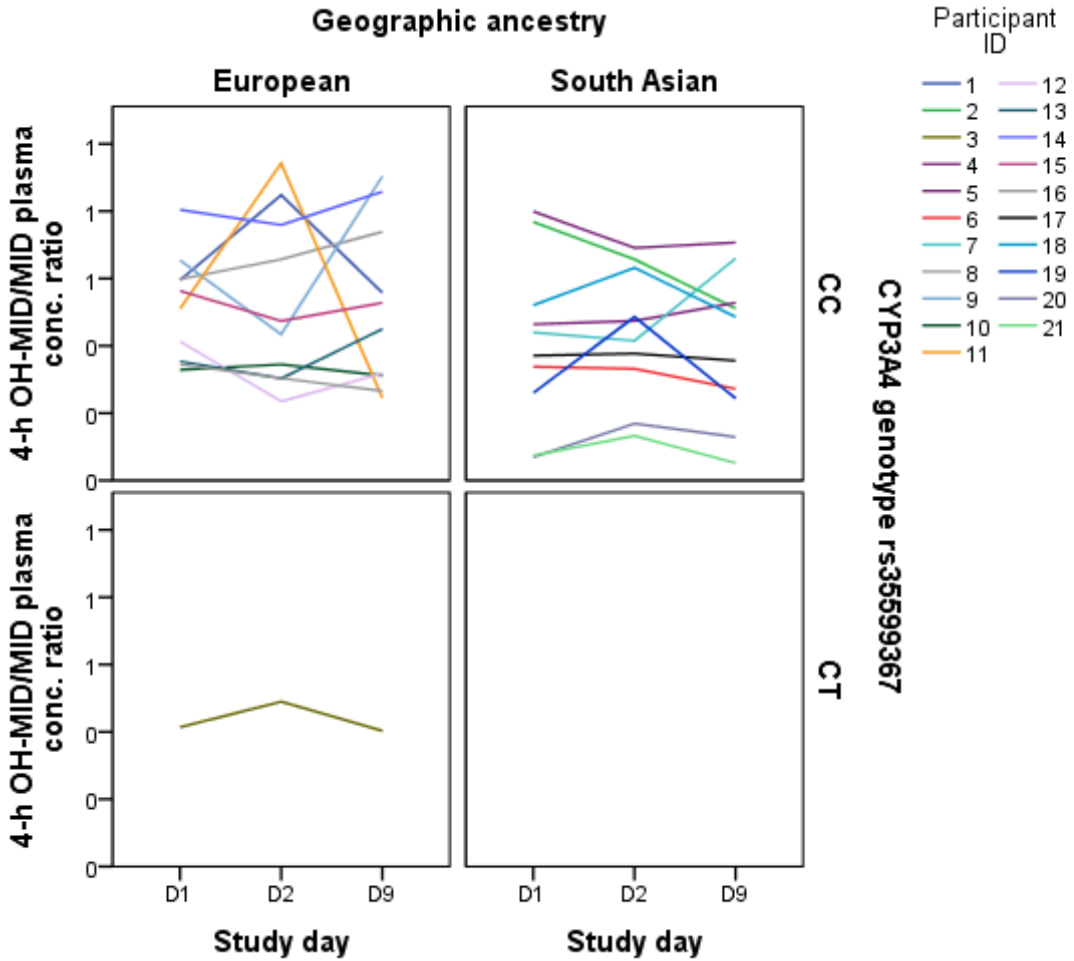
For better visualisation, individual changes in CYP3A4 activity by ancestry and genotype across study days are depicted below in Figure 5.16.

Table 5.24: Back-transformed geometric EMM ratios of CYP3A4 activity with 90% CIs across study days by ancestry group.

	D2/D1	D9/D1	D9/D2
Europeans (*n* = 11)	0.98 (0.81, 1.19)	0.94 (0.78, 1.15)	0.96 (0.79, 1.17)
South Asians (*n* = 10)	1.22 (0.99, 1.49)	0.99 (0.81, 1.21)	0.81 (0.66, 0.99)[1]
Overall (*n* = 21)	1.05 (0.91, 1.22)	0.96 (0.83, 1.11)	0.91 (0.79, 1.05)

1. P = 0.096

Figure 5.16: CYP3A4 activity across study days by ancestry and *CYP3A4*22* (rs35599367) genotype.



5.3.5 Genetics

Frequencies of drug-metabolising gene variants by ancestry group are shown in Appendix 8.19. Statistical analyses on differences in allele frequencies between ancestry groups were not performed because this study was not designed nor powered around these comparisons. Further, with *n* = 21 participants overall, there were few subjects with variants, complicating any interpretation of statistical comparisons between ancestry groups. However, numerous variant genotypes were significant predictors of CYP enzyme activity and SUL exposure, and these have been reported above for the relevant mixed-effects models studied described in this chapter.

5.4 Discussion

This study is the first report of a simultaneous investigation into how diet, genetics and geographic ancestry affect the activities of five CYP enzymes, and how these effects can be related to SUL exposure. In this study, genetics and a predominantly curry diet were strong predictors of SUL exposure. In Chapter 2, studies were identified that showed a relationship between *GSTM1*, *GSTP1* and *UGT1A1* genotypes and ITC exposure, alongside corresponding increases in drug-metabolising enzyme activity. In particular, work by Peterson *et al.* (2009) report data showing that the *GSTM1*-null allele is associated with higher amounts of isothiocyanates excreted in urine, presumably due to inhibition of ITC metabolism. While none of the natural log-transformed CYP metrics significantly correlated with SUL D2 $AUC_{0\text{-}8\ h}$, this does not preclude an effect, as local exposure following absorption on intestinal enzymes and transporters could contribute to these observations.

There were no significant differences in SUL exposure between ancestry groups. However, as increased SUL exposure was strongly associated with a predominantly curry diet, curry constituents could inhibit the phase II enzymes that metabolise SUL (as they do with CYP1A2). It is worth noting that this study only measured one ITC, SUL (Chapter 4), and that phenethyl isothiocyanate (PEITC), indoles and glucosinolates themselves are also reported to affect drug-metabolising enzyme activity (Chapter 1). Future studies should measure the plasma concentrations of other ITCs and their active metabolites to further explore the mechanisms of ITC-induced increases in drug-metabolising enzyme activity. This study reports for the first-time data on optimal sampling times for SUL plasma concentrations as metrics for ITC exposure. SUL D2 $AUC_{0\text{-}8\ h}$ significantly correlated with all other SUL concentration-time points, with the 3- and 4-hour samples having nearly 1:1 correlation.

These results are useful for future studies when measuring SUL exposure; one 4-h SUL sample provides information about systematic exposure equivalent to the 6 samples required to calculate the $AUC_{0\text{-}8\ h}$.

Broccoli consumption significantly increased CYP1A2 in the European cohort, but not in the South Asians, in agreement with observations in a previous study (Perera *et al.*, 2012a) and consistent with other controlled trials (Chapter 2). Further, the parent drug (caffeine) decreased and its CYP1A2-mediated metabolite (paraxanthine) increased in the European group, supporting a case for increased CYP1A2 enzyme activity in Europeans who consume broccoli-enriched diets. There was no evidence of short-term inhibition on D2 immediately following a broccoli meal. Genetics contributed to variability in CYP1A2 activity overall, with the *CYP1A2*1F* variant having the greatest effect. There were however no significant Ancestry*Genotype interactions in the model, therefore the difference in activity between ancestry groups were not explained in terms of a difference in SNP frequencies. Curry consumption was significantly higher in the South Asians and heavy CYP-inducer consumption was higher in the Europeans. Curry constituents are potential CYP1A2 inhibitors (Chapter 1), and their higher consumption in South Asians could explain lower CYP1A2 activity in this group after broccoli consumption.

There were issues encountered when studying CYP2C19 activity, namely erratic and unpredictable absorption of omeprazole from the commercial tablet used (and therefore erratic and unpredictable formation of 5-hydroxyomeprazole). Other studies have reported similar issues with using omeprazole as an *in vivo* probe for CYP2C19 activity in humans (Chapter 3). However, one of the strengths of the mixed-effects modelling approach for analysing pharmacokinetic crossover data is that the model can account for missing data

across the included factors. Therefore, even with some cases missing, an analysis was still made possible. There was evidence of short-term inhibition of CYP2C19 immediately after a broccoli meal, which then increased to approximately the same initial activity after 6 days of broccoli consumption. This was observed in both ancestry groups. Genotype played a large role in accounting for CYP2C19 variability, with activity differences for all three variants (*CYP2C19*17*, *CYP2C19*1C* and *CYP2C19*2*) studied in the model. Interestingly, there was also an ancestry group difference by genotype: Europeans tended to have higher activity than South Asians when their higher-activity variant genotypes were the same. One hypothesis to explain this is the increased curry consumption in the South Asian cohort: the CYPs share common molecular regulatory mechanisms, therefore if curry constituents can inhibit CYP1A2, they may also inhibit other CYP enzymes (Chapter 1). If an enzyme is being inhibited at the active site, induction will not produce an increased activity response, as any new enzyme formed will be inhibited by the presence of the dietary constituent. Moreover, CYP2C19 activity was positively associated with high CYP1A2 inducer consumption and negatively associated with a high CYP1A2 inhibitor diet. The CYP1A2 inducer diets were more prevalent in Europeans and the inhibitor diets more prevalent in South Asians, offering an explanation of these observations. In the mixed effects model, there was no overall effect of ancestry, nor an overall difference between ancestry groups across study days, indicating that individual gene variants and diet must be considered together when explaining the variability in CYP2C19 in dietary intervention trials. This study represents the first reported attempt to simultaneously explore the effects of diet, ancestry and genetics on CYP2C19 (Chapter 2).

Similarly, the effects of cruciferous vegetable-enriched diets, genetics and geographic ancestry on CYP2C9 activity have not yet been reported (Chapter 2). Interestingly, activity

increased immediately after a broccoli meal, and then decreased following 6-days of broccoli consumption, as indicated by an increase in losartan and a decrease in EXP concentrations. South Asians had nearly 2-fold higher overall CYP2C9 activity than the Europeans, but this result could not be explained by differences in genotype, as the ancestry group difference was still noted in the variant genotype sub-groups. It remains uncertain why a marked difference in CYP2C9 activity between the ancestry groups was observed, especially given that it could not be explained by differences in the genotypes studies or diet data captured between Europeans and South Asians. Further, the various diet categories and SUL exposure did not correlate with CYP2C9 activity, complicating the interpretation of these findings. Lastly, losartan and EXP relative exposure patterns were the same in each ancestry group, suggesting that the changes were not a chance finding.

The activity of CYP2D6 is largely influenced by genetics rather than environmental factors such as diet (Chapter 1), and therefore induction from cruciferous vegetables was not expected. However, there is some research suggesting potential inhibition by the SUL found in broccoli (section 1.7.1). In the current study, acute inhibition followed by an increase in activity between D2 to D9 (similar to CYP2C19) was observed, however only the D2 to D9 difference was statistically significant. There were no overall differences in changes in relation to the broccoli-enriched diet between ancestry groups, either in general or by study day. Interestingly, despite the well-documented variability in CYP2D6 activity between variant genotypes (Chapter 1), genotype and gene copy number did not significantly account for variability in the various mixed-effects models used in this study. However, for a given variant genotype, and also for participants with one copy of the *CYP2D6* gene, Europeans had 2- to 3-fold higher CYP2D6 activity than South Asians. Consequently, when genotype and ancestry are both considered factorially in the models, there is still a

difference between ancestry groups. Seeing as the only measured differentiating factor between the ancestry groups was a higher proportion of predominantly curry consumers in the South Asian cohort, perhaps the CYP-inhibiting constituents in the high-curry diet could explain the higher activity in Europeans when genotypes are equivalent.

As expected, CYP3A4 displayed marked variability even within each ancestry group, and no significant differences in activity were observed between study days. Further, no resolution was possible in the mixed-effects model to determine if there were differences between ancestries across the study days, and there were no significant differences in CYP3A4 activity by *CYP3A4*22* genotype. However, *post hoc* sample size calculations (section 5.2.7.1) suggested that this study was too small to meaningfully assess CYP3A4 across some of this study's endpoints, and further investigation in larger subject cohorts is warranted.

The main weakness of this study is its relatively small sample size (quality characteristics of dietary intervention trials were discussed in Chapter 2). While appropriate design measures and mixed-effects models were used, ultimately more participants are needed across the various genetic and diet sub-categories to perform sufficiently powerful comparisons in each sub-group. Further, some studies, for example, Peterson *et al.* (2009) and Navarro *et al.* (2009b), actively recruited by genotype to test specific hypotheses, which was not possible in this smaller study. It is clear though, that some of the CYPs—especially CYP2C19, CYP2C9 and CYP2D6—have interesting ancestry-diet-genotype interactions as reported in this study, and future investigations should consider recruiting sufficient participants of relevant genotypes for appropriately powered sub-group analyses. Also, with respect to design, this study recruited only male healthy volunteers, and therefore the effects of *Cruciferae*-enriched diets on CYP activity on females cannot be commented on from these

results. Larger, well-designed studies in this area recruited near-equal numbers of males and females, also incorporating sex as a factor or covariate into their mixed-effects models (see work by Navarro *et al*., Peterson *et al.* and Lampe *et al*. in Chapter 2). Future investigators are encouraged to take this approach of investigating the effect of sex on enzyme activity and response to dietary interventions into their study designs if resources allow.

A further limitation of this study is a lack of methods to assess epigenetic effects on CYP activity and how these might differ between subjects of European and South Asian ancestries. As discussed in Chapter 1, epigenetics as a source of variability in drug-metabolising enzymes is a largely uncharted landscape, and much research is required before a strong clinical foundation of these important concepts is established. Inheritable (or otherwise) epigenetic differences between ancestry groups could be the key to explaining some observations in this study. The activity of CYP2C9 in South Asians was consistently higher than Europeans, even when stratified by genotypes. Perhaps the answer lies in yet-undiscovered regulatory genes and proteins, or ancestry group differences in histone methylation. Future studies should incorporate these considerations into their study designs and testable hypotheses, perhaps through epigenetic *ex vivo* analysis of liver biopsy tissue. Similarly, constituents of foodstuffs have been shown to affect the activity of drug transporting proteins (Dolton *et al.*, 2012) (section 1.4), and exploring differences in their expression and activity following cruciferous vegetable-enriched diets is warranted in future research.

Lastly, with respect to design weaknesses, this study did not employ a standardised basal diet. Although participants were asked to make no major changes to their diet during the study period (which was confirmed qualitatively by comparing the two food diaries), having

a basal diet to which the cruciferous vegetable intervention is added allows for more robust control of dietary constituents, which if different at baseline, could confound results. While this study was able to consider the consumption of CYP1A2 inducers, inhibitors and high curry consumption for some of the enzymes, controlling them altogether in a control diet would be beneficial from a design standpoint, and future studies are encouraged to do so if resources and time allow.

5.5 Conclusions

The significance of the research performed here can be framed in the context of the overall objectives of this thesis: to better understand how diet, genetics and geographic ancestry contribute to variability in drug metabolism. This trial is the first to report on how these factors affect five CYP enzymes simultaneously using the phenotyping cocktail approach. Further, data are presented from a prospective, controlled study suggesting that CYP1A2 inducer foods, CYP1A2 inhibitor foods, a predominantly curry diet and a collection of genetic variants contribute to the interindividual variation in CYP activity, in particular for CYP1A2, CYP2C19 and CYP2C9. Some of the enzyme activity effect sizes observed are significant, ranging from approximately 20% to 4-fold differences between participant sub-groups. Effect sizes of this magnitude can be clinically significant for drugs with narrow therapeutic ranges that are substrates for these enzymes. Further research is needed to determine if differences in pharmacokinetics translate to differences in drug efficacy and safety.

The results presented demonstrate successful and effective use of the analytical techniques developed and validated in Chapters 3 and 4. Further, a clear evidence framework has been provided explaining how cruciferous vegetable-enriched diets affect CYP1A2, and to a lesser extent, CYP2C19. The activity of these enzymes varies between individuals of European and South Asian ancestry in terms of genetics and other dietary constituents. There could be implications for patients eating appreciable amounts of cruciferous vegetables when taking medicines metabolised by these enzymes. These data should help investigators when designing future trials in this area of clinical pharmacology.

6 Overall conclusions and closing comments

The research described in this thesis has explored the effects of various intrinsic and extrinsic factors on variability in response to medicines, specifically, on drug-metabolising enzyme activity (as a contributor to variability in pharmacokinetics). Chapter 1 described and explored the relevant background literature covering variability in response to medicines, and this was used to build a thematic, evidence-based framework from which testable hypotheses were formed and investigated. A systematic review with meta-analyses was conducted that assessed the results of studies investigating cruciferous vegetable interventions and drug metabolism (Chapter 2). The results of this review informed the design of a clinical study that investigated the effects of diet, genetics and geographic ancestry on the activities of CYP1A2, CYP2C19, CYP2C9, CYP2D6 and CYP3A4. Before this trial could be conducted, appropriate bioanalytical methods were developed and validated to analyse biological samples collected. The assays for the CYP-phenotyping cocktail and sulforaphane (SUL) are presented in Chapters 3 and 4, respectively. Finally, a 3-period crossover trial in Europeans and South Asians investigated CYP activity between ancestry groups by genotype and diet (Chapter 5).

This body of work contributes to a better understanding of the variability in response to medicines between European and South Asian populations. The systematic review identified literature indicating that the activity of CYP1A2 and GST-α are significantly affected by *Cruciferae*-enriched diets. Further, the effects of cruciferous vegetable consumption on CYP2C19, CYP2C9, CYP2D6 and CYP3A4 activity have not been reported. The study characteristics that are considered appropriate for dietary intervention trials with pharmacokinetic endpoints were profiled. Of significance, none of the studies identified in

the systematic review were designed or powered to explore differences in the effects of *Cruciferae*-enriched diets on drug metabolising enzyme activity between geographic ancestry groups.

For the pharmacokinetic study performed, the Inje and Ghassabian CYP-phenotyping cocktails were used as a platform for the development and validation of an optimised UHPLC-MS/MS CYP assay to measure analytes probing for CYP1A2, CYP2C19, CYP2C9, CYP2D6 and CYP3A4 activities. This assay was successfully used to phenotype the 21 healthy subjects of the study. Similarly, a simplified, optimised SUL assay was developed and validated to measure SUL concentrations following broccoli consumption. The use of these assays allowed for SUL systematic exposure to be measured at the same time as CYP-phenotyping, enabling further exploration of the relationships between diet, genetics, ancestry and CYP activity in Europeans and South Asians.

Since these assays were developed and used, a series of validation studies have been reported that use dry blood spots from capillary, finger-prick whole blood samples as their biological matrix of interest (Bosilkovska *et al.*, 2014a; Bosilkovska *et al.*, 2016; Bosilkovska *et al.*, 2014b). While some of the probe drugs used in these studies are not available in Australia, and use cola beverages as a source of caffeine (see Chapter 3), the minimally-invasive methods presented by Bosilkovska *et al*. are the way forward for *in vivo* phenotyping of CYP enzyme activity. Future pharmacokinetic studies should consider adopting a capillary blood-sampling approach, as using this method greatly reduces sampling volumes, sample handling and storage issues, as well as reducing risk to study participants. Further, with respect to the bioanalysis of ITCs, attempts were made to also incorporate phenethyl isothiocyanate (PEITC) into the SUL bioanalysis. However, due to

poor ionisation in both electrospray and atmospheric chemical ionisation modes (ESI and APCI, respectively), this was not possible. Previous reports indicate that ESI in particular struggles to produce a stable product ion or adduct for analyte quantification, which can be fixed by using APCI instead (Zheng & Zheng, 2015). Unfortunately, the methods and suggestions by Zheng *et al*. were not replicable on the Agilent UHPLC-MS/MS system used in the studies reported in this thesis, and PEITC was unable to be included in the analysis. There is a body of literature that suggests PEITC can affect drug-metabolising enzyme activity (section 1.7.1), therefore efforts to find ways of reliably quantifying this ITC in human biological matrices are encouraged.

The clinical study performed represents the culmination of this research programme (Chapter 5). This study showed that CYP1A2 activity is increased in Europeans, but not South Asians, after a broccoli-enriched diet intervention, supporting previous reports (Perera *et al.*, 2012a). This population difference in CYP1A2 activity could not be explained by ancestry group differences in the *CYP1A2* variants explored. However, diet, and in particular, the consumption of CYP1A2 inducers relative to the consumption of CYP1A2 inhibitors, and differences in the preparation of curry between the ancestry groups could contribute to differences in enzyme activity. Further, GSTs can be inhibited by ITCs (section 1.7.1), and evidence supporting these previous reports is presented here. Sulforaphane exposure was positively correlated with a predominantly curry diet, suggesting that curry constituents can inhibit the GSTs responsible for their clearance. Further, the *GSTM1* and *GSTP1* null alleles were significant predictors of SUL exposure, and when ancestry and genotype were incorporated into the mixed model together, no significant difference was observed between Europeans and South Asians. *GSTT1*, *GSTT2* and *UGT1A1* variant genotypes did not significantly affect SUL exposure, nor did SUL correlate with any of the CYP enzymes studied.

The 4-h SUL plasma concentration was correlated with the $AUC_{0\text{-}8\ h}$, and future studies are encouraged to explore the use of this single time point as a metric for SUL exposure.

This study is the first reported to simultaneously investigate the effects of diet and genetics on CYP2C19 activity between European and South Asian ancestry groups. Evidence of acute inhibition followed by return to baseline following broccoli consumption was presented in this study. Several drugs with narrow therapeutic concentration ranges are metabolised by CYP2C19, such as clopidogrel, cyclophosphamide, phenytoin, voriconazole and the R-isomer of warfarin (AMH, 2018). Therefore, variability in the activity of this enzyme caused by environmental and genetic influences can directly affect patient outcomes in those taking CYP2C19 substrates for various diseases (Amsden & Gubbins, 2017; Hicks *et al.*, 2013; Li *et al.*, 2012; Stingl & Viviani, 2015). It is interesting that CYP2C19 activity was higher in Europeans than South Asians with the same variant genotypes for *CYP2C19*1C* and *CYP2C19*17*. Allele frequencies of these variants were comparable to those found in the 1000 genome project (Table 1.8 and Appendix 8.19). One hypothesis explaining this observation is that a heavy CYP1A2 inducer diet was more common in the Europeans than the South Asians, which could explain the overall higher activity when variant genotypes were equal. Relatively few studies have investigated the effects of genetics on CYP2C19 activity in South Asian populations, but the ones that do report a similar gene-activity relationship with *CYP2C19*17* and *CYP2C19*2* compared to Europeans, suggesting diet (and other extrinsic factors) are responsible for explaining the difference in activity between these two ancestry groups (Arya *et al.*, 2015; Jose *et al.*, 2016; Mahadevan *et al.*, 2014; Shalia *et al.*, 2013; Sridharan *et al.*, 2016; Tantray *et al.*, 2017; Xavier *et al.*, 2016). Future studies should also explore the effects of transporters and epigenetics on differences in CYP2C19 activity between these ancestry groups (sections 1.4, 1.5 and 5.4).

Similarly, there are no reports of how cruciferous vegetable-enriched diets and genetics affect CYP2C9 activity differently in Europeans relative to South Asians. Overall, enzyme activity increased immediately after a broccoli meal, and decreased slightly following 6 days of broccoli consumption. An ancestry group difference was present: South Asians had nearly 2-fold higher CYP2C9 activity than Europeans throughout the study, even when stratified by the same variant genotypes. Further, none of the diet categories or SUL exposure correlated with CYP2C9 activity. Variant allele frequencies for *CYP2C9*2* and *CYP2C9*3* were comparable to those reported for Europeans and South Asians in the 1000 genomes project (Table 1.8 and Appendix 8.19). As CYP2C9 activity is regulated by genetics more than extrinsic factors, few studies have investigated the effects of diet on this enzyme. Therefore, it is difficult to attribute the higher activity in South Asians compared to Europeans of the same variant genotypes to differences in dietary practices. However, a recent clinical study has shown that overnight fasting before phenotyping significantly reduces CYP2C9 activity by up to 25% (Lammers *et al.*, 2015). Perhaps the nature of this 'fasting effect' on CYP2C9 activity could be different between those of different geographic ancestries, and should be explored further. Because CYP2C9 is involved in the metabolic clearance of medicines with narrow therapeutic indices, such as bosentan, cyclophosphamide, phenytoin, ruxolitinib, voriconazole and the S-isomer of warfarin, the 2-fold difference in enzyme activity between South Asians and Europeans presented in this study is of potential clinical significance, and ancestry group differences in the pharmacokinetics and dynamics of these substrate drugs should be investigated.

The effects of cruciferous vegetables, dietary constituents, genetics and ancestry on CYP2D6 and CYP3A4 have not previously been studied simultaneously before. In this research, CYP2D6 activity was 2- to 3-fold higher in Europeans than South Asians in *CYP2D6*4* and

*CYP2D6*10* homozygotes, and those with one copy of the *CYP2D6* gene. Diet was unable to explain this difference between the ancestry groups, although there was evidence of enzyme inhibition following broccoli consumption in both groups. CYP2D6 is highly polymorphic, and haplotypes can be constructed from the numerous variant genotypes, which then correspond to activity phenotype sub-groups (Hicks *et al.*, 2014; Hicks *et al.*, 2013). Because this study was relatively small, some variant genotype sub-groups had few-to-no cases in them, and allele frequencies in Europeans and South Asians were different compared to those reported in the 1000 genome project (Table 1.8 and Appendix 8.19). Therefore, despite the gain in power from using mixed-effects models, the difference in CYP2D6 activity between ancestry groups within the same variant genotypes could be due to chance, rather than a true observed difference as a result of the small sample size. However, the ancestry group difference in activity is large, and therefore warrants further investigation in appropriately powered studies that recruit based on *CYP2D6* variant haplotypes. Lastly, the difference in activity between Europeans and South Asians could be explained by differential involvement of other enzymes. While the conversion of dextromethorphan (DXM) to dextrorphan (DXR) has been shown to be mostly mediated by CYP2D6, other CYP enzymes also contribute, including CYP2C9 and CYP3A4 (Yu & Haining, 2001). Perhaps ancestry-group differences in CYP2C9, as reported in this study, could be driving the observed difference in CYP2D6 activity between Europeans and South Asians of the same variant genotypes.

CYP3A4 displayed marked interindividual variability in both ancestry groups, and *post hoc* sample size and power calculations suggest that a much larger sample size in each ancestry group would be needed to assess the studied effects further for this enzyme. CYP3A4 is far less polymorphic compared to CYP2C19, CYP2C9 and CYP2D6, and this was reflected in the

current study, with only one participant out of the 21 studies having a different genotype for *CYP3A4*22*, in alignment with data from the 1000 genome project (Table 1.8 and Appendix 8.19). From the results of this study, it remains unclear how intrinsic and extrinsic factors interact to explain differences in CYP3A4 activity between Europeans and South Asians. Future research should recruit large enough numbers in both ancestry groups to conduct appropriately powered sub-group comparisons of enzyme activity.

Overall, this research project has explored factors contributing to variability in response to medicines, with a focus on how diet, genetics and geographic ancestry contribute to the activity of drug-metabolising enzymes. Specifically, the effects of cruciferous vegetables, CYP variant genotypes and dietary practices can contribute to variability in the activities of CYP1A2, CYP2C19, CYP2C9, CYP2D6 and CYP3A4. Future dietary intervention trials are encouraged to adopt a basal control diet, and appropriately power the study to account for intra- and inter-individual variability in CYP activity. Simultaneously investigating dietary and genetic information when aiming to understand ancestry group differences in CYP activity are recommended. Ideally, subjects of relevant enzyme variant genotypes should be considered. While numerous pharmacokinetic effects and interactions were explored in this research, it remains unclear if these differences in concentration and systemic exposure translate into clinically significant variability in patients. Ultimately, more trials are needed to assess whether *Cruciferae*-enriched diets can affect the safe and efficacious use of CYP substrates in pharmacotherapy. Some of the ancestry group differences in CYP activity reported in this research were large, and further investigations should explore how cruciferous vegetables and variant genotypes affect important therapeutic CYP substrates, such as theophylline, warfarin and voriconazole, in Europeans and South Asians.

7 References

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8 Appendices

8.1 Systematic review search terms and strategy

The Cochrane Central Register of Controlled Trials (CENTRAL)

CENTRAL was accessed online via the Ovid platform. Several keywords were used to search for relevant studies. These included ($ = truncation): cruciferous$, Brassica, Brassicaceae, Raphanus, Nasturtium, broccoli, cauliflower, watercress, radish, drug-metaboli$, drug metaboli$, GST$, UGT$, cytochrome$, and CYP$. Search results within each group (cruciferous vegetables and drug metabolism) were combined with the Boolean operator *OR* and the two groups then merged with *AND*.

Embase

Embase was accessed online via the Ovid portal. Medline Subject Headings (MeSH) were identified and chosen to encompass broad subject areas and were implemented in tandem with keywords to maximise the sensitivity of the search. MeSHs and keywords were divided into the two groups and combined using *OR* and *AND* as above.

The MeSHs and keywords chosen within each group were:

Drug metabolism MeSHs:

- Cytochrome P-450 Enzyme System
- Glucuronosyltransferase
- Glutathione Transferase
- Glutathione *S*-Transferase pi

Drug metabolism keywords:

- Drug metaboli$ ($ = truncation)
- Drug-metaboli$

Cruciferous vegetables MeSHs:

- Brassica
- Brassicaceae
- Glucosinolates
- Isothiocyanates
- Raphanus
- Nasturtium

Cruciferous vegetables keywords:

- Cruciferous$

i. Medline

Medline was accessed online via the Ovid platform. Medline Subject Headings (MeSH) were used in the same way as for Embase searches (above). MeSHs and keywords were divided into the same two groups: drug metabolism and cruciferous vegetables. All relevant MeSHs and keywords within these groups were combined with the Boolean operator *OR* and the two groups then merged with *AND*. The MeSHs and keywords chosen were the same as used for the Embase search (above).

8.2 Systematic review data extraction sheet

Author (year): _____ Enzyme(s) studied: _____

Sample size: ____ Males: ____ Females: _____ Mean age: _____
Mean weight: _____ Mean height: _____ Mean BMI: ___

Participant group similarity: statistically significant difference(s) in participant demographic characteristics? Yes □ No □ Not stated explicitly □

Study design: Pre-test/post-test □ Crossover □ Parallel □

Randomisation: Yes □ No □ n/a (no bias plausible from design) □

Aside from intervention, groups treated equally? Yes □ No □

Sample size/power/effect size calculation stated? Yes □ No □

Dietary intervention details:

Metric(s) chosen to measure PK endpoints: _____
Assay validated? Yes □ No □

Difference in enzyme activity (value, % change, SD, CI, *P*-value):

8.3 National Ethics Application Form (NEAF) approval

Contact Sydney Local Health District Human Research Ethics Committee – CRGH
Concord Repatriation General Hospital (CRGH)
Concord NSW 2139
Telephone (02) 9767 5622
Email ethicscrgh@gmail.cr.nsw.gov.au

Our Ref: (HREC/12/CRGH/206)

CONCORD
REPATRIATION GENERAL
HOSPITAL

4 April 2013

Professor Andrew McLachlan
Building 76
CONCORD RGH

Dear Professor McLachlan,

Re: HREC/12/CRGH/206 CH62/6/2012-159
The effect of broccoli consumption on the activity of drug metabolising enzymes in people of European and South Asian ancestry.

Thank you for submitting the above project for single ethical and scientific review. This project was first considered by the Sydney Local Health District Human Research Ethics Committee – CRGH at its meeting held on 22 November 2012. This Human Research Ethics Committee (HREC) has been accredited by the NSW Ministry of Health as a lead HREC under the model for single ethical and scientific review.

This lead HREC is constituted and operates in accordance with the National Health and Medical Research Council's *National Statement on Ethical Conduct in Human Research* and the *CPMP/ICH Note for Guidance on Good Clinical Practice*.

I am pleased to advise that the Committee has granted ethical approval of this research project.

The documents reviewed and approved include:

National Ethics Application Form (NEAF) – submission code AU/1/60AF010		
Protocol (including advertisement, data collection form, consent form & food diary)	Version: 3	Date: **27/03/2013**
Participant Information Sheet	Version: 3	Date: **6/02/2013**

The HREC has provided ethical and scientific approval for the following sites:
1. Concord Repatriation General Hospital

Please note the following conditions of approval:

1. You will immediately report anything which might warrant review of ethical approval of the project in the specified format, including unforeseen events that might affect continued ethical acceptability of the project, (including Serious Adverse Events).

2. Proposed changes to the research protocol, conduct of the research, or length of HREC approval will be provided to the HREC for review in the specified format.

3. You will notify the HREC, giving reasons, if the project is discontinued at a site before the expected date of completion.

8.4 Site-specific Approval (SSA) approval

Contact: *Sydney Local Health District (SLHD)*
Research Office
Concord Repatriation General Hospital (CRGH)
Level 1, Building 75, Hospital Road
Concord NSW 2139
Telephone: (02) 9767 5622
Email: ethicscrgh@email.cs.nsw.gov.au

Our Ref: *(SSA Authorisation)*

CONCORD
REPATRIATION GENERAL
HOSPITAL

20 June, 2013

Professor Andrew McLachlan
Building 76
CONCORD RGH

Dear Professor McLachlan,

HREC reference number: HREC/12/CRGH/206
SSA reference number: SSA/13/CRGH/111
Project title: *The effect of broccoli consumption on the activity of drug metabolising enzymes in people of European and South Asian ancestry.*

Thank you for submitting an application for authorisation of this project.

I am pleased to inform you that the delegate of the Chief Executive has granted authorisation for this study to take place at the following site:

- *Concord Repatriation General Hospital*

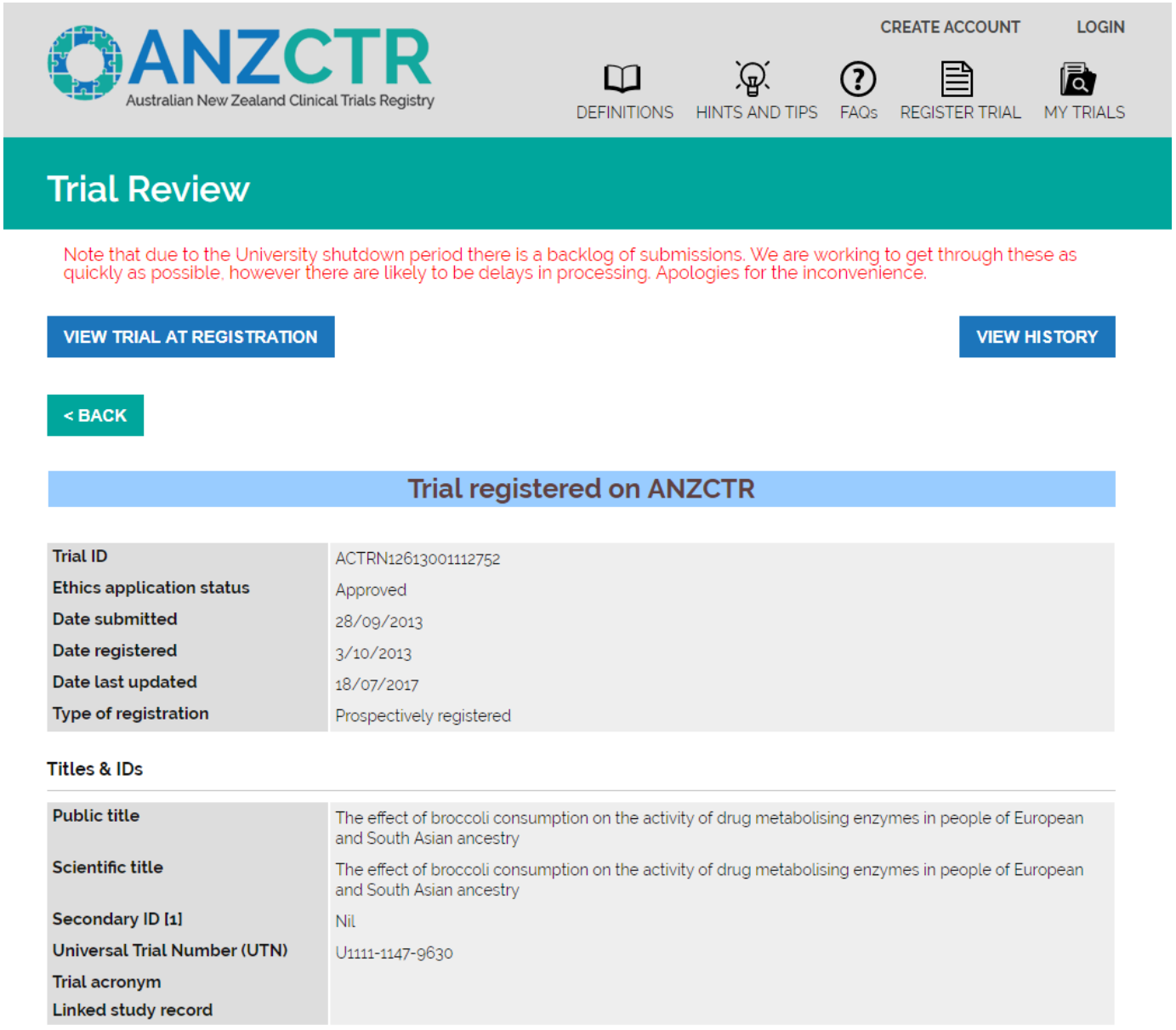
The participant documents approved for use at this site are:

- *Participant Information Sheet Version 3 dated 6/02/2013*

The following conditions apply to this research project. These are additional to those conditions imposed by the Human Research Ethics Committee that granted ethical approval:

1. Proposed amendments to the research protocol or conduct of the research which may affect the ethical acceptability of the project, and which are submitted to the lead HREC for review, are copied to this office.
2. Proposed amendments to the research protocol or conduct of the research which may affect the ongoing site acceptability of the project are to be submitted to this office.
3. A copy of the TGA acknowledgment of the Clinical Trial Notification (CTN) form must be submitted to the CRGH Clinical Trials Pharmacist for inclusion on file.
4. Where appropriate, I recommend that you consult with your Medical Defence Union to ensure that you are adequately covered for the purposes of conducting this study. If you (or your co-investigators) are undertaking this research on behalf of the University of Sydney or as part of a conjoint appointment to the University, you must inform the University of Sydney Risk

8.5 ANZCTR registration details



ANZCTR
Australian New Zealand Clinical Trials Registry

CREATE ACCOUNT LOGIN

DEFINITIONS HINTS AND TIPS FAQs REGISTER TRIAL MY TRIALS

Trial Review

Note that due to the University shutdown period there is a backlog of submissions. We are working to get through these as quickly as possible, however there are likely to be delays in processing. Apologies for the inconvenience.

VIEW TRIAL AT REGISTRATION VIEW HISTORY

< BACK

Trial registered on ANZCTR

Trial ID	ACTRN12613001112752
Ethics application status	Approved
Date submitted	28/09/2013
Date registered	3/10/2013
Date last updated	18/07/2017
Type of registration	Prospectively registered

Titles & IDs

Public title	The effect of broccoli consumption on the activity of drug metabolising enzymes in people of European and South Asian ancestry
Scientific title	The effect of broccoli consumption on the activity of drug metabolising enzymes in people of European and South Asian ancestry
Secondary ID [1]	Nil
Universal Trial Number (UTN)	U1111-1147-9630
Trial acronym	
Linked study record	

8.6 TGA CTN approval

Australian Government

Department of Health and Ageing
Therapeutic Goods Administration

File Number: 2013/010107

Mr Shane Eagles, c/o Professor Andrew McLachlan
Building 4 (ANZAC 3)
Concord Repatriation General Hospital
Hospital Road
CONCORD, NSW 2139

CTN Scheme (Drugs): Acknowledgement of New Trial

Your notification to conduct a clinical trial under the Clinical Trial Notification (CTN) Scheme, pursuant to Schedule 5A of Regulation 12 of the Therapeutics Goods Regulations, has been received by the Office of Scientific Evaluation (OSE).

Trial Number: 2013/0379
Protocol Number: HREC/12/CRGH/206

Drug(s):

Drug Active Name	Trade Name	Code Name	Strength
Caffeine	NoDoz	N/A	100mg
Dextromethorphan	Benadryl Dry Forte	N/A	30mg
Losartan	Cozaar	N/A	25mg
Midazolam	Hypnovel	N/A	2mg
Omeprazole	Losec	N/A	20mg

It is noted that:

i. the approval of the goods for this trial was given in accordance with Item 3 of Schedule 5A of the Therapeutic Goods Regulations by the body or organisation conducting the trial at each additional site.

ii. the representative of the Ethics Committee for each additional site has certified that the Committee is constituted and operates in accordance with the NHMRC "National Statement on Ethical Conduct in Human Research" has considered this clinical trial, and has provided advice to the body or organisation conducting the trial.

The Therapeutic Goods Administration has not carried out an assessment of the quality, safety or efficacy of any drug product in relation to this notification.

Please note that, in the event that the Secretary of the Commonwealth Department of Health and Ageing becomes aware that to undertake or continue the clinical trial would be contrary to the public interest, the Secretary has the authority to direct that use of the drug product(s) for this clinical trial must cease.

A form "CTN Scheme (Drugs): Trial Completion Advice" is enclosed. Please fill out and return this form after the Clinical Trial has completed.

Riannon Cuschieri
Experimental Products Section
Office of Scientific Evaluation
23 July, 2013

Experimental Products Section, TGA, PO Box 100, Woden ACT 2606 ABN 40 231 424 814
Phone: 02 6232 8121 Fax: 02 6232 8112 Email: eds@tga.gov.au www.tga.gov.au

TGA Therapeutic Goods Administration

8.7 Eligibility questionnaire

Faculty of Pharmacy
The University of Sydney

Pharmacy Aged Care Research Lab
Concord Repatriation General Hospital

Broccoli and Drug Metabolism

DATA COLLECTION SHEET

1. Patient Demographics

1.1 Patient initials: ____

1.2 Date of Birth: ___/___/___

1.3 Study Code: ____ (Investigator)

1.4 Age: _____

1.5 Weight: _____ (kg)

1.6 Height: _____cm

2. Ethnicity - General

2.1 What is your ethnic ancestry? _____ (European or South Asian)

2.1.1 Where were you born? _____

2.2 Were all four of your biological grandparents born in the same country? □ Y □ N

2.2.1 Please fill out the following table with country of birth:

	Grandmother	**Grandfather**
Maternal (Mum's side)		
Paternal (Dad's side)		

2.3 How many years have you resided in Australia? _____

2.3.1 Do your parents reside in Australia? If so, for how many years? _____

2.4 Is English your first language? □ YES □ NO

2.4.1 What other languages do you speak at home? _____

3. Ethnicity – South Asian

3.1 If Indian Ancestry - What area of the Indian sub-continent is your family background?

□ **North** □ **South**

3.1.1 How would you define your heritage?

□ Himachal Pradesh □ Uttar Pradesh □ Punjab □ Andhra

□ Pradesh □ Tamil □ Orissa □ Rajasthan

□ Maharashtra □ Sri Lankan/Sinhalese □ Sri Lankan/Tamil

4. Medication/Medical History

4.1 Would you consider yourself healthy? _____

4.1.1 Please list any previous or ongoing health problems you have (e.g. diabetes, high blood pressure)

_____

_____

4.2 How regularly would you take over the counter medicines (such as pain relieving medications e.g. paracetamol)?

□ Two to three times a week □ Once a week □ Once a month

□ Less than once a month

4.3 How regularly would you take complementary medicines (such as health vitamins e.g. fish oil)?

□ Two to three times a week □ Once a week □ Once a month

□ Less than once a month

4.4 Do you take any over-the-counter, complementary, herbal (such as gingko) or ayurvedic medicines? □ Y □ N

4.4.1 If so, please list

_____

_____

5. Alcohol & Illicit Drugs

5.1 How would you describe your alcohol consumption?

□ Over three drinks per day □ 1-2 drinks per day □ 1-2 drinks per week

□ Over six drinks per week □ 3-6 drinks per week

□ Don't drink alcohol

5.2 Do you regularly use illicit drugs (e.g. marijuana, ecstasy, cocaine, etc.)?

□ Y □ N

8.8 Participant consent form

Consent form

Faculty of Pharmacy
The University of Sydney

Pharmacy Aged Care Research Lab
Concord Repatriation General Hospital

RESEARCH STUDY INTO

Broccoli and Drug Metabolism

PARTICIPANT CONSENT FORM

I, …… [name]

of …… [address]

have read and understood the Information for Participants for the above-named research study and have discussed the study with the study researchers.

- I have been made aware of the procedures involved in the study, including any known or expected inconvenience, risk, discomfort or potential side effect and of their implications as far as they are currently known by the researchers.
- I freely choose to participate in this study and understand that I can withdraw at any time.
- I also understand that this research study is strictly confidential.
- I hereby agree to participate in this research study.

Name (Please Print): ……

Signature: …… **Date:** ……

Name of Person who conducted informed consent discussion (Please Print):

Signature: …… **Date:** ……
of Person who conducted informed consent discussion

8.9 Participant Information Sheet (PIS)

Faculty of Pharmacy
The University of Sydney

Pharmacy Aged Care Research Lab
Concord Repatriation General Hospital

Broccoli and drug metabolism

INFORMATION FOR PARTICIPANTS

You are invited to take part in a research study in healthy volunteers that will investigate the impact of eating broccoli on the activity of five enzymes that 'break down' (metabolise) medicines in the body.

What is this study about?

The aim of this study is to investigate the effect that eating broccoli can have on five enzymes that metabolise medicines in the body. Previous studies suggest that materials found in broccoli may increase or decrease the ability of the body to break down some medicines. This study aims to understand how this can happen and what it might mean for people taking these medicines. You are being asked to take part because you are healthy, male, aged between 18 and 55 years and of either South Asian (based on the information that both sets of grandparents have South Asian ancestry) or European ancestry (both sets of grandparents have European ancestry).

The study is being conducted by Mr Shane Eagles (PhD Student), Adjunct Associate Professor Annette Gross and Professor Andrew McLachlan from the University of Sydney and Concord Hospital.

Who can enter this study (inclusion and exclusion criteria)?

Inclusion: Men who are of European and South Asian ancestry between the ages of 18 and 55 years who are healthy (i.e. no current short-term or long-term health problems).
Exclusion: People who are suffering from any current illness or long-term illness or are taking prescription medication, over-the-counter medicines or herbal/complementary medicines. People who are current cigarette smokers or ex-smokers who have quit smoking in the last 6 months. People with a known allergy or previous reaction to any of the following medications: caffeine, omeprazole, losartan, dextromethorphan and midazolam.
What does this study involve?

If you agree to participate in this study, you will be required to have a face-to-face interview (approx. 20 minutes duration) with the researchers at a time and place that suits you.

In this study you will be asked to participate in four main activities:

1. Record the type and amount of all foods/beverages consumed completing a summary sheet leading up to your first visit and by completing a food diary for the last three days of the study;

2. Add 200 g (approximately 2 cups) of broccoli twice daily (with lunch and dinner) to your usual diet for six days;

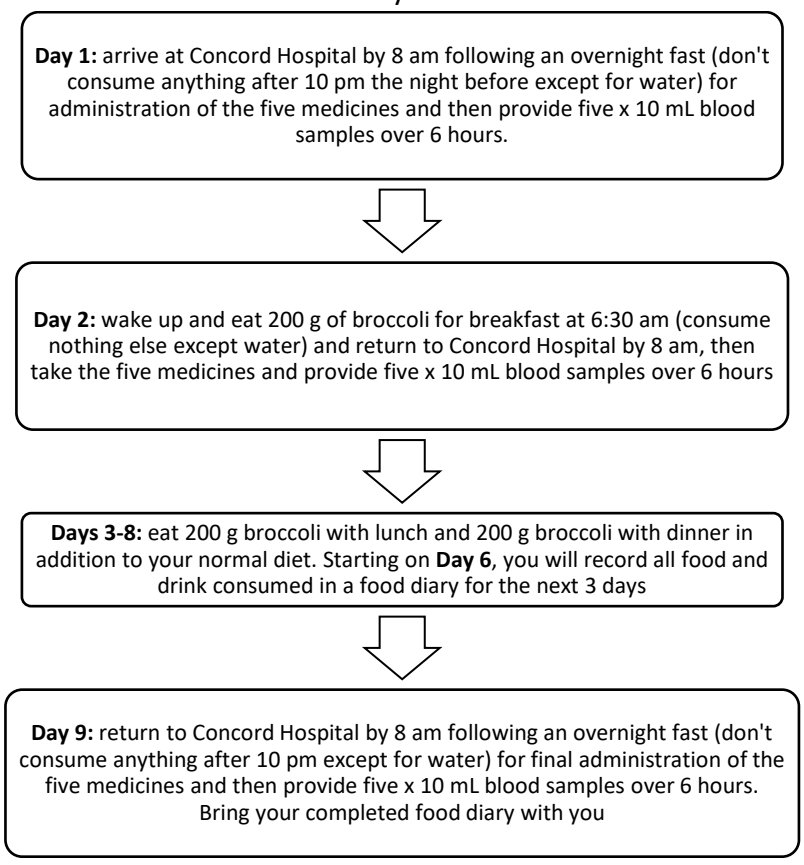
3. Provide a blood sample (10 mL) to allow for the collection of your DNA, which will be used to study the genes that influence drug metabolising activity in your body;

4. Come to Concord Hospital on three separate occasions. On each occasion you will take a dose of five medications and provide venous blood samples (five x 10 mL samples) which will be used to measure the concentration of the medicines and their breakdown products in your blood over a 6-hour period. This information tells us the *activity* of the enzymes in the body. Each visit will take approximately 8 hours.

Throughout the duration of the study you will be asked to follow your normal diet and broccoli will be eaten in addition to this for lunch and dinner. You are encouraged not to make any major changes to your diet whilst participating in this study.

Please note that food and beverages will be provided during your visits to Concord Hospital.

The diagram below summarises the timeline of the study, outlining the order in which you will be asked undertake each activity:



Why do I have to provide a list of food and drink I consume?
In the same way that broccoli can affect drug-metabolising enzyme activity in our bodies, most of what we eat and drink on a daily basis can have a similar effect on the way our body responds to medicines. The food diary will assist the investigators in explaining the different responses to eating broccoli amongst the participants, as certain foods and beverages have been shown to directly affect the enzymes being investigated in this study.

What foods and beverages should I avoid while participating in this study?
Grapefruit and grapefruit juice are known to affect the activity of some drug-metabolising enzymes being investigated in this study. Consuming grapefruit products during the study may change the results and participants are required to avoid them until after the study is complete. Also, you are required to avoid drinking any caffeinated beverages such as coffee (all types), tea (all types), Coke/Pepsi (all types) and energy drinks (all types) after 6 pm the night before coming to Concord on study days, and to avoid these beverages on the study days also. You may however consume caffeinated beverages during the 6 days of broccoli consumption.

Why will I be asked to take five medicines on three separate occasions?
Each of the five medicines used in this study are selectively broken down by one of the five enzymes being investigated in the study. The amount of the medicines and their metabolism by-products in your blood allows the researchers to measure the activity of the enzymes involved. These medicines have been chosen because they are specific for the breakdown pathway of interest, they have been proven to be safe, commonly used and have been shown not to interact or affect each other.

The administration of the five medications and collection of blood samples needs to take place on three separate occasions so that the effect of eating broccoli on enzyme activity can be measured. The first occasion will be a 'baseline' measurement, the second occasion will also involve you eating 200 g of broccoli just before taking the medicines (to measure the short-term effects of eating broccoli) and the third occasion will be after six days of broccoli consumption (to measure the medium-term effects of eating broccoli).

How safe are the medicines in this study?
These medicines have been selected because they have been widely used and much is known about their effects on the body. All medicines have some risk of unwanted effects but in this study the chances of experiencing these effects are low because only a single dose is taken on each occasion. The medicines and their possible effects are summarized in the table below.

Medicine	Usual use in humans	Usual dose range	Dose used in this study	Possible side effects (occur in between 1% to 10% of people)
Caffeine	Stimulant present in beverages (e.g. tea, coffee, energy drinks)	Varies greatly—as a guide, an average cup of coffee contains 80-150 mg of caffeine	100 mg	Stomach upset, sleeplessness, restlessness, nervousness, shakes, headache and lightheadedness
Omeprazole	Treatment of 'heartburn' or acid reflux from the stomach	10-40 mg per day	20 mg	Stomach upset, headache, dizziness, mild tingling or 'pins-&-needles' in arms/legs, mild skin rash
Losartan	Treatment of high blood pressure (hypertension)	50-100 mg per day	25 mg	Dizziness, muscle cramps, leg pain, nasal congestion
Dextromethorphan	Over-the-counter cough suppressant (e.g. Bisolvon, Benadryl)	30–120 mg per day	30 mg	Diarrhoea, sedation
Midazolam	Sedative usually used in the hospital setting during short surgical procedures	1-3.5 mg as a single dose	2 mg oral liquid which you will drink	Drowsiness, altered alertness, slowed breathing rate, short-term changes in blood pressure and heart rate, headache

What is involved with providing blood samples?

To measure the amount of the medicines and their by-products in your blood we will need you to provide blood samples. This will take place at Concord Hospital following an overnight fast (no food/drink besides water after 10 pm the night before) immediately before and over the six hours after you take the five medicines (at 0, 1, 2, 4 and 6 hours post-dose for a total of five samples taken on each occasion). 2 hours after the medicines are administered you will be provided with a muffin and water to break your fast, and a light lunch (sandwich/roll) will be provided later in the day. Samples will be taken via an intravenous cannula, which is a small tube that is inserted inside the vein or intravenous needle by trained nursing and/or pathology staff under the supervision of a medical doctor. The same research team will be with you at all times over this six-hour period and will answer and address any concerns you have whilst providing the blood samples.

Two of the medicines, midazolam and dextromethorphan, can cause mild sedation and drowsiness so driving is not recommended to and from the hospital on the days you take these medicines. We suggest that a friend/family member drop you off and pick you up. If this is not possible then a taxi can be provided to take you to and from the hospital. While under the sedative effects of midazolam you are advised to avoid operating machinery and catching public transport.

How will my blood samples be used?
Blood samples will only be used to measure the amounts of the five medicines and their respective by-products to determine the activity of the enzymes that they are involved with.

A sample of your blood (approximately 10 mL) will be collected for DNA testing. Everyone's DNA is different to some degree. The purpose of this testing is to investigate the genes that control the ability of your body to breakdown the medicines in this study.

How will my DNA sample be used?
Your DNA sample will ONLY be used to analyse the genes that can influence the activity of the drug-metabolising enzymes of interest in this study. Certain variations in these genes can influence the activity of the drug-metabolising enzymes. The researchers will be attempting to match any increases/decreases in enzyme activity with the presence of these particular gene variants. Your DNA sample will NOT be used for any other purpose.

How do I store and eat the broccoli?

After your second visit to Concord Hospital (after the second occasion of taking the five medicines and providing blood samples) the investigators will provide you with sealed, pre-weighed "snap-lock" labeled bags containing 200 g (approximately 2 cups) of broccoli. You will be given 13 bags—enough to eat 200 g at lunch and 200 g at dinner for the next six days and one bag for breakfast on Day 2 of the study. These bags need to be kept in a refrigerator until use. If you are taking the broccoli to work with you only take the relevant portion needed for that day and ensure it is kept refrigerated until use; use a cooler bag with an ice pack when travelling.

All participants are required to microwave one 200 g bag of broccoli in the microwave-safe container provided. Add a small amount of water underneath the white steaming tray, but ensure the water level stays below the tray, as nutrients can leak from the broccoli if it is touching water. Microwave for a time based on your microwave's power settings (see table below). Take care in removing the broccoli from the microwave and when eating as the contents of the container may be hot. Proceed to eat the ENTIRE contents. All broccoli must be consumed. The broccoli is to be eaten in addition to your normal diet (continue eating/drinking what you normally would).

Broccoli is a rich source of dietary fibre, and a sudden increase of fibre in your diet can slow down the movement of material through your gastrointestinal tract and in some cases

cause constipation. You are advised to drink plenty of water for the duration of the study to reduce the chance of this occurring.

Table 8.1: Recommended cooking times based on microwave wattages.

Microwave power (watts)	Recommended cooking time (seconds)
700	150 (2 min 30 s)
800	135 (2 min 15 s)
900	130 (2 min 10 s)
1000	120 (2 min)
1200	110 (1 min 50 s)

Healthy volunteer statement

People often volunteer to take part in medical research because they have a medical condition and the research may offer a chance of improving their health. Your role in this study is different. You are a healthy volunteer. As such, you need to carefully consider the risks associated with the research before you consent to take part. There is no expected benefit to your health from participation in this study.

Will I be reimbursed for my time?

You will be compensated for your time and inconvenience with a $500 payment on the provision that the study is completed in full.

What are the risks associated with this study?

Possible side effects that can arise from taking the five medications used in this study are listed above in this information sheet. None of these are considered serious or life-threatening and will pass after the medicines are cleared from your body. While at Concord Hospital for the administration of these medicines/to provide blood samples, you will be monitored by the researchers and will be cared for should anything unexpected occur.

Risks associated with intravenous blood sampling are usually limited to mild redness, swelling, and/or bruising around the site where the needle breaks the skin. Infection of the puncture site and/or associated veins is possible but is an extremely unlikely complication associated with this procedure. All blood samples will be taken under standard hospital conditions according to NSW Health guidelines to ensure your safety in this regard.

There are no known risks associated with eating 400 g of broccoli daily for six days, however this dietary intake of broccoli may cause flatulence and constipation in some people.
What are the benefits of this study?

While we intend that this research study furthers medical knowledge, it may not be of direct benefit to you.

Who owns my samples?

By signing the attached consent form, you relinquish all rights to ownership of your samples.

Can I obtain the results of tests on my sample?
The results of any tests done on your sample will not be made known to you, your family members or any other person. The results of these tests will not affect any present or future insurance policies, or your ability to get or keep a job.

Can I withdraw from the study?

Participation in this study is entirely voluntary. You are in no way obliged to participate and - if you do participate - you can withdraw at any time. Whatever your decision, please be assured that it will not affect your relationship with medical or research staff.

Confidentiality

All details obtained from participants will remain confidential. A report of this study may be submitted for publication, but individual participants will not be identifiable in such a report.

Compensation

Every reasonable precaution will be taken to ensure your safety during the course of the study. In the event that you suffer any injury as a result of participating in this research project, hospital care and treatment will be provided at no extra cost to you.

Further Information

When you have read this information, Mr Shane Eagles will discuss it with you further and answer any questions you may have. If you would like to know more at any stage, please feel free to contact Professor Andrew McLachlan, on 9767 7373 or Mr Shane Eagles from the University of Sydney on 0431 635 958. This information sheet is for you to keep.

This study has been approved by the Human Research Ethics Committee - CRGH of the Sydney Local Health District. If you have any concerns or complaints about the conduct of the research study, you may contact the Secretary of the Concord Hospital Human Research Ethics Committee, on (02) 9767 5622. Alternatively, if you wish to speak with an independent person within the Hospital about any problems or queries about the way in which the study was conducted, you may contact the Patient Representative on (02) 9767 7488.

8.10 Approved clinical study advertisement

Faculty of Pharmacy
The University of Sydney

Pharmacy Aged Care Research Lab
Concord Repatriation General Hospital

AN INVITATION TO PARTICIPATE

Are you interested in taking part in a clinical study investigating the effect of broccoli on drug metabolism?

We are looking for healthy **MALE** volunteers aged between 18 to 55 years of European or South Asian geographic ancestry (ethnicity or origin):

- European Ancestry (all countries of Europe including the UK and Ireland)
- South Asian Ancestry (India, Pakistan and Sri Lanka)

If you are interested please contact either:

Mr Shane Eagles	**Professor Andrew McLachlan**
seag2551@uni.sydney.edu.au	andrew.mclachlan@sydney.edu.au
(phone 0431 635 958)	(phone 9767 7373)

(Eligible participants will be reimbursed for their time on completion of the study)

A project conducted by researchers at the *Faculty of Pharmacy, University of Sydney* in collaboration with *Concord Repatriation General Hospital*

8.11 First participant 3-day food diary

Faculty of Pharmacy
Research Lab
The University of Sydney

Pharmacy Aged Care

Concord Repatriation General Hospital

Broccoli and Drug Metabolism

3-day diet summary sheet

Over the next three days, please fill out the following diet summary sheet.

To the best of your ability, accurately record what you eat/drink for breakfast, lunch and dinner for the three days leading up to your first Concord visit.

Write the name/type of the food or drink on the lines provided along with the amount/quantity consumed. If you are unsure of the amount in grams or mL/L, try to estimate or describe the amount, e.g. one can of… one tin of… 6 slices of… and so on. Please describe the items in as much detail as possible. For example, instead of “ham sandwich” write “ham, lettuce, tomato and cheese sandwich with butter (white bread)”.

The researchers need this information to identify other items in your everyday diet that may affect the study’s results in a similar way to the broccoli.

Breakfast Day 1	**Breakfast Day 2**	**Breakfast Day 3**
Lunch Day 1	**Lunch Day 2**	**Lunch Day 3**
Dinner Day 1	**Dinner Day 2**	**Dinner Day 3**
Snacks & Drinks	**Snacks & Drinks**	**Snacks & Drinks**

8.12 Second 3-day food diary (D6-D8)

Faculty of Pharmacy
The University of Sydney

Pharmacy Aged Care Research Lab
Concord Repatriation General Hospital

Broccoli and Drug Metabolism

FOOD DIARY

Participant instructions:

From days 6-8 of the study you will be required to accurately record of all of the food and drinks you consume in this diary. The researchers need this information to identify other items in your everyday diet that may affect the study's results in a similar way to the broccoli.

Write the name/type of the food or drink on the lines provided along with the amount/quantity consumed. If you are unsure of the amount in grams or mL/L, try to estimate or describe the amount, e.g. one can of… one tin of… 6 slices of… and so on. Please describe the items in as much detail as possible. For example, instead of "ham sandwich" write "ham, lettuce, tomato and cheese sandwich with butter (white bread)".

Each page is divided into easy to manage sections based around the three main meals of the day: breakfast, lunch and dinner.

It is recommended that you record an entry in the diary **IMMEDIATELY AFTER** eating or drinking something to maximise the accuracy of the entries.

There is a section for snacks in between each meal of the day—all food and drinks, no matter how small a serving should be recorded.

There is also a broccoli check-box to help you to remember if you have eaten your broccoli portions. Please initial these sections as you microwave and eat each 200 g serving.

Breakfast Day 6	**Breakfast Day 7**	**Breakfast Day 8**
Lunch Day 6 **Broccoli eaten? Y N**	**Lunch Day 7** **Broccoli eaten? Y N**	**Lunch Day 8** **Broccoli eaten? Y N**
Dinner Day 6 **Broccoli eaten? Y N**	**Dinner Day 7** **Broccoli eaten? Y N**	**Dinner Day 8** **Broccoli eaten? Y N**
Snacks & Drinks	**Snacks & Drinks**	**Snacks & Drinks**

8.13 Study day medical support protocol

Responsibilities of medical staff in preparation for study days:

- Write a clinical trials prescription for all participants
- Arrange for the on-call clinician to be available to come to Andrology and assess any participants presenting with mild ADRs/medical complaints

Responsibilities of medical staff on the day:

- Insert/withdraw cannula
- Check on any participants presenting with mild ADRs/medical complaints

Responsibilities of researchers on the day:

- Welcome participants and assess eligibility to participate on the day (i.e. not recently unwell, not currently unwell, successfully fasted since 10 pm the night before, baseline BP > 80/60 mmHg)
- Drawing blood via cannula/handing of samples
- Recording onto the data recording sheet
- Monitoring clinical signs/symptoms of hypotension and sedation every hour
- Monitoring clinical signs/symptoms of ADRs
- Ensuring participants receive morning snack and lunch
- Escorting participants to their means of transportation

IF

Mild drop in BP/HR accompanied by clinical signs of an ADR (light headedness, dizziness, sweaty/clammy/pale complexion) and/or increase in sedation score

THEN

Contact on-call clinician to assess participant in Andrology

IF

Moderate to severe as above or presents with nausea/vomiting

THEN

Arrange for participant to go to ED

IF

Severe ADR/medical emergency (anaphylaxis, extreme sedation, extremely low BP/HR)

THEN

Call 222 and report Code Blue

Assessing sedation:

Use the Sedation Score for opioid use as per the Australian Medicines Handbook (AMH) 2013, page 46.

Sedation score:

0 – wide awake

1 – easy to rouse

2 – easy to rouse, but cannot stay awake

3 – difficult to rouse.

Aim to keep the sedation score < 2; a score of 2 represents early respiratory depression.

If any participant persistently has a sedation score of 2 or more the on-call clinician will be contacted to assess the participant in Andrology.

Assessing BP:

As there is no universal 'cut-off' value for determining when clinical hypotension is present, this will be assessed primarily by the presence of the clinical signs of hypotension: dizziness, light headedness, sweating/clammy with pale pallor and in extreme circumstances fainting. A drop in systolic BP of >30 will be used as a guide however the clinical signs will determine the course of action taken.

BP drop > 30 and/or participant complains of dizziness, light headedness or presents as clammy, pale, etc. then contact the on-call clinician to assess the participant in Andrology.

Assessing HR:

A HR persistently below 50 bpm will be considered as a warning sign of bradycardia (BMJ Best Practice Guidelines – Bradycardia). Clinical signs of bradycardia such as dizziness/light headedness fainting or shortness of breath accompanied with a HR close to or below 50 will be considered criteria to contact the on-call clinician to assess the participant in Andrology.

Assessing a medical emergency:

If signs of anaphylaxis (shortness of breath, hives/acute rash, etc.) or extreme medical dysfunction/distress are observed in a participant, the researchers will transport the participant to the ED. If immediate assistance is required 222 will be called to report a Code Blue emergency.

8.14 Participant broccoli hand-out

Broccoli handling/cooking instructions

Each bag contains 200 g of washed and prepared broccoli. You must eat one WHOLE bag with lunch and dinner for 6 days as directed. ALL broccoli must be consumed. If you are full try spreading the two bags out over the whole day rather than with lunch and dinner.

Broccoli must be kept at 4-6˚C at ALL times unless microwave cooking. If taking it to work/school/university etc., use the eski and cooler brick provided as a means of transporting the broccoli.

If, for some reason, a portion of the broccoli is not consumed, record this in as much detail as possible (e.g. how much eaten, amount lost or forgotten, etc.) in the second food diary at the bottom.

Please note: we will be analysing your blood samples for broccoli constituent levels, and will be able to determine if broccoli is not being eaten. If the investigators have sufficient reason to believe the broccoli has not been consumed as agreed to, the participant will be deemed to have not completed the study in full – thus not receive payment for their participation.

Microwaving the broccoli

1. Ensure the white tray is in the bottom of the container, and add a small amount of tap water up to just below the line of the tray – *ensure the broccoli is not touching the water*
2. Secure the lid tightly and close the steam hole, then microwave at the specified time as per the wattage of your microwave as below:

Microwave power (watts)	Recommended cooking time (seconds)
700	150 (2 min 30 s)
800	135 (2 min 15 s)
900	130 (2 min 10 s)
1000	120 (2 min)
1200	110 (1 min 50 s)

3. The broccoli will be hot so be careful in removing it from the container.
4. Eat the broccoli – if it is really unpalatable you may add a small amount (< 10 mL) of salad dressing to add flavour

8.15 DNA purification and extraction

Pages 17-19 of "Genomic DNA from blood: User manual" (Macherey-Nagel, Dec. 2015/Rev. 15). Provided by Trent Peters courtesy of the Australian Genome Research Facility Ltd

Genomic DNA purification with NucleoSpin® Blood L

Before starting the preparation:

- Check if Buffer BQ2 and Proteinase K were prepared according to section 3.
- Set an incubator or water bath to 56 °C.
- Preheat Elution Buffer BE to 70 °C.
- For centrifugation, a centrifuge with a **swing-out rotor** and appropriate buckets
- capable of reaching 4,000–4,500 x *g* is required.

1. **Lyse blood sample**

Pipette up to **2 mL blood** (or body fluid) sample (equilibrated to room temperature) and **150 µL Proteinase K** into a 15-mL tube (not provided).

If processing buffy coat, do not use more than 1 mL and add PBS to adjust the volume to 2 mL.

If cultured cells are used, resuspend up to 2 x 107 cells in a final volume of 2 mL PBS.

If old or clotted blood samples are processed, see section 6.1 for recommendations.

Add **2 mL Buffer BQ1** (if processing less than 2 mL blood, add one volume of Buffer BQ1) to the samples and vortex the mixture vigorously for 10 s.

Note: Vigorous mixing is important to obtain high yield and purity of DNA.

Incubate samples at **56 °C** for **15 min**.

Let the samples cool down to room temperature before proceeding with addition of ethanol.

The lysate should become brownish during incubation with Buffer BQ1. Increase incubation time with Proteinase K (up to 20 min) and vortex once or twice during incubation if processing older or clotted blood samples.

2. **Adjust DNA binding conditions**

Add **2 mL ethanol (96–100 %)** (if processing less than 2 mL blood, add 1 volume of ethanol) to each sample and mix by inverting the tube 10 times.

Note: High local ethanol concentration must be avoided by immediate mixing after addition. Be sure that the lysate has cooled down to room temperature before loading it onto the column. Loading of hot lysate may lead to diminished yields.

3. **Bind DNA**

For each preparation, take one **NucleoSpin® Blood L Column** placed in a Collection Tube and load **3 mL of lysate**. Do not moisten the rims of the columns. Close the tubes with screw caps and centrifuge **3 min** at **4,500 x *g***.

Usually the lysate will start to flow-through the columns even before centrifugation. This will not adversely affect DNA yield or purity. Keep NucleoSpin® Blood L Column in an upright position as liquid may pass through the ventilation slots on the rim of the column even if the caps are closed.

Load **all of the remaining lysate** in a second step to the respective NucleoSpin® Blood L Column, avoiding moistening the rim. Centrifuge **5 min** at **4,500 x *g***. Discard the flow-through and place the column back into the Collection Tube.

Remove the Collection Tube with the column carefully from the rotor to avoid that the flow-through comes in contact with the column outlet. Be sure to wipe off any spilled lysate from the Collection Tube before placing the column back.

4. **Wash silica membrane**

Add **2 mL Buffer BQ2**. Centrifuge **2 min** at **4,500 x *g***.

It is not necessary to discard the flow-through after the first washing step.

Add **2 mL Buffer BQ2**. Centrifuge **10 min** at **4,500 x *g***. Remove the column carefully from the rotor in order to avoid that the flow-through comes in contact with the column outlet.

By prolonged centrifugation during this second washing step, residual ethanolic washing Buffer BQ2 is removed from the silica membrane of the NucleoSpin® Blood L Column.

5. **Dry silica membrane**

The drying of the NucleoSpin® Blood L Column is performed by prolonged centrifugation time (10 min) in the 2nd wash step.

6. **Elute highly pure DNA**

Insert the column into a new Collection Tube (15 mL) and apply **200 µL preheated Buffer BE (70 °C)** directly to the center of the silica membrane. Incubate at **room temperature** for **2 min**. Centrifuge at **4,500 x *g*** for **2 min**.

For alternative elution procedures see section 2.4.

8.16 List of ADME iPLEX gene variants

GENE	NUMBER OF HAPLOTYPE GROUPS‡	HAPLOTYPES (Haplotypes which are indistinguishable with the iPLEX ADME PGx Pro Panel are shown in parentheses)
ABCB1	11	*1, (*1e;g;h;p), (*2;*12;*15), *4, *6, (*8;*16), (*8A;*16A), *9, (*10;*13;*17), *11, *18
ABCC2	7	(*1A;*1B;*3), *1C, *2, *4, *5, *6, *7
ABCG2	3‡	WT, Q141K, Q126X
COMT¶	8	*1, *2, A, B, C, D, E, F
CYP1A1	9	*1, *2, *3, *4, *5, *6, *7, *8, *9
CYP1A2	6	*1A, *1C, *1K, *1L, (*1F;J), *7
CYP2A6	13 plus CNV	*1, *1X2b, *2, *5, *6, (*7;*10;*19;*36;*37), *8, (*9;*13;*15), *11, *12, *17, *20, *26
CYP2B6	7 plus CNV	*1, (*2;*10), (*6;*7;*19;*20;*29), *8, *13, (*16;*18), *28
CYP2C8	7	*1, *2, *3, *4, *5, *7, *8
CYP2C9	15	*1, *2, (*3;*18), *4, *5, *6, *8, *9, *10, *11, *12, *13, *15, *25,*27
CYP2C19	12	*1, (*1B;C;*9), *2, *3, *4, *5A, *5B, *6, *7, *8, *12, *17
CYP2D6	32 plus CNV	*1A, (*2A;*31;*51), (*2L;*35;*71), *3, *4, *4M, *6, *7, *8, *9, (*10;*36;*37; *47;*49;*52;*54;*57;*65;*72), *11, *12, *14A, *14B, *15, *17, *18, *19, *20, *21A, *21B, *30, *40, *41, *42, *44, *56A, *56B, *58, *64, *69
CYP2E1	3	*1, *2, *7
CYP3A4	5	*1, *2, *6, *20, *22
CYP3A5	6	*1, *3, *5, *6, *7, (*3K;*10)
DPYD	6	*1, *2, *7, *8, *9, *10
GSTM1	2	*A, *B
GSTP1	4	A, B, C, D
GSTT1	CNV only	
GSTT2b	CNV only	
NAT1	8	*4, *5, *14, *17, *19, *22, *15, *11
NAT2	33	*4, *5, *5A, *5C, *5D, *5E, *5G, *5J, *5K, *5P, *6, *6B, *6C, *6E, *6F, (*6I;J), *7A, *7B, *7C, *11, *12, (*12B;E), *12C, *13, *14, (*14B;H), *14C, *14D, *14E, *14F, *14G, *14I, *19
SLC15A2	3	*1, *2, *3
SLC22A1	16‡	WT, AAGTTGGT, TGGTAAGT, R61C, C88R, G220V, P283L, R287G, P341L, G401S, M408V, M420X-1, M420X-2, M420X-3, MI420I, G465R
SLC22A2	6‡	WT, P54S, M165V, S270A, R400C, K432Q
SLC22A6	2‡	WT, R50H
SLCO1B1	11	(*1A,*4,*6,*7,*8), (*1B;*14), *2, *3, *5, *9, *10, *11, *12, *13, (*15;*16;*17)
SLCO1B3	3‡	WT, S112A, M233I
SLCO2B1	2‡	WT, S464F
SULT1A1	4 plus CNV	(*1;*5;*6), (*2;*7), *3, *4
TPMT	7	*1, *2, (*3A;*3D), *3B, *3C, *4, *8
UGT1A1	7	*1, *6A, *6B, *7, *27, *29, *60
UGT2B15	2‡	WT, Y85D
UGT2B17	CNV only	
UGT2B7	2	*1, *2
VKORC1	4	*1, *2, *3, *4
TOTAL	266	

8.17 SPSS mixed-effects model syntaxes

CYP1A2

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CYP2C19

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CYP3A4

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  /EMMEANS=TABLES(rs35599367a) COMPARE ADJ(LSD)
  /EMMEANS=TABLES(Day*Ethnicity) COMPARE(Day) ADJ(LSD)
  /EMMEANS=TABLES(Day*Ethnicity) COMPARE(Ethnicity) ADJ(LSD)
  /EMMEANS=TABLES(Ethnicity*rs35599367a) COMPARE(Ethnicity) ADJ(LSD)
  /EMMEANS=TABLES(Ethnicity*rs35599367a) COMPARE(rs35599367a) ADJ(LSD).
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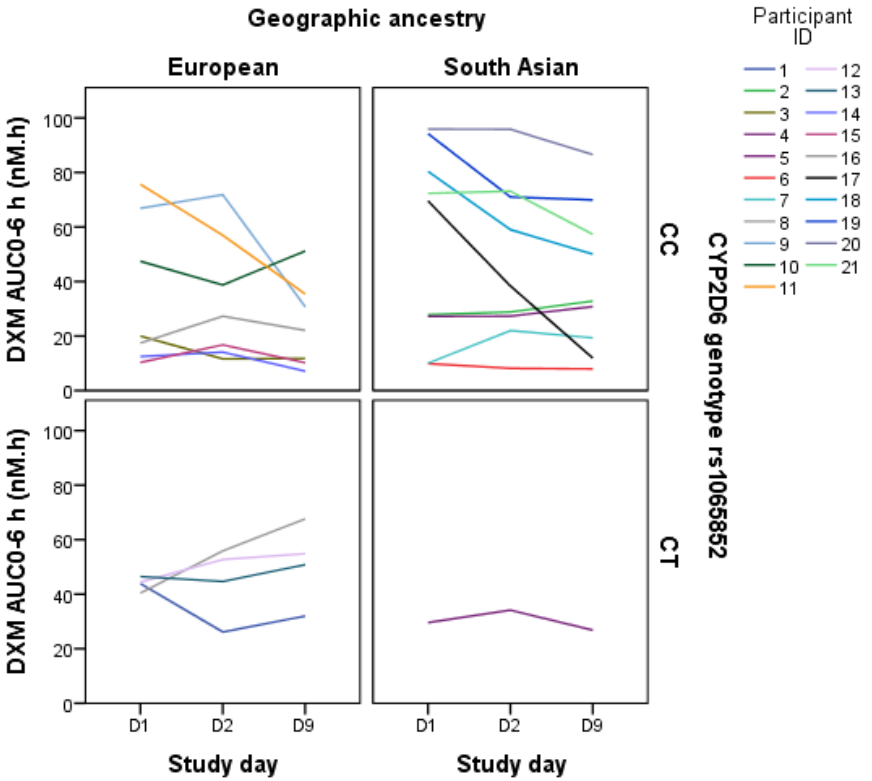
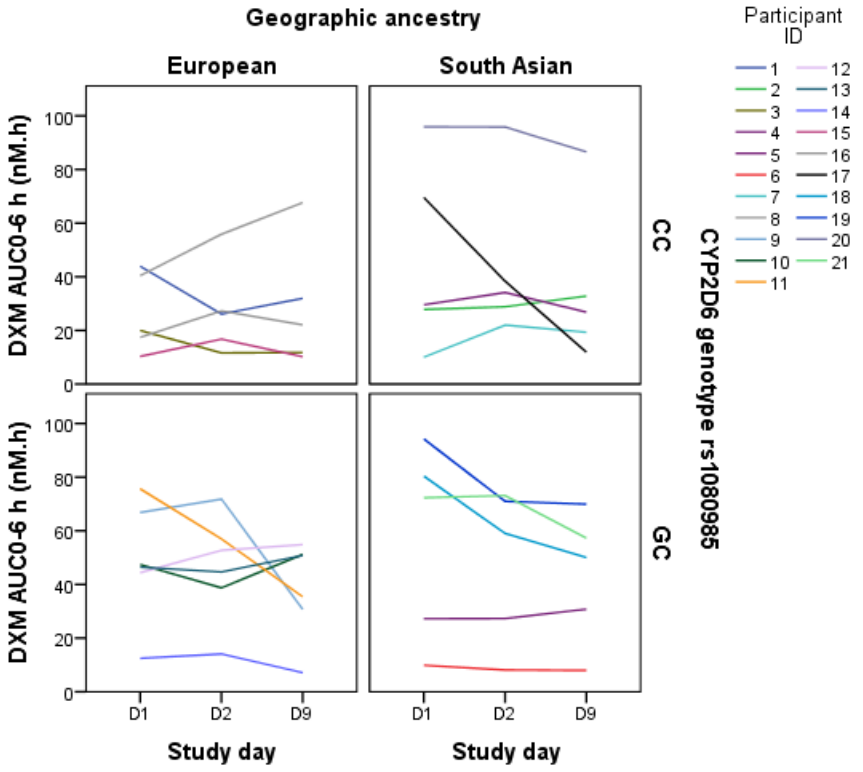
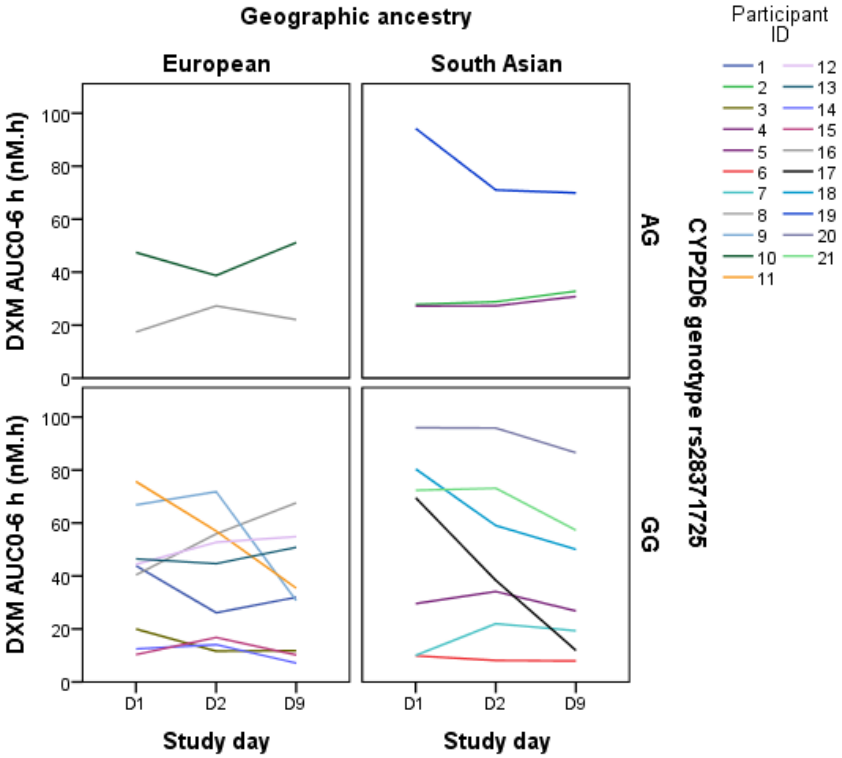
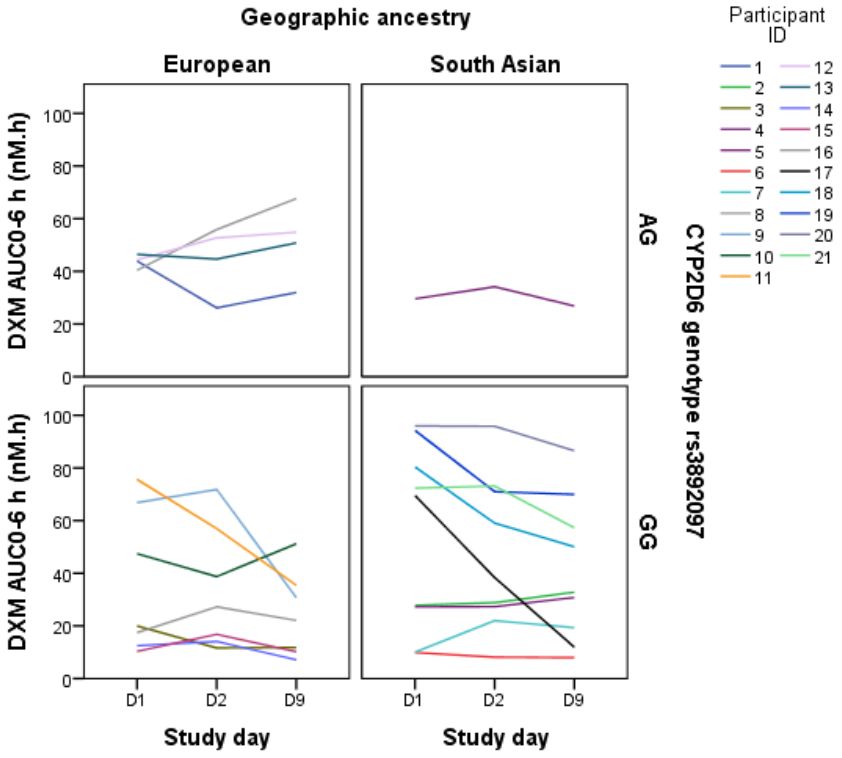
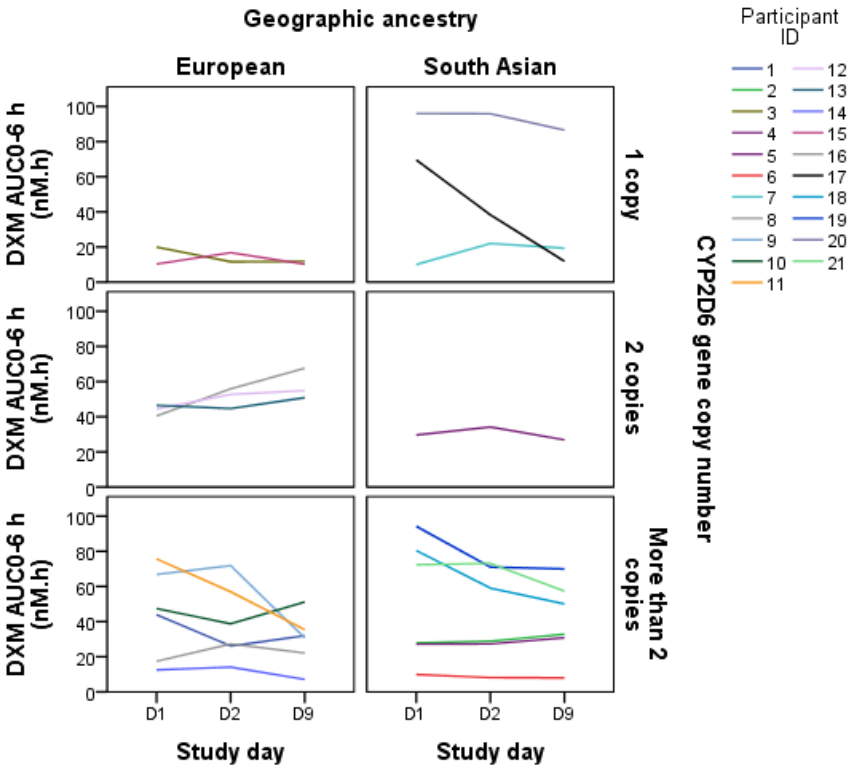
8.18 Dextromethorphan and dextrorphan AUC data and CYP2D6 activity by genotype

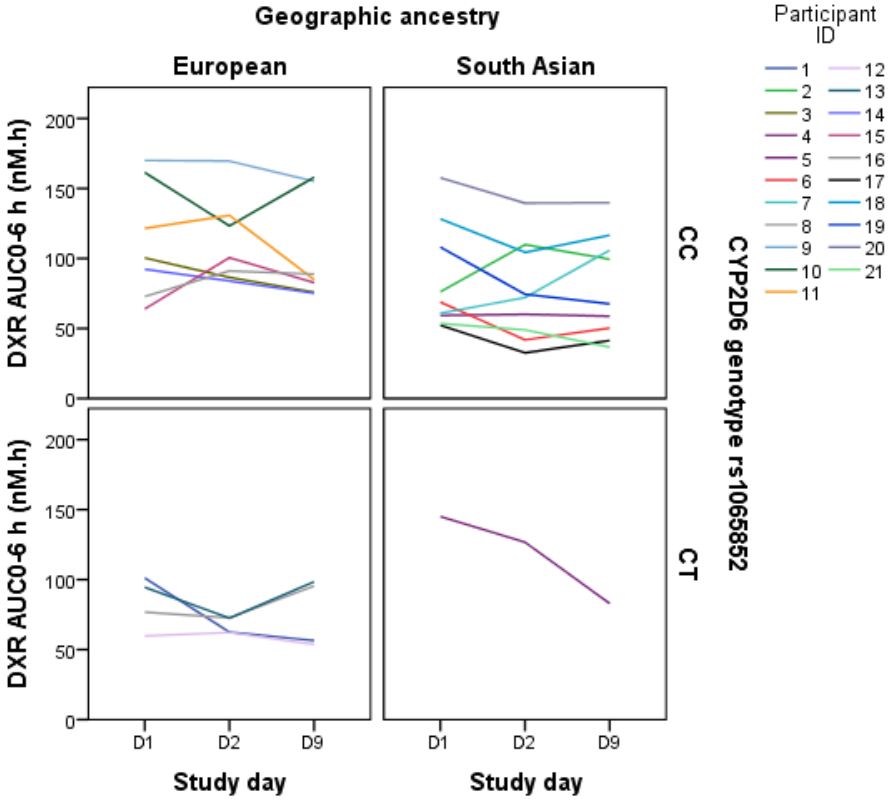


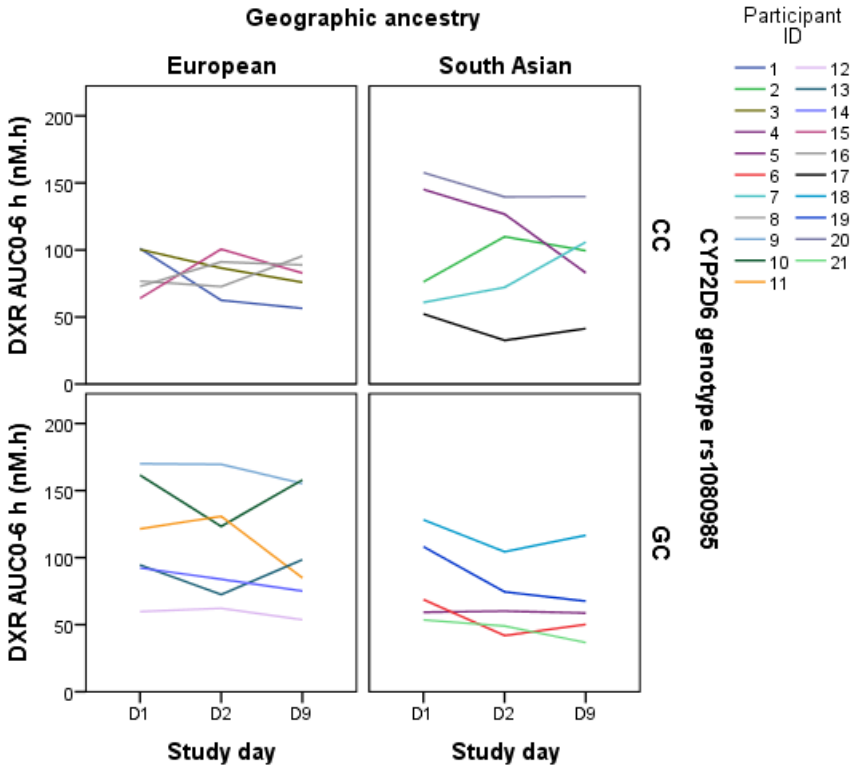



Geographic ancestry
European
South Asian
DXM AUC0-6 h (nM.h)
100
80
60
40
20
0
AG
GG
CYP2D6 genotype rs28371725
D1
D2
D9
Study day
Participant ID
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21


Geographic ancestry
European
South Asian
DXM AUC0-6 h (nM.h)
100
80
60
40
20
0
AG
GG
CYP2D6 genotype rs3892097
D1
D2
D9
Study day
Participant ID
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21


Geographic ancestry
European
South Asian
DXM AUC0-6 h (nM.h)
1 copy
2 copies
More than 2 copies
CYP2D6 gene copy number
Participant ID
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
0 20 40 60 80 100
D1 D2 D9
Study day


Geographic ancestry
European
South Asian
DXR AUC0-6 h (nM.h)
CC
CT
CYP2D6 genotype rs1065852
Participant ID
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
0 50 100 150 200
D1 D2 D9
Study day


Geographic ancestry
European
South Asian
DXR AUC0-6 h (nM.h)
200
150
100
50
0
CC
GC
CYP2D6 genotype rs1080985
D1
D2
D9
Study day
Participant ID
1
2
3
4
5
6
7
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9
10
11
12
13
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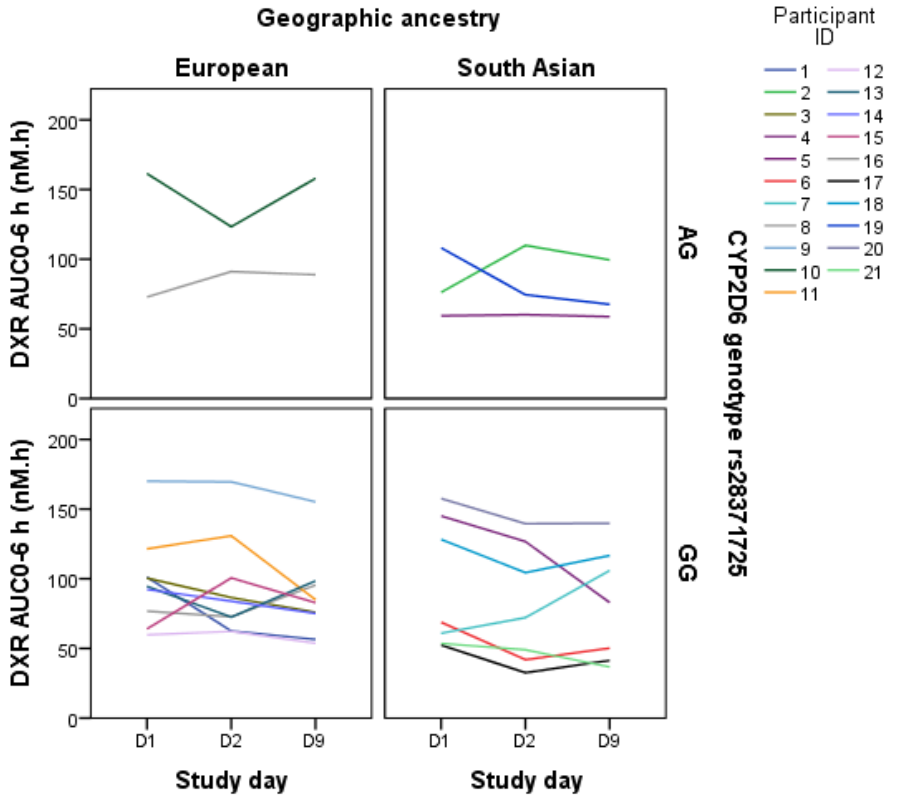
15
16
17
18
19
20
21


Geographic ancestry
European
South Asian
DXR AUC0-6 h (nM.h)
200
150
100
50
0
AG
GG
CYP2D6 genotype rs28371725
D1
D2
D9
Study day
Participant ID
1
2
3
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6
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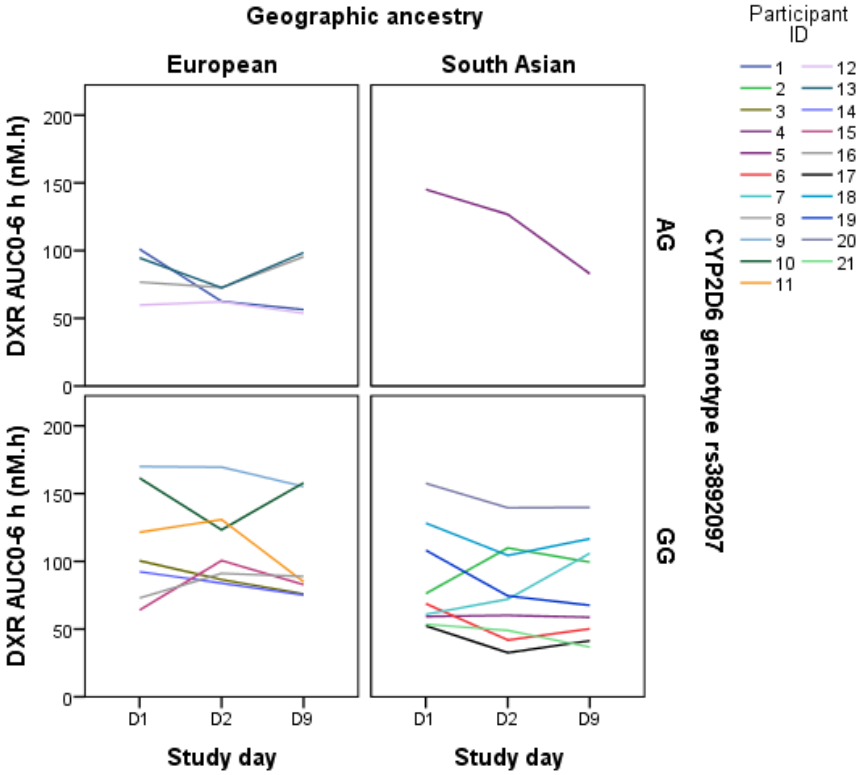
16
17
18
19
20
21


Geographic ancestry
European
South Asian
DXR AUC0-6 h (nM.h)
200
150
100
50
0
AG
GG
CYP2D6 genotype rs3892097
D1
D2
D9
Study day
Participant ID
1
2
3
4
5
6
7
8
9
10
11
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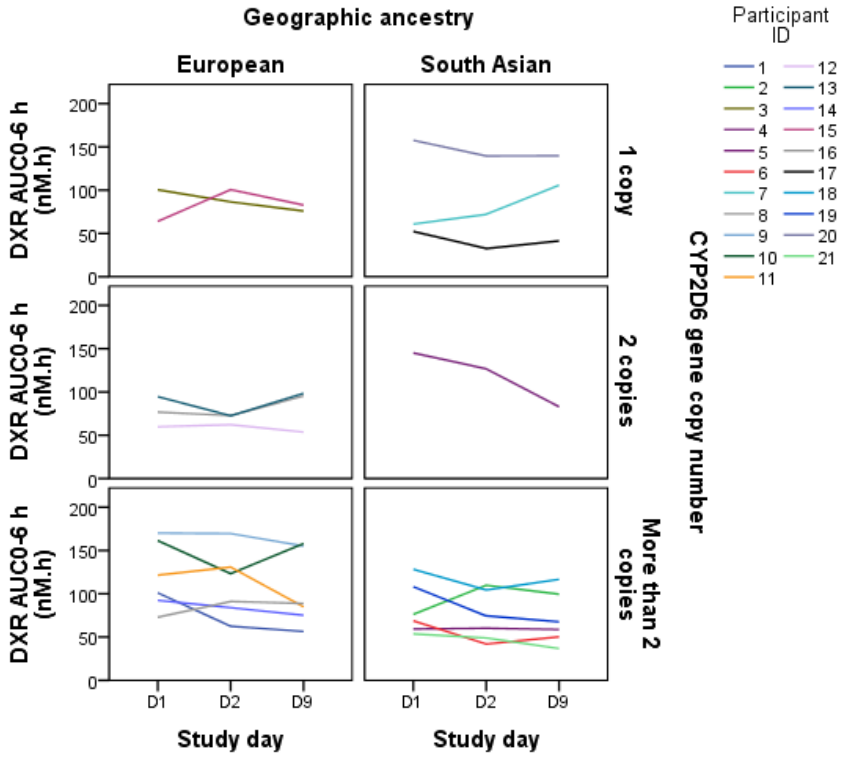
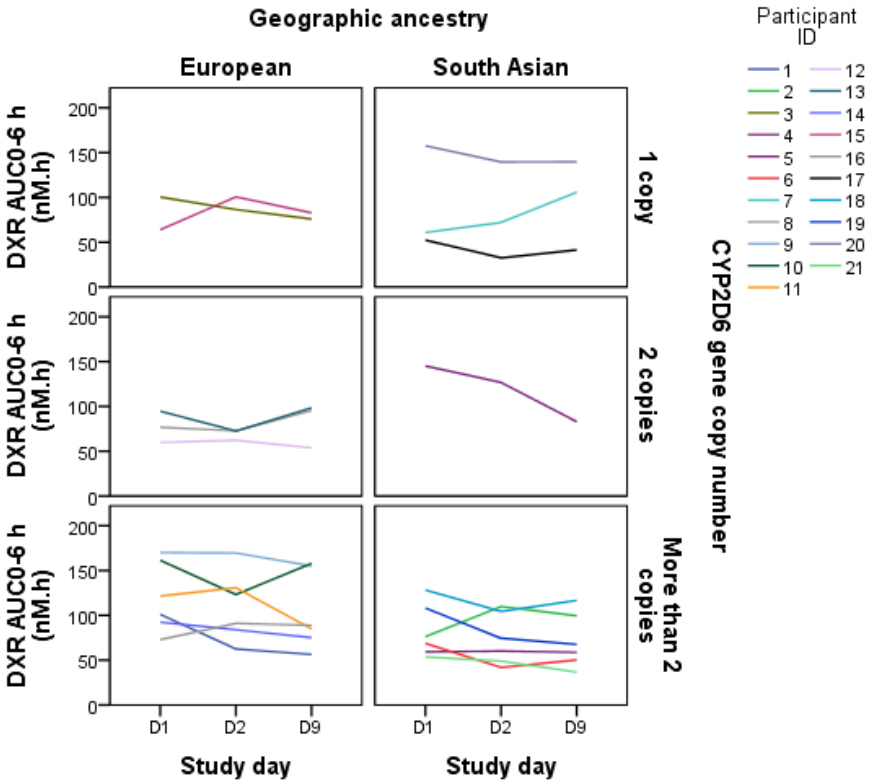
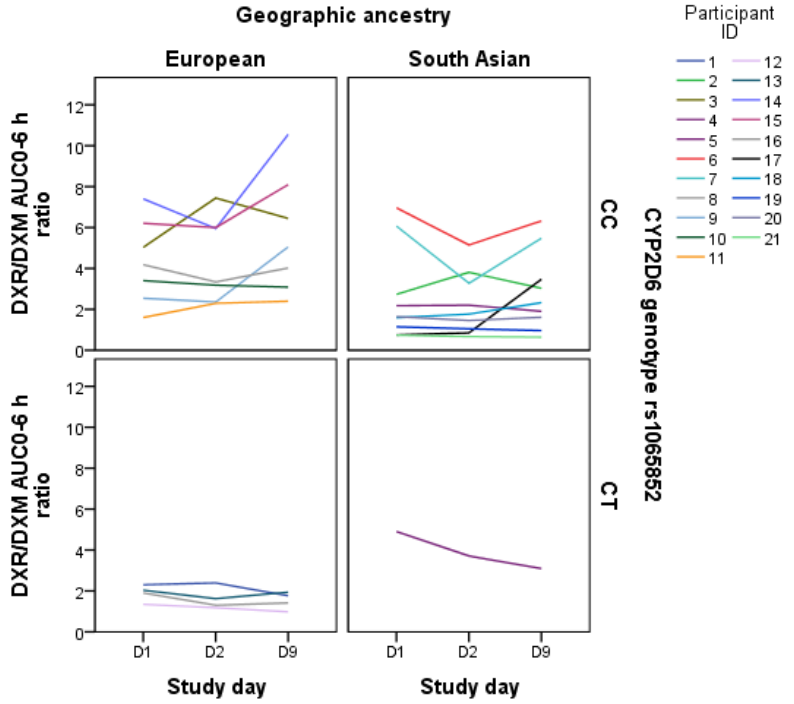
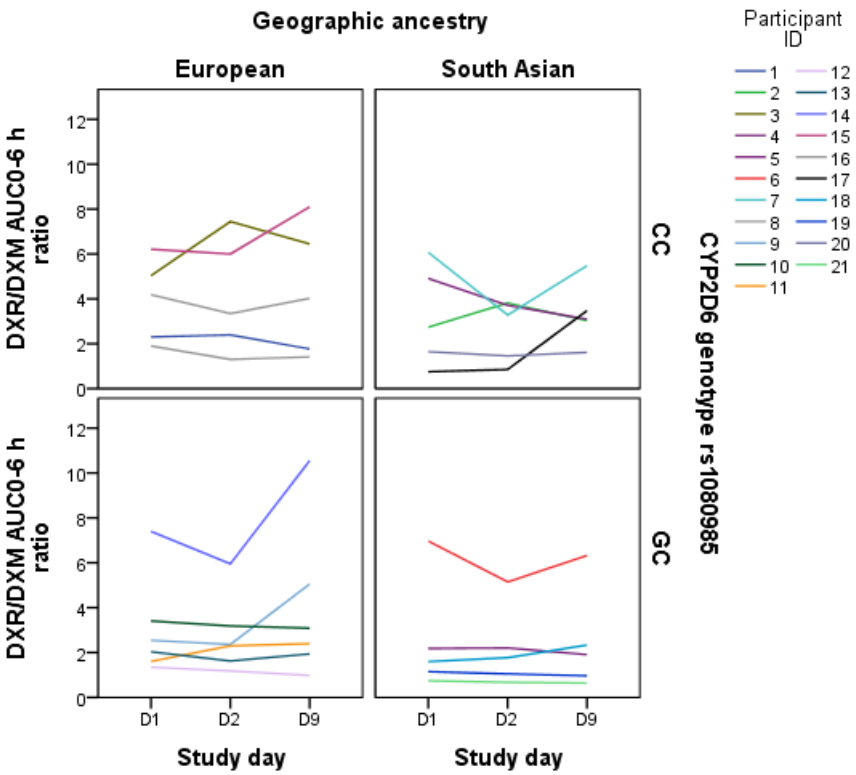
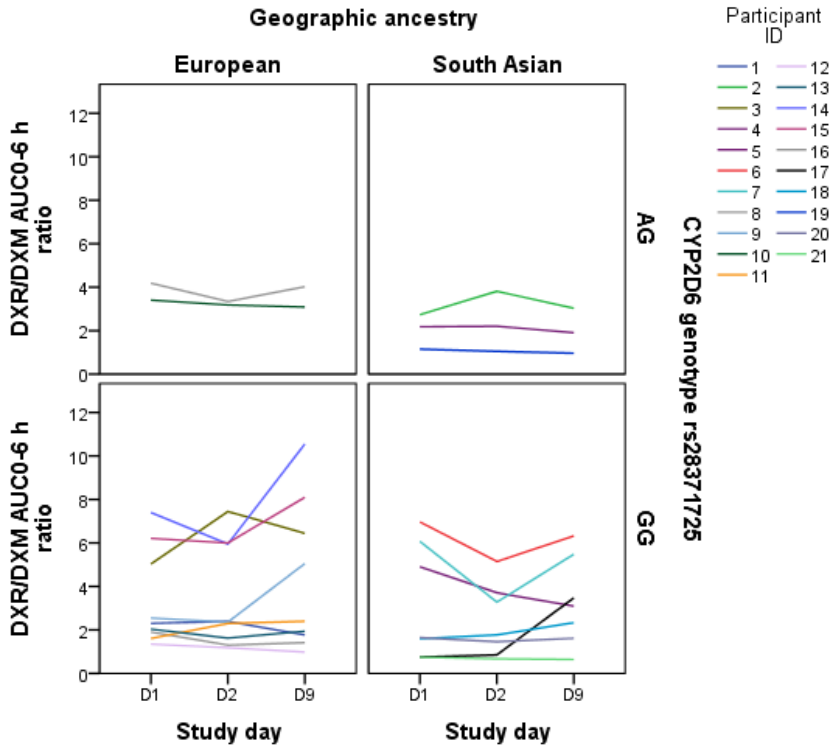
15
16
17
18
19
20
21

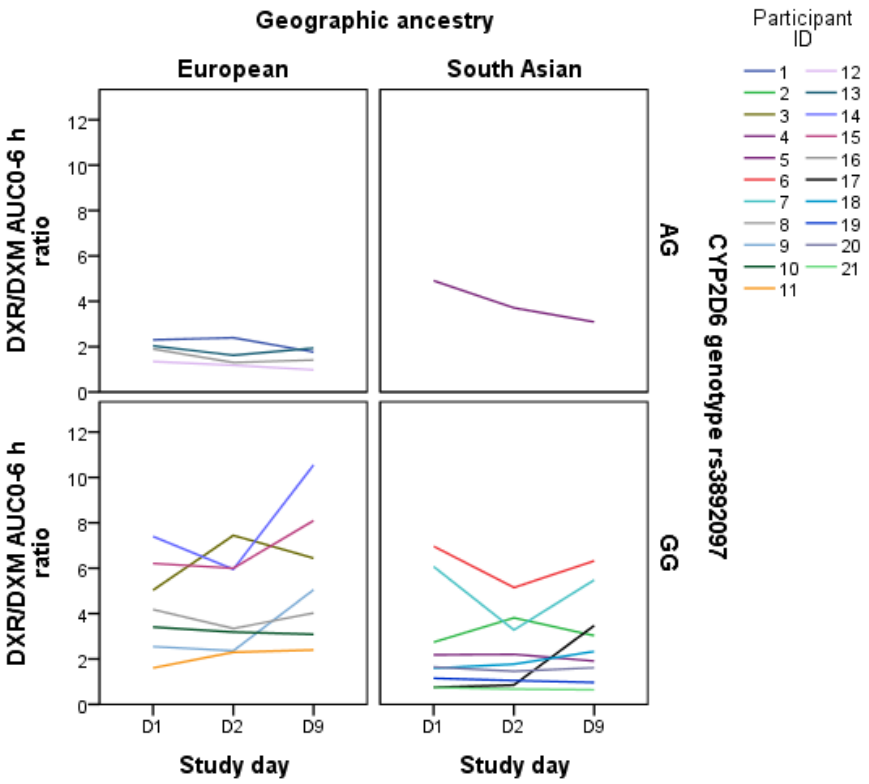

Geographic ancestry
European
South Asian
DXR AUC0-6 h (nM.h)
200
150
100
50
0
1 copy
2 copies
More than 2 copies
CYP2D6 gene copy number
D1
D2
D9
Study day
Participant ID
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21

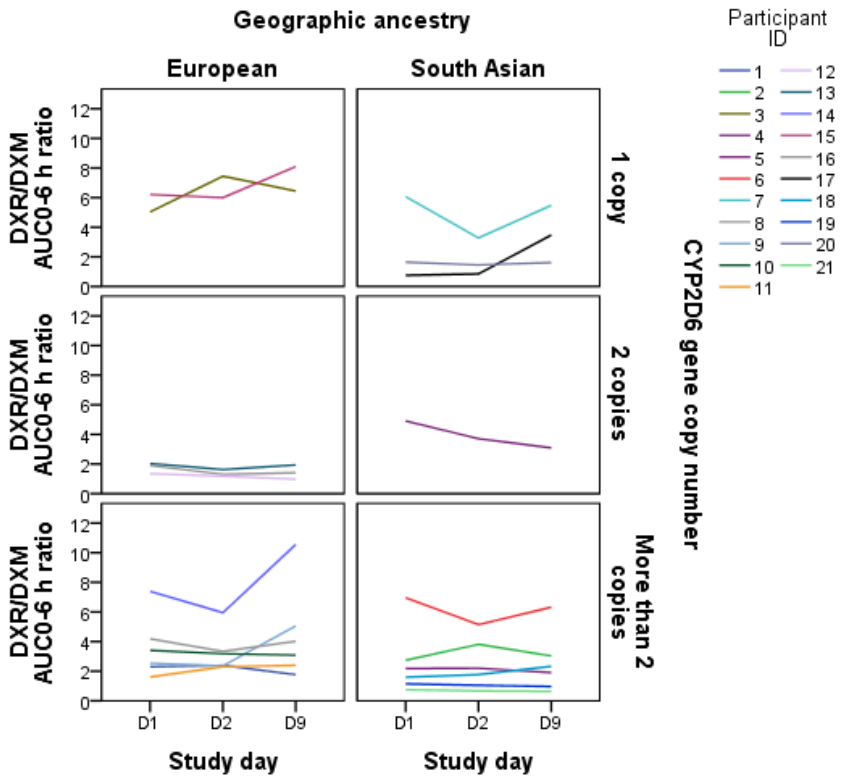

Geographic ancestry
European
South Asian
DXR AUC0-6 h (nM.h)
1 copy
2 copies
More than 2 copies
CYP2D6 gene copy number
Participant ID
Study day
D1
D2
D9


Geographic ancestry
European
South Asian
DXR/DXM AUC0-6 h ratio
CC
CT
CYP2D6 genotype rs1065852
Participant ID
Study day
D1
D2
D9


Geographic ancestry
European
South Asian
DXR/DXM AUC0-6 h ratio
CC
GC
CYP2D6 genotype rs1080985
Participant ID
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
D1 D2 D9
Study day


Geographic ancestry
European
South Asian
DXR/DXM AUC0-6 h ratio
AG
GG
CYP2D6 genotype rs28371725
Participant ID
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
D1 D2 D9
Study day


Geographic ancestry
European
South Asian
DXR/DXM AUC0-6 h ratio
CYP2D6 genotype rs3892097
AG
GG
Participant ID
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
D1 D2 D9
Study day


Geographic ancestry
European
South Asian
DXR/DXM AUC0-6 h ratio
CYP2D6 gene copy number
1 copy
2 copies
More than 2 copies
Participant ID
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
D1 D2 D9
Study day

8.19 Allele frequencies by ancestry

CYP1A2 genotype *CYP1A2*1C* * Geographic ancestry Crosstabulation

			Geographic ancestry		
			European	South Asian	Total
CYP1A2 genotype rs2069514	GA	Count	0	3	3
		% within CYP1A2 genotype rs2069514	0.0%	100.0%	100.0%
		% within Geographic ancestry	0.0%	30.0%	15.0%
		% of Total	0.0%	15.0%	15.0%
	GG	Count	10	7	17
		% within CYP1A2 genotype rs2069514	58.8%	41.2%	100.0%
		% within Geographic ancestry	100.0%	70.0%	85.0%
		% of Total	50.0%	35.0%	85.0%
Total		Count	10	10	20
		% within CYP1A2 genotype rs2069514	50.0%	50.0%	100.0%
		% within Geographic ancestry	100.0%	100.0%	100.0%
		% of Total	50.0%	50.0%	100.0%

CYP1A2 genotype *CYP1A2*1F* * Geographic ancestry Crosstabulation

			Geographic ancestry		
			European	South Asian	Total
CYP1A2 genotype rs762551	AA	Count	5	4	9
		% within CYP1A2 genotype rs762551	55.6%	44.4%	100.0%
		% within Geographic ancestry	45.5%	40.0%	42.9%
		% of Total	23.8%	19.0%	42.9%
	CA	Count	5	4	9
		% within CYP1A2 genotype rs762551	55.6%	44.4%	100.0%
		% within Geographic ancestry	45.5%	40.0%	42.9%
		% of Total	23.8%	19.0%	42.9%
	CC	Count	1	2	3
		% within CYP1A2 genotype rs762551	33.3%	66.7%	100.0%
		% within Geographic ancestry	9.1%	20.0%	14.3%
		% of Total	4.8%	9.5%	14.3%
Total		Count	11	10	21
		% within CYP1A2 genotype rs762551	52.4%	47.6%	100.0%
		% within Geographic ancestry	100.0%	100.0%	100.0%
		% of Total	52.4%	47.6%	100.0%

CYP2C19 genotype *CYP2C19*1C* * Geographic ancestry Crosstabulation

			Geographic ancestry		
			European	South Asian	Total
CYP2C19 genotype rs3758581	GA	Count	2	1	3
		% within CYP2C19 genotype rs3758581	66.7%	33.3%	100.0%
		% within Geographic ancestry	18.2%	10.0%	14.3%
		% of Total	9.5%	4.8%	14.3%
	GG	Count	9	9	18
		% within CYP2C19 genotype rs3758581	50.0%	50.0%	100.0%
		% within Geographic ancestry	81.8%	90.0%	85.7%
		% of Total	42.9%	42.9%	85.7%
Total		Count	11	10	21
		% within CYP2C19 genotype rs3758581	52.4%	47.6%	100.0%
		% within Geographic ancestry	100.0%	100.0%	100.0%
		% of Total	52.4%	47.6%	100.0%

CYP2C19 genotype *CYP2C19*2* * Geographic ancestry Crosstabulation

			Geographic ancestry		
			European	South Asian	Total
CYP2C19 genotype rs4244285	AA	Count	0	1	1
		% within CYP2C19 genotype rs4244285	0.0%	100.0%	100.0%
		% within Geographic ancestry	0.0%	10.0%	4.8%
		% of Total	0.0%	4.8%	4.8%
	GA	Count	3	5	8
		% within CYP2C19 genotype rs4244285	37.5%	62.5%	100.0%
		% within Geographic ancestry	27.3%	50.0%	38.1%
		% of Total	14.3%	23.8%	38.1%
	GG	Count	8	4	12
		% within CYP2C19 genotype rs4244285	66.7%	33.3%	100.0%
		% within Geographic ancestry	72.7%	40.0%	57.1%
		% of Total	38.1%	19.0%	57.1%
Total		Count	11	10	21
		% within CYP2C19 genotype rs4244285	52.4%	47.6%	100.0%
		% within Geographic ancestry	100.0%	100.0%	100.0%
		% of Total	52.4%	47.6%	100.0%

CYP2C19 genotype *CYP2C19*17* * Geographic ancestry Crosstabulation

			Geographic ancestry		
			European	South Asian	Total
CYP2C19 genotype rs12248560	CC	Count	8	7	15
		% within CYP2C19 genotype rs12248560	53.3%	46.7%	100.0%
		% within Geographic ancestry	72.7%	70.0%	71.4%
		% of Total	38.1%	33.3%	71.4%
	CT	Count	2	3	5
		% within CYP2C19 genotype rs12248560	40.0%	60.0%	100.0%
		% within Geographic ancestry	18.2%	30.0%	23.8%
		% of Total	9.5%	14.3%	23.8%
	TT	Count	1	0	1
		% within CYP2C19 genotype rs12248560	100.0%	0.0%	100.0%
		% within Geographic ancestry	9.1%	0.0%	4.8%
		% of Total	4.8%	0.0%	4.8%
Total		Count	11	10	21
		% within CYP2C19 genotype rs12248560	52.4%	47.6%	100.0%
		% within Geographic ancestry	100.0%	100.0%	100.0%
		% of Total	52.4%	47.6%	100.0%

CYP2C9 genotype *CYP2C9*2* * Geographic ancestry Crosstabulation

			Geographic ancestry		
			European	South Asian	Total
CYP2C9 genotype rs1799853	CC	Count	8	10	18
		% within CYP2C9 genotype rs1799853	44.4%	55.6%	100.0%
		% within Geographic ancestry	72.7%	100.0%	85.7%
		% of Total	38.1%	47.6%	85.7%
	CT	Count	3	0	3
		% within CYP2C9 genotype rs1799853	100.0%	0.0%	100.0%
		% within Geographic ancestry	27.3%	0.0%	14.3%
		% of Total	14.3%	0.0%	14.3%
Total		Count	11	10	21
		% within CYP2C9 genotype rs1799853	52.4%	47.6%	100.0%
		% within Geographic ancestry	100.0%	100.0%	100.0%
		% of Total	52.4%	47.6%	100.0%

CYP2D6 genotype *CYP2D6*10* * Geographic ancestry Crosstabulation

			Geographic ancestry		
			European	South Asian	Total
CYP2D6 genotype rs1065852	CC	Count	7	9	16
		% within CYP2D6 genotype rs1065852	43.8%	56.3%	100.0%
		% within Geographic ancestry	63.6%	90.0%	76.2%
		% of Total	33.3%	42.9%	76.2%
	CT	Count	4	1	5
		% within CYP2D6 genotype rs1065852	80.0%	20.0%	100.0%
		% within Geographic ancestry	36.4%	10.0%	23.8%
		% of Total	19.0%	4.8%	23.8%
Total		Count	11	10	21
		% within CYP2D6 genotype rs1065852	52.4%	47.6%	100.0%
		% within Geographic ancestry	100.0%	100.0%	100.0%
		% of Total	52.4%	47.6%	100.0%

CYP2D6 genotype *CYP2D6*2A* * Geographic ancestry Crosstabulation

			Geographic ancestry		
			European	South Asian	Total
CYP2D6 genotype rs1080985	CC	Count	5	5	10
		% within CYP2D6 genotype rs1080985	50.0%	50.0%	100.0%
		% within Geographic ancestry	45.5%	50.0%	47.6%
		% of Total	23.8%	23.8%	47.6%
	GC	Count	6	5	11
		% within CYP2D6 genotype rs1080985	54.5%	45.5%	100.0%
		% within Geographic ancestry	54.5%	50.0%	52.4%
		% of Total	28.6%	23.8%	52.4%
Total		Count	11	10	21
		% within CYP2D6 genotype rs1080985	52.4%	47.6%	100.0%
		% within Geographic ancestry	100.0%	100.0%	100.0%
		% of Total	52.4%	47.6%	100.0%

CYP2D6 genotype *CYP2D6*41* * Geographic ancestry Crosstabulation

			Geographic ancestry		
			European	South Asian	Total
CYP2D6 genotype rs28371725	AG	Count	2	3	5
		% within CYP2D6 genotype rs28371725	40.0%	60.0%	100.0%
		% within Geographic ancestry	18.2%	30.0%	23.8%
		% of Total	9.5%	14.3%	23.8%
	GG	Count	9	7	16
		% within CYP2D6 genotype rs28371725	56.3%	43.8%	100.0%
		% within Geographic ancestry	81.8%	70.0%	76.2%
		% of Total	42.9%	33.3%	76.2%
Total		Count	11	10	21
		% within CYP2D6 genotype rs28371725	52.4%	47.6%	100.0%
		% within Geographic ancestry	100.0%	100.0%	100.0%
		% of Total	52.4%	47.6%	100.0%

CYP2D6 genotype *CYP2D6*4* * Geographic ancestry Crosstabulation

			Geographic ancestry		
			European	South Asian	Total
CYP2D6 genotype rs3892097	AG	Count	4	1	5
		% within CYP2D6 genotype rs3892097	80.0%	20.0%	100.0%
		% within Geographic ancestry	36.4%	10.0%	23.8%
		% of Total	19.0%	4.8%	23.8%
	GG	Count	7	9	16
		% within CYP2D6 genotype rs3892097	43.8%	56.3%	100.0%
		% within Geographic ancestry	63.6%	90.0%	76.2%
		% of Total	33.3%	42.9%	76.2%
Total		Count	11	10	21
		% within CYP2D6 genotype rs3892097	52.4%	47.6%	100.0%
		% within Geographic ancestry	100.0%	100.0%	100.0%
		% of Total	52.4%	47.6%	100.0%

***CYP2D6* gene copy number * Geographic ancestry Crosstabulation**

			Geographic ancestry		
			European	South Asian	Total
CYP2D6 copy number	1 copy	Count	2	3	5
		% within CYP2D6 copy number	40.0%	60.0%	100.0%
		% within Geographic ancestry	18.2%	30.0%	23.8%
		% of Total	9.5%	14.3%	23.8%
	2 copies	Count	3	1	4
		% within CYP2D6 copy number	75.0%	25.0%	100.0%
		% within Geographic ancestry	27.3%	10.0%	19.0%
		% of Total	14.3%	4.8%	19.0%
	More than 2 copies	Count	6	6	12
		% within CYP2D6 copy number	50.0%	50.0%	100.0%
		% within Geographic ancestry	54.5%	60.0%	57.1%
		% of Total	28.6%	28.6%	57.1%
Total		Count	11	10	21
		% within CYP2D6 copy number	52.4%	47.6%	100.0%
		% within Geographic ancestry	100.0%	100.0%	100.0%
		% of Total	52.4%	47.6%	100.0%

CYP3A4 genotype *CYP3A4*22* * Geographic ancestry Crosstabulation

			Geographic ancestry		
			European	South Asian	Total
CYP3A4 genotype rs35599367	CC	Count	10	10	20
		% within CYP3A4 genotype rs35599367	50.0%	50.0%	100.0%
		% within Geographic ancestry	90.9%	100.0%	95.2%
		% of Total	47.6%	47.6%	95.2%
	CT	Count	1	0	1
		% within CYP3A4 genotype rs35599367	100.0%	0.0%	100.0%
		% within Geographic ancestry	9.1%	0.0%	4.8%
		% of Total	4.8%	0.0%	4.8%
Total		Count	11	10	21
		% within CYP3A4 genotype rs35599367	52.4%	47.6%	100.0%
		% within Geographic ancestry	100.0%	100.0%	100.0%
		% of Total	52.4%	47.6%	100.0%

GSTM1 rs1065411 * Geographic ancestry Crosstabulation

			Geographic ancestry		
			European	South Asian	Total
GSTM1 rs1065411	CC	Count	4	4	8
		% within GSTM1 rs1065411	50.0%	50.0%	100.0%
		% within Geographic ancestry	36.4%	40.0%	38.1%
		% of Total	19.0%	19.0%	38.1%
	CG	Count	1	1	2
		% within GSTM1 rs1065411	50.0%	50.0%	100.0%
		% within Geographic ancestry	9.1%	10.0%	9.5%
		% of Total	4.8%	4.8%	9.5%
	GG	Count	6	5	11
		% within GSTM1 rs1065411	54.5%	45.5%	100.0%
		% within Geographic ancestry	54.5%	50.0%	52.4%
		% of Total	28.6%	23.8%	52.4%
Total		Count	11	10	21
		% within GSTM1 rs1065411	52.4%	47.6%	100.0%
		% within Geographic ancestry	100.0%	100.0%	100.0%
		% of Total	52.4%	47.6%	100.0%

GSTP1 rs1695 * Geographic ancestry Crosstabulation

			Geographic ancestry		
			European	South Asian	Total
GSTP1 rs1695	AA	Count	8	6	14
		% within GSTP1 rs1695	57.1%	42.9%	100.0%
		% within Geographic ancestry	72.7%	60.0%	66.7%
		% of Total	38.1%	28.6%	66.7%
	AG	Count	3	3	6
		% within GSTP1 rs1695	50.0%	50.0%	100.0%
		% within Geographic ancestry	27.3%	30.0%	28.6%
		% of Total	14.3%	14.3%	28.6%
	GG	Count	0	1	1
		% within GSTP1 rs1695	0.0%	100.0%	100.0%
		% within Geographic ancestry	0.0%	10.0%	4.8%
		% of Total	0.0%	4.8%	4.8%
Total		Count	11	10	21
		% within GSTP1 rs1695	52.4%	47.6%	100.0%
		% within Geographic ancestry	100.0%	100.0%	100.0%
		% of Total	52.4%	47.6%	100.0%